SECOND EDITION

HANDBOOK OF TOXICOLOGY OF CHEMICAL WARFARE AGENTS

EDITED BY

RAMESH C. GUPTA

HANDBOOK OF TOXICOLOGY OF CHEMICAL WARFARE AGENTS

THE UNCERTAINTY OF THE DANGER BELONGS TO THE ESSENCE OF TERRORISM

Jürgen Habermas (1929–Present)

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Dedication

This book is dedicated to my beloved wife Denise, daughter Rekha,
and parents, the late Chandra and Triveni Gupta

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SECTION I

INTRODUCTION, HISTORICAL PERSPECTIVE, AND EPIDEMIOLOGY

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CHAPTER

1

Introduction

Ramesh C. Gupta

For centuries, extremely toxic chemicals have been used in wars, conflicts, terrorist and extremist activities, malicious poisonings, and executions. Natural toxins from plants or animals were one of the earliest forms of chemical warfare agents (CWAs). These were used to coat arrowheads and were commonly referred to as "arrow poisons." Ancient use of some CWAs and riot control agents (RCAs) dates back to the fifth century BC, during the Peloponnesian War, when the Spartans used smoke from burning coal, sulfur, and pitch to temporarily incapacitate and confuse occupants of Athenian strongholds. The Spartans also used bombs made of sulfur and pitch to overcome the enemy. The Romans used irritant clouds to drive out adversaries from hidden dwellings. In the fifteenth century, Leonardo da Vinci proposed the use of an arsenic sulfide powder as a chemical weapon. Modern use of CWAs and RCAs or incapacitating agents dates back to World War I (WWI).

With advancements in science and chemistry in the 19th century, the possibility of chemical warfare increased tremendously. The first full-scale use of CWAs began in April 1915, when German troops launched a poison gas attack in Ypres, Belgium, using 168 tons of chlorine gas, killing approximately 5000 Allied (British, French, and Canadian) soldiers. During WWI, the deployment of CWAs, including toxic gases (chlorine, phosgene, cyanide, and mustard), irritants, and vesicants in massive quantities (approximately 125,000 tons), resulted in approximately 90,000 fatalities and 1.3 million nonfatal casualties. The majority of deaths during WWI were a result of exposure to chlorine and phosgene gases. During the Holocaust, the Nazis used carbon monoxide and the insecticide Zyklon-B, which contains hydrogen cyanide, to kill several million people in extermination camps. Poison gases were also used during the Warsaw Ghetto Uprising in 1943. In November 1978, religious cult leader Jim Jones murdered more than 900 men, women, and children with cyanide.

Before, during, and after World War II, anticholinesterase organophosphate (OP) nerve agents/gases were developed in Germany, the United States, the United Kingdom, and Russia. They were also produced in large volumes in many other countries. During the "Cold War" period, they were maximally produced and stockpiled. These nerve agents have been used in wars and by dictators, extremists, cult leaders, and terrorist groups as chemical weapons of mass destruction (CWMD) on many occasions. In 1980, Iraq attacked Iran using mustard gas and OP nerve gas. During the period of the Iraq and Iran conflict (1980–1988), Iran sustained 387 attacks and more than 100,000 troops were victims, as were a significant number of civilians. Thousands of victims still suffer from long-term health effects. Soon after the end of the Iraq–Iran war in 1988, the brutal dictator of the Iraqi regime, Saddam Hussein, used multiple CWAs against the Kurdish minorities in a Halabja village, killing more than 10% of the town's 50,000 residents. To date, mustards have been used in more than a dozen conflicts, killing and inflicting severe injuries in millions of military personnel and civilians.

During the Persian Gulf War, exposure to OP nerve agents occurred from the destruction of munitions containing 8.5 metric tons of sarin/cyclosarin housed in Bunker 73 at Khamisyah on March 4, 1991, and during additional destruction of these nerve agents contained in rockets in a pit at Khamisyah on March 10, 1991. Although exposure levels to nerve agents were too low to produce signs of acute toxicity, military personnel serving in and around the Khamisyah area still suffer from long-term adverse health effects, most notably "Gulf War Syndrome." In 1996, approximately 60,000 veterans of the Persian Gulf War claimed to suffer from "Gulf War Syndrome" or "Gulf Veterans' Illnesses," possibly because of low-level exposure to nerve agents, mustard, pyridostigmine bromide, and pesticides. Exposed veterans had an increased incidence of chronic

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myelocytic leukemia and increased risk of brain cancer deaths compared with unexposed personnel.

In the mid 1990s, two terrorist attacks by a fanatic religious cult Aum Shinrikyo (Supreme Truth), known as Aleph since 2000, took place in Japan (Matsumoto in 1994 and Tokyo Subway in 1995). In both incidents, the OP nerve agent sarin was used as a CWA. An estimated 70 tons of sarin was manufactured by Aum Shinrikyo in Kamikuishiki, Japan. Although the total fatality count involved not more than 20 civilians, injuries were observed in more than 6000 and millions were terrified. These acts of chemical terrorism were unprecedented and the impact propagated not only throughout Japan but also throughout the entire world. In the past few decades, many incidents have also occurred with biotoxins such as ricin and anthrax. Publicity surrounding frequent recent use attributable to easy access and copycat crimes increase the possibility of future use of these chemicals and biotoxins, thus warranting advancement in emergency preparedness planning at the federal, state, and local government levels.

It is interesting to note that toxic chemicals have been used by governmental authorities against rebels or civilians. In the 1920s, Britain used chemical weapons in Iraq "as an experiment" against Kurdish rebels seeking independence. Winston Churchill strongly justified the use of "poisoned gas against uncivilized tribes." The Russian Osnaz Forces used an aerosol containing fentanyl anesthetic during the Moscow theater hostage crisis in 2002. RCAs were frequently used in the United States in the 1960s to disperse and control crowds.

At present, more than 25 countries and possibly many terrorist groups possess CWAs, and many other countries and terrorist groups are seeking to obtain them because of their easy access. Some of these agents are stockpiled in enormous quantities and their destruction and remediation are not only expensive but also associated with significant health risks. There is also the possibility of accidental release of CWAs or CWMD at the sites of their production, transportation, dissemination, and deployment. The intentional or accidental release of highly toxic chemicals, such as the nerve agent VX (Dugway Proving Ground, Utah, USA, 1968), Agent Orange (Vietnam, 1961–1972), PBB (Michigan, USA, 1973), dioxin (Seveso, Italy, 1976), and methyl isocyanate (Bhopal, India, 1984), has caused injuries in more than one million people and deaths in several thousands. A 1968 accident with VX nerve gas killed more than 6000 sheep in the Skull Valley area of Utah.

After September 11, 2001, the chances for the use of CWMD by extremist and terrorist groups, such as Al Qaeda, have been greater than ever, thus presenting great risks to humans, domestic animals, and wildlife in many parts of the world. On November 26, 2008, Pakistani Islamic terrorists attacked Mumbai City in India at 10 different sites, including two luxury hotels, a Jewish center, a train station, hospitals, and cafes. Approximately 200 innocent people died and approximately 300 people were injured by bullets and smoke. It is more likely that terrorist groups such as these may use toxic industrial chemicals (agents of opportunity) either in this way or as a precursor for more deadly CWMD. At present, many countries have established Defense Research Institutes with two major missions: (i) to understand the toxicity profile of CWAs/CWMDs and (ii) to develop strategic plans for prophylactic and therapeutic countermeasures. By the turn of the twenty-first century, the United States established the Department of Homeland Security. Many other countries also developed similar governing branches and agencies at the state and national levels to protect people and property from terrorist attacks. Among chemical, biological, and radiological weapons, the possibility of CWMD is more likely because of their easy access and delivery system. It is important to mention that understanding the toxicity profile of CWAs/CWMD is very complex, because these chemical compounds are of a diverse nature and, as a result, treatment becomes very difficult or, in some cases, impossible.

In the past, many accords, agreements, declarations, documents, protocols, and treaties have been signed at the international level to prohibit the development, production, stockpiling, and use of CWAs, yet dictators and terrorists produce and/or procure these chemicals to harm or kill enemies, create havoc, and draw national and international attention. In 1907, The Hague Convention outlawed the use of chemical weapons; however, during WWI, many countries used these chemicals. The first international accord on the banning of chemical warfare was agreed upon in Geneva in 1925. Despite the General Protocol, the Japanese used chemical warfare against China in 1930. In 1933, the Chemical Weapon Convention banned the development, possession, and use of CWAs. The document was signed and implemented by more than 100 countries. However, during WWII many chemicals of warfare were developed, produced, and used by several countries. In 1993, another global convention banning the production and stockpiling of CWAs was signed by more than 100 countries.

It is highly likely that these agents will be used in wars, conflicts, and terrorist attacks with malicious intent. In such scenarios, these extremely toxic agents continuously pose serious threats.

This first edition of *Handbook of Toxicology of Chemical Warfare Agents* was prepared to offer the most comprehensive coverage of every aspect of the deadly toxic chemicals that can be used as CWAs/CWMD. In addition to the chapters on radiation, several chapters were included on deadly biotoxins (ricin, abrin, strychnine, anthrax, and botulinum toxins) that can be weaponized in chemical, radiological, and biological warfare. Many

special and unique topics were offered that were not covered in other books. This was the first book to offer detailed target organ toxicity in the area of toxicology. In every chapter, all factual statements were substantiated with appropriate references.

Since the publication of the first edition of this handbook, concerns regarding the use and misuse of CWAs and biological warfare agents (BWAs) are greater than ever before. The delayed health effects from CWAs used during the Iraq–Iran conflict of the 1980s, during the sarin subway attacks in Japan and during the first Gulf War in the 1990s are still not well-understood. Recently, the Syrian government stockpiled more than 1300 metric tons of chemical agents, including sarin, VX, and sulfur mustard. In August 2013, the Syrian military repeatedly attacked civilians with chemical weapons, including sarin. More than 1300 people died and thousands were injured. Again, during April 11–13, 2014, Syrian military forces attacked civilians in Hama province with chlorine gas, killing and injuring an unaccounted number of people. Of course, the Syrian government has denied use of either sarin or chlorine gas.

The second edition of the *Handbook of Toxicology of Chemical Warfare Agents* is prepared to meet today's challenges of academicians and lay persons alike. The format used is user-friendly and easy to understand. Standalone chapters on individual chemical and biological agents, target organ toxicity, biosensors and biomarkers, risks to humans, animals, and wildlife, and prophylactic and therapeutic countermeasures are just a few of the many novel topics covered in this volume. The chapters are enriched with historical background as well as the latest information and up-to-date references. With 76 chapters, this book serves as a reference source for biologists, toxicologists, pharmacologists, forensic scientists, analytical chemists, local/state/federal officials in the Department of Homeland Security, Department of Defense, Defense Research Establishments, Department of Veterans Affairs, physicians at medical and veterinary emergency care units of hospitals, poison control centers, medical and veterinary diagnostic laboratories, environmentalists and wildlife interest groups, researchers in the areas of nuclear, chemical, and biological agents, and college and university libraries.

Contributors to the chapters in this book are the most qualified scientists in their particular areas of CWAs and BWAs. These scientists are from around the globe and are regarded as authorities in the fields of pharmacology, toxicology, and military medicine. The editor sincerely appreciates the authors for their dedicated hard work and invaluable contributions to this volume. The editor gratefully acknowledges Robin B. Doss and Michelle A. Lasher for their technical assistance. Finally, the editor remains indebted to Molly McLaughlin, Rhys Griffiths, and Kristine Jones, the editors at Elsevier, and Caroline Johnson, Susan McClung and Heather Turner in the production department of Elsevier for their immense contributions to this book.

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CHAPTER

2

Historical Perspective of Chemical Warfare Agents

Nathan H. Johnson, Joseph C. Larsen and Edward Meek

The opinions and assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the US Department of Defense, the US Air Force, or the US Department of Health and Human Services.

INTRODUCTION

The employment of chemicals in war has a long history (Silvagni et al., 2002; Romano et al., 2008). Just as the use of chemicals brought about tremendous advances in society, the concept of using chemicals to help win wars has been pursued for centuries (Joy, 1997; Smart, 1997). There are many examples of the exploitation of chemicals in warfare and conflict dating back to ancient times. Primitive humans may have been the first to use chemical compounds in both hunting and battle scenarios. The use of smoke from fires to drive animals or adversaries from caves may have been the earliest use of chemical weapons. Natural compounds derived from plants, insects, or animals that were observed to cause sickness or death were likely used by our distant ancestors in attempts to gain or maintain superiority (Hammond, 1994). Natural toxins from plants or animals on arrowheads, as well as the poisoning of water or food, could increase casualties and cause fear in opposing military forces or civilian populations. These early uses of chemicals paved the way for more lethal chemical weapons. For example, in the fourth century BC, smoke containing sulfur was used in the war between Sparta and Athens (Joy, 1997). Chinese manuscripts indicate arsenic-based compounds were used in battle (Joy, 1997). A few hundred years later, toxic smoke was employed by the Romans in Spain (Coleman, 2005). During the second siege of Constantinople, the Byzantine emperor Leo III used "Greek fire" in his quest for military victory (Coleman, 2005). During the ensuing years, there were many instances of the limited and attempted use of chemicals and toxins on the battlefield. Many of these examples may have been influenced by the intentional poisonings occurring in civilian settings (Joy, 1997; Smart, 1997; Newmark, 2004; Coleman, 2005). The earliest known treaty to ban poisons in warfare was signed between the French and Germans in the seventeenth century (Smart, 1997). In the siege of Groningen, European armies employed incendiary devices to release belladonna, sulfur, and other compounds. This led to the Strasbourg Agreement in 1675, which prohibited poison bullets (Smart, 1997; Coleman, 2005).

As science and chemistry advanced in the nineteenth century, the possibilities of chemical warfare increased exponentially. Advancements were made in industrial applications of sulfur, cyanide, and chlorine (Joy, 1997). In addition, the concept of delivering chemicals via projectiles was introduced. During the Crimean War, the British refused to use cyanide-based artillery shells against the Russians on the grounds that it was a "bad mode of warfare" (Smart, 1997). This was an early example of the ethical questions surrounding chemical use in warfare that continued into the twentieth century (Vedder and Walton, 1925). During the US Civil War, both the Northern and Southern armies seriously considered using various chemicals in their pursuit of operational victories (Smart, 1997). Early attempts at international treaties met with mixed results. The United States prohibited any use of poison during the Civil War. The Brussels Convention on the Law and Customs of War of 1874 prohibited poisons or poison-related arms (Smart, 1997). The first Peace Conference at the Hague

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prohibited projectiles filled with asphyxiating or deleterious gases (Smart, 1997). The employment of chemicals as asphyxiating warfare agents was vigorously discussed there (Joy, 1997). However, some countries, including the United States, were not signatories to this agreement. Arguments again were made against chemicals based on moral grounds. However, counterarguments were made based on the assumption that chemicals lead to death without suffering (Vedder and Walton, 1925; Joy, 1997; Coleman, 2005). Individuals who advocated chemicals did not view their use as an unfair advantage; rather, it was just one of a series of technological advances which, if mastered, could provide strategic, operational, and tactical advantages on the battlefield. The second Peace Conference at the Hague, held eight years later, prohibited both poisons and poisoned weapons (Smart, 1997). The British use of picric acid–filled shells during the Boer War and the Japanese use of arsenical rag torches in the Russo-Japanese War further illustrate that chemical warfare was considered by some a legitimate form of warfare at the turn of the twentieth century (Smart, 1997). During the early twentieth century, technological advancements in the chemical industry made the possibility of sustained military operations using chemicals a realistic possibility. The murder of Archduke Franz Ferdinand at Sarajevo in June 1914, which sparked World War I, set the stage for what would become the first widespread use of chemical weapons to date (Harris and Paxman, 2002).

THE FIRST SUSTAINED USE OF CHEMICALS AS AGENTS OF WAR

The talk and rhetoric of the late nineteenth century should have prepared the countries on both sides of World War I for chemical warfare. However, that was not the case (Smart, 1997). World War I clearly demonstrated the deadly and destructive nature of chemicals in modern warfare. Both sides of the war experimented with novel forms of warfare, including chemical weapons, and followed the lead of their adversary (Hay, 2000). It is little wonder that this war is known as the "chemist's war" (Fitzgerald, 2008). Initially, the French used gas grenades with little effect, followed by the German use of shells filled with tear gas (Joy, 1997). The Germans, capitalizing on their robust chemical industry, produced shells filled with dianisidine chlorosulfate (Smart, 1997). These shells were used in October 1914 against the British at Neuve-Chapelle, but with little effect. In the winter of 1914–1915, the Germans fired 150-mm howitzer shells filled with xylyl bromide (Smart, 1997). These shells were fired on both the eastern and western fronts, with disappointing effects. Despite this inauspicious start, efforts were continued to develop new uses of chemical warfare. It would soon be evident that chemical weapons would be devastating on the battlefield (Coleman, 2005; Tucker, 2006). In late 1914, Fritz Haber, a German scientist who later won the Nobel Prize in Chemistry, proposed the possibility of releasing chlorine gas from cylinders (Joy, 1997). Chemical warfare was attractive to Germans for two reasons: the shortage of German artillery shells and the ability to defeat the trench system of the enemy (Smart, 1997). After consideration and debate, the Germans released chlorine in April 1915 at Ypres, Belgium (Coleman, 2005). The German military was not prepared for the tremendous operational advantage the chlorine release provided, however. It did not take long for the British and French forces to respond in kind to the German offensive (Vedder and Walton, 1925; Joy, 1997; Smart, 1997; Coleman, 2005). In the fall of 1915, a British officer, William Livens, introduced a modified mortar (Figure 2.1) that could project gas-filled shells of chlorine or phosgene, the two agents of choice at that time (Joy, 1997). Both chlorine and phosgene caused extreme respiratory problems to those soldiers who were exposed to them (Vedder and Walton, 1925; Joy, 1997; Smart, 1997; Coleman, 2005; Hurst et al., 2007) (Figure 2.2).

FIGURE 2.1 British Livens Projector, western front, World War I.

As the United States entered the war in the spring of 1917, an obvious concern of its military command was the effect of chemical warfare on standard operations. Chemistry departments at universities were tasked with investigating and developing novel chemical agents (Joy, 1997). Protective equipment (Figure 2.3) and basic studies of the biological effects of chemical agents were assigned to the US Army Medical Department (Joy, 1997). In the fall of 1917, the army began to build an industrial base for producing chemical agents at Edgewood Arsenal, MD (Joy, 1997). As the effects of chlorine and phosgene became diminished by the advent of gas masks (Figure 2.4), the Germans turned to dichlorethyl sulfide (mustard) at Ypres against the British (Joy, 1997). As opposed to the gases, mustard remained persistent in

FIGURE 2.2 Australian infantry in trench with gas masks donned, Ypres, Belgium, September 1917.

FIGURE 2.3 US Army captain wearing a gas mask in training, 1917.

FIGURE 2.4 World War I soldier and horse, both wearing gas masks.

FIGURE 2.5 British soldiers temporarily blinded by tear gas awaiting treatment at the Battle of Estaires, April 1918.

the area, and contact avoidance was the major concern (Joy, 1997). It is worth noting that almost 100 years after it was first used on the battlefield, mustard still has no effective treatment; research continues into developing effective therapeutics (Babin et al., 2000; Baskin et al., 2000; Casillas and Kiser, 2000; Hay, 2000; Schlager and Hart, 2000; Hurst et al., 2007; Romano et al., 2008). It has been estimated that there were over 1 million chemical casualties (Figure 2.5) of World War I, with almost 8% being fatal (Joy, 1997). The Russians on the eastern front had a higher percentage of fatalities than other countries in the war, primarily due to the later introduction of a protective mask (Joy, 1997). The relatively low rate of chemical deaths in World War I demonstrated the most insidious aspect of the use of such weapons—namely, the medical and logistical burden that it placed on the affected army. The eventual Allied victory brought a temporary end to chemical warfare. In 1919, the Treaty of Versailles prohibited the Germans from producing or using chemical weapons.

INITIAL COUNTERMEASURES

The concept of a protective mask against chemical attack dates back over 500 years, to Leonardo da Vinci (Smart, 1997). By the mid-nineteenth century, protective masks were proposed in the United States and Europe for both industrial and military use. The modern gas mask was developed by the Germans with sodium thiosulfate- and bicarbonate-soaked pads, and it was used during World War I (Joy, 1997). The French and English soon followed with their own versions of gas masks (Joy, 1997). In 1916, the Germans introduced a mask that incorporated a canister through which the soldiers breathed (Joy, 1997). Initially, the American forces in World War I used gas masks obtained from allies already fighting in the war (Smart, 1997). In 1918, the Americans introduced Richardson, Flory, and Kops (RFK) mask, a modified version of the British mask. In addition, masks were developed for the animals, such as horses, that supported the war efforts. Decontamination efforts during World War I were rudimentary and included chemical neutralization and aeration of clothing and equipment. Although the need to detect chemical agents was clearly identified, very little progress was made during World War I. Medical treatment included removal of the patient from the source, decontamination, and palliative care (Smart, 1997).

EVENTS AFTER WORLD WAR I

By the time World War I ended, the world had been introduced to chemical warfare on an unprecedented level. While some groups thought that humanity had learned a lesson about the cruel nature of chemical warfare, others prudently went to work on improving chemical defenses (Vedder and Walton, 1925). The thoughts of many professional military officers were that future wars would be fought under the new paradigm of chemical warfare (Vedder and Walton, 1925; Vedder, 1926; Smart, 1997). New gas masks were developed, and training in chemical environments was introduced (Vedder and Walton, 1925; Vedder, 1926; Joy, 1997). Textbooks and manuals, such as those written by US Army Colonel Edward B. Vedder (Figure 2.6), were introduced into the military medical community (Vedder and Walton, 1925). In addition, the civilian medical community gained valuable insight into toxicology from the events of World War I (Vedder, 1929; Johnson, 2007). Despite the firsthand experience with chemical warfare, some countries, including the United States, struggled to fund their offensive and defensive programs adequately during demobilization (Smart, 1997).

It did not take long for chemical warfare to appear in other conflicts. Chemical agents were used to subdue rioters and suppress rebellions. For example, the British used chemical agents to suppress uprisings in Mesopotamia in the early 1920s by dropping bombs in cities throughout the area (Coleman, 2005). The Soviet Union used chemical agents to quell the Tambov peasant rebellion in 1921, and France and Spain used mustard-gas bombs to subdue the Berber rebellion in the 1920s (Werth et al., 1999). Italy and Japan used mustard in several small regional conflicts (Joy, 1997). The Italian conflict in Ethiopia was particularly noteworthy because mustard was sprayed and dropped from planes, and some experts think that the agent's use contributed significantly to the Italian victory (Smart, 1997). This use demonstrated the contemporary belief that chemicals were viable alternatives to traditional combat.

FIGURE 2.6 Captain Edward B. Vedder, the "father" of the United States Army Medical Research Institute of Chemical Defense (USAMRICD).

The Japanese also used chemical weapons during the 1930s against regional foes. Mustard gas and the vesicant lewisite were released against Chinese troops and were also used in Southeast Asia (Coleman, 2005). Lewisite is an arsine that was usually produced as an oily brown liquid that was said to smell like geraniums (Spiers, 1986; Hammond, 1994). It was developed in the United States by Winford Lee Lewis in 1918 and was found to be effective at penetrating clothing. The United States produced approximately 20,000 tons of lewisite but only used small quantities of the chemical in World War I (Coleman, 2005). Dimercaprol, more commonly called *British antilewisite*, was developed as an effective treatment for the vesicant (Goebel, 2008). In the period between the two world wars, mustard was a key part of defensive planning (Coleman,

2005). New stores of mustard were produced in many countries. Work continued on many fronts to improve protective equipment. For example, the US Chemical Warfare Service introduced the M1A2 mask, an improvement on the M1 mask (Smart, 1997). In the Geneva Protocol of 1925, 16 of the world's major nations pledged never to use gas as a weapon of warfare; however, it was not ratified in the United States until 50 years later, in 1975 (Hammond, 1994). There has long been vigorous debate on the merits of treaties with nations that balance military needs against the potential irrational concept of chemical warfare (Vedder, 1926).

WORLD WAR II

In the lead-up to World War II, the Germans forever changed chemical warfare with the discovery of the organophosphorus nerve agents (Goebel, 2008). These agents inhibit cholinesterase enzymes in the nerve synapse responsible for the breakdown of the neurotransmitter acetylcholine (ATSDR, 2008). This results in the accumulation of the neurotransmitter in the synapse and overstimulation of the nervous system. This can result in subsequent respiratory failure and death (ATSDR, 2008).

In 1936, Gerhard Schrader, a German chemist working on the development of insecticides for IG Farben, developed a highly toxic organophosphate compound, which he named *tabun* (Hersh, 1968; Hammond, 1994). Schrader and an assistant became casualties of their discovery when a drop of the neurotoxicant was spilled in the lab, exposing both of them (Tucker, 2006). Had the amount of tabun spilled been greater, both researchers would have certainly succumbed to the effects of the poison. Tabun was the first of a series of compounds termed *nerve gases* (Coleman, 2005). The correct terminology, however, is *nerve agents*, as these substances are not gases; rather, they are liquids dispersed as fine aerosols. Tabun was extremely toxic in small amounts, and it was invisible and virtually odorless (Tucker, 2006). The compound could be inhaled or absorbed through the skin. These characteristics made it too dangerous to be used as an insecticide by farmers. German law required that any discovery having potential military applications be reported to military officials (Tucker, 2006). Schrader was not overly excited about producing chemical agents for the military; however, the Germans placed him in a secret military research facility with the emphasis on producing these nerve agents and discovering new agents (Tucker, 2006). Subsequently, Schrader and his team of researchers discovered a more lethal organophosphate compound similar to tabun, which he named *sarin* in honor of the team members: Schrader, Ambrose, Rudriger, and van der Linde (Coleman, 2005).

FIGURE 2.7 Gas mask production—Detroit, Michigan, 1942.

FIGURE 2.8 A private trains using protective gear during World War II.

At the onset of World War II, both the Allies and the Germans anticipated that chemical agents would be deployed on the battlefield (Tucker, 2006). This expectation intensified research into the development of new agents, delivery systems, and methods of protection (Figures 2.7 and 2.8). The Allied forces were unaware of the Germans' new nerve agent, tabun, at the beginning of the war. The German Army advanced very rapidly across Europe using their Blitzkrieg method of maneuvering. As a result, German military leaders were reluctant to use chemical weapons, fearing that their forces would lose momentum waiting for contaminated areas

to clear. (Tucker, 2006). Nevertheless, the Germans produced and stockpiled large amounts of nerve agents throughout the war (Spiers, 1986). The production of these organophosphate agents was complex, required custom equipment, and was hazardous to those involved in its production (Tucker, 2006). If workers got exposed, they would be dunked in a bath of sodium bicarbonate (Harris and Paxman, 2002; Goebel, 2008). It is also interesting to note that some members of the German workforce were given rations containing higher percentages of fat (Harris and Paxman, 2002). This was done because authorities observed that workers with higher-quality rations seemed protected against exposure to low levels of tabun. Many detainees were used in the manufacture and testing of chemical agents in Germany (Harris and Paxman, 2002; Tucker, 2006). It is not known how many chemical casualties resulted from prisoners of war being forced to work at producing nerve agents, but some fatalities were documented. The discovery of tabun and sarin was followed by the discovery of soman in 1944 by Richard Kuhn and Konrad Henkel at the Kaiser Wilhelm Institute for Medical Research (Tucker, 2006). This class of nerve agents is collectively termed "G" agents; the *G* stands for *German*, since German researchers discovered this class of compounds. A second letter is included as the specific identifier of each compound: GA (tabun), GB (sarin), GD (soman), and GF (cyclosarin) (ATSDR, 2008). These agents were mass-produced by the Nazi regime throughout the war, but they were not used (Tucker, 2006). There has been considerable debate about why the Germans did not employ their chemical weapons in World War II. While it may never be known conclusively, several possible reasons include lack of intelligence regarding the German superiority in chemical weapons, fear of retaliation, and Adolf Hitler's personal exposure to chemical agents on the battlefield in World War I (Harris and Paxman, 2002; Tucker, 2006).

Other chemical agents that had been produced during and following World War I were still being produced. On December 2, 1943, German planes sank several American ships off the coast of Italy. At least one of the ships contained mustard, which was to be used in retaliation if the Germans unleashed a large-scale chemical weapons attack (Tucker, 2006). Many casualties resulted from exposure to the mustard, some of which included civilian merchant seamen (US Navy, 2008). The presence of the agent on the ship was classified, resulting in physicians incorrectly treating many of the victims (Tucker, 2006).

POST–WORLD WAR II

By the conclusion of World War II, both the Allies and Germany had stockpiled large amounts of chemical agents (Tucker, 2006). The Allied forces divided up the stockpiles of agents discovered in German facilities. Following the end of the war, many of the Allied countries continued to conduct research on the German nerve agents. The rise of the Soviet Union as a power and adversary prompted the United States and other countries to continually search for novel chemical and biological warfare agents (Tucker, 2006). The research and resources that were allotted for these efforts were not trivial, even though they were often overshadowed by the research and development of thermonuclear weapons (Hersh, 1968; Goebel, 2008).

The post–World War II era ushered in the nuclear age. Some felt the age of chemical warfare was over (Smart, 1997), but subsequent events would prove this to be a hasty conclusion. In the United States, research on the G-series agents and medical countermeasures against these agents was accomplished by the late 1940s. Research and intelligence gathering was further hastened by the impressive gains that the Soviet Union made in chemical warfare capability in the years after World War II. By the early 1950s, production of sarin had been initiated in the United States (Smart, 1997). At nearly the same time, Ranajit Ghosh, a chemist at the British company Imperial Chemical Industries plant, developed a new organophosphate compound to use as a potential insecticide (Tucker, 2006). Like with Gerhard Schrader, this compound was deemed too toxic to be used in the field as a pesticide. The compound was sent to researchers in Porton Down, England, synthesized, and developed into the first of a new class of nerve agents, the V agents (Goebel, 2008). Like the G agents, the V agents have a second letter designation: VE, VG, VM, and VX (Coleman, 2005). Of these, VX was the most common. The V series of agents are generally more toxic than the G agents (ATSDR, 2008). In a deal brokered between the British and US governments, the British traded the VX technology for the thermonuclear weapons technology of the United States (Tucker, 2006). The United States produced and stockpiled large quantities of VX after that (Hersh, 1968; Hammond, 1994).

Throughout the 1950s and 1960s, advancements were made in the production and delivery of chemical weapons to include sarin and VX (Smart, 1997). While work on improving masks continued, a renewed concern was the inability to detect nerve agents. Several prototypes were developed in the mid-1950s. Great advancements were made in the therapeutics of agents that inhibited the enzyme acetylcholinesterase (Taylor, 2006; Gupta, 2008; Klaassen, 2008). Atropine was introduced in the early 1950s. Oximes were added as an adjunct to speed up reactivation of the enzyme (Smart, 1997). The autoinjector was developed to overcome user fear of self-injection of atropine. Major advances were made in the use of chemical weapons in artillery (Figure 2.9). For example, the United States developed both short- and long-range rockets filled with chemical agents. But it

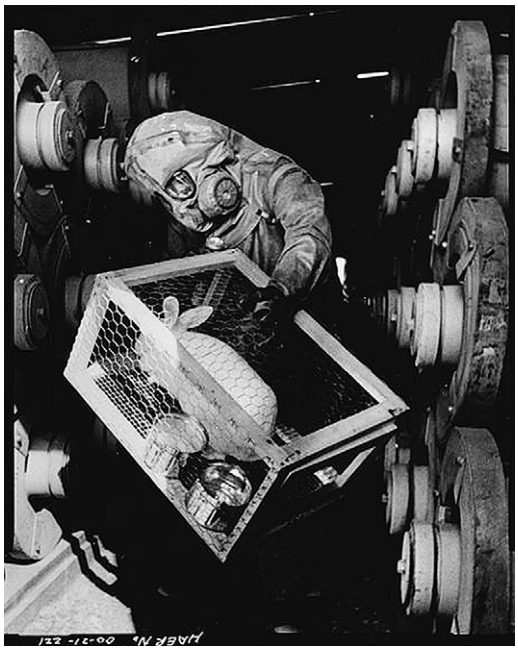

FIGURE 2.9 Testing for leaks at sarin production plant, 1970.

disposed of stockpiles of its chemical weapons in the late 1960s in an operation termed CHASE (which stood for "cut holes and sink 'em") in the sea (Coleman, 2005). In 1969, nerve agent stockpiles were discovered in US depots in Japan after several US military members became ill while doing maintenance (Tucker, 2006). This stockpile, which had been kept secret from the Japanese, created an uproar that later resulted in the disposal of the agents in the Johnston Atoll in the Pacific Ocean.

Defensive equipment, such as improved field alarms and drinking tubes for gas masks, were introduced in the 1960s (Smart, 1997). Great strides were also made in collective protection during the 1960s and 1970s. Although not used extensively since World War I, chemical agents have nonetheless been used for military purposes. The Egyptians allegedly used mustard and possibly nerve agents in the North Yeman civil war in the 1960s (Joy, 1997; Smart, 1997). This was the first reported use of nerve agents in armed conflict. There were allegations that chemical agents were used by the Vietnamese in Laos and Kampuchea in the late 1970s (Coleman, 2005). In the Vietnam War, the United States used defoliants and tear gas, and the Soviet Union was accused of using chemical agents in their war in Afghanistan (Joy, 1997).

INCAPACITANTS AND TOXINS

Incapacitating agents have long been considered an intermediate between chemical and traditional warfare. The Germans investigated the military use of lacrimators in the 1880s followed shortly thereafter by the French (Smart, 1997). The English and French considered using lacrimators in World War I (Smart, 1997). Japanese forces used tear gas against the Chinese in the late 1930s. The US Army used riot control agents and defoliants in the Vietnam War (Smart, 1997). The defoliant known as *Agent Orange* was later potentially linked to several forms of cancer (Stone, 2007). During the 1950s and 1960s, the United States had an active incapacitant program (Smart, 1997). These agents were thought of as more humane than traditional chemical agents because the intent was not to kill. These agents were designated *K agents* and included tetrahydrocannabinol and lysergic acid (Smart, 1997). One of the most extensively studied incapacitating agents was 3-quinuclidinyl benzilate, designated *BZ* by the US Army (Ketchum, 2006). Like many incapacitating agents, BZ was not adopted due to difficulty producing reproducible effects, unwanted side effects, latency in its effects, and difficulty in producing a dissemination that was free of smoke (Smart, 1997; Ketchum, 2006).

There have been multiple attempts to use the toxins from plants and living organisms to develop viable weapon systems. Two that are noteworthy are ricin and botulinum toxin. Ricin, a very potent toxin derived from the castor bean plant, has been recognized as a potential biological weapon since World War I. While the British were developing the V agents, US military researchers patented a procedure for purifying ricin (Harris and Paxman, 2002). The development of a method of disseminating ricin as a chemical weapon proved problematic, which made its use very limited. In 2003, ricin was detected on an envelope processed in a postal facility in Greenville, South Carolina. Postal workers did not develop symptoms of ricin exposure, and the individual who mailed the letter remains at large (Shea, 2004). The development and use of botulinum neurotoxin as a biological weapon was initiated at least 60 years ago (Smart, 1997; Arnon et al., 2001). In the 1930s, during Japan's occupation of Manchuria, the Japanese biological warfare group Unit 731 purportedly fed cultures of *Clostridium botulinum* to prisoners, killing them. The US Army biological weapons program produced botulinum neurotoxin during World War II in response to Germany's biological weapons program (Coleman, 2005). In fact, more than 100 million toxoid vaccine doses were prepared in time for the D-Day invasion of Normandy (Arnon et al., 2001).

RECENT EXPERIENCE

The 1980s proved to be a very significant time for the employment of chemical weapons on the battlefield. In 1980, Iraq invaded Iran (Smart, 1997). The Iraqi armed forces, advised by the Soviet Union, possessed chemical agents and were trained in their use. The war was

FIGURE 2.10 Aftermath of Iraqi chemical weapon attack (1980s).

unequivocally barbarous, and neither side gained an advantage. In many ways, this war had similarities to World War I. By 1983, Iran formally protested to the United Nations (UN) about the Iraqi use of chemical agents. The general consensus was that Iraq used mustard agents and possibly tabun in this war (Figure 2.10). It is estimated that 5% of Iranian casualties, totaling approximately 45,000, can be attributed to chemical agents; the Iraqi Army used chemical agents against the Kurdish minority in northern Iraq as well; and Libya was suspected of using chemical agents when it invaded Chad in 1986 (Smart, 1997).

The late 1980s also saw improvements in defensive equipment, such as the M40 gas mask developed by the United States (Smart, 1997). Other advancements were made in collective protection, decontamination, and detection. In 1984, US president Ronald Reagan issued a statement calling for an international ban on chemical weapons (Tucker, 2006). Subsequently, on June 1, 1990, President George H.W. Bush and Soviet leader Mikhail Gorbachev signed a treaty banning the production of chemical weapons and initiated the destruction of the stockpiles of both nations (Tucker, 2006). In 1993, the Chemical Weapons Convention was convened and signed, and it was implemented in 1997 (Hammond, 1994). As of 2008, the vast majority of UN member states had joined the Chemical Weapons Convention (OPCW, 2008).

In 1990, the Iraqi Army invaded neighboring Kuwait. Subsequently, the United States, at the request of Saudi Arabia, led a coalition to send forces to the area (Smart, 1997). These forces were the largest to operate in a potential chemical environment since World War I. They were provided with atropine autoinjectors, an acetylcholinesterase reactivator, and a nerve agent pretreatment (pyridostigmine bromide). Fortunately, chemical weapons apparently were not used in this conflict, although multiple false alarms were reported. The failure of the Iraqi military to use chemical weapons could be attributed to fear of retaliation, breakdown of communication, changing wind patterns, the surprising speed of the coalition attack, or the fact that Iraqi chemical infrastructure was attacked during the initial portion of the conflict. Since the conflict ended, many coalition veterans have reported a myriad of symptoms that have been commonly referred to as *Gulf War syndrome.* The etiology of this syndrome is unclear despite multiple epidemiological studies (Coleman, 2005). The most recent example of chemical weapons use is the ongoing Syrian civil war (Pellerin, 2013).

TERRORIST USE

One of the reasons why chemical weapons have been used relatively infrequently in combat over the past century is the fear of retaliation by opposing countries. In less organized asymmetrical conflicts, this fear is not as dangerous. At the same time, the potential exploitation of chemical weapons by terrorists is of great worldwide concern. The appeal of these weapons to terrorists lies largely in the fact that many of these chemical agents are cheap and relatively easy to produce, transport, and release. These characteristics, along with the fear associated with the idea of a chemical attack, make chemicals an ideal weapon for terror attacks (Romano and King, 2001). In 1974, Muharem Kurbegovic attacked several public buildings with firebombs in California and claimed to have developed sarin and some other nerve agents (Tucker, 2006). The search of his home resulted in the discovery of various precursor materials for chemical agents and a large amount of sodium cyanide. In 1994, the Aum Shinrikyo, a Japanese religious cult, carried out several attacks both in the subway and in residential areas using sarin produced by the cult's members (Tucker, 2006). A total of 19 people were killed, and over 6,000 received medical attention. Some of those who sought medical attention may have done so due to a fear of exposure. Psychological stress is a common aftermath of a chemical or biological attack (Romano and King, 2001). In the twenty-first century, chemicals that once had been used exclusively by the military have reemerged as contemporary threats. In the fall of 2006, Al Qaeda and associated groups used chlorine combined with traditional car and truck bombings to spread panic in Iraq (Garamone, 2007). These attacks were followed by similar incidents in the subsequent months.

CONCLUDING REMARKS AND FUTURE RESEARCH

So long as there are legitimate uses for chemicals in our society, the risk of chemical agents in conflict and

terrorist activity will always be present. Research continues across the globe for better detection, protection, and treatment of chemical agents. While many countries have denounced and indeed are signatories to various treaties to limit the use and production of chemical warfare agents, nonstate and terror organizations are under no such restrictions. Luckily, chemical weapon use has been limited in both warfare and less formal conflicts. As we progress into the twenty-first century, the use of established chemical warfare agents is a real possibility. The potential use of legitimate industrial chemicals (e.g., the Iraqi burning of petroleum fields in the first Gulf War) and the potential synthesis of new agents should also be recognized. History has demonstrated that chemicals have been used in both organized and asymmetrical conflicts, and preparations for defense and therapy for such encounters is prudent. Chemicals represent a unique force multiplier that simply cannot be ignored in the twenty-first century.

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CHAPTER

3

Global Impact of Chemical Warfare Agents Used Before and After 1945

Jiri Bajgar, Josef Fusek, Jiri Kassa, Kamil Kuca and Daniel Jun

INTRODUCTION

The threat of chemical weapons (CWs), used either by States or Parties to the Chemical Weapons Convention (CWC; Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on their Destruction) or by terrorists, has never attracted so much public attention as it has in the past 10 years. Despite the existing legal documents dealing with prohibition of CWs, for example, Geneva Protocol 1925 and CWC, some incidents of the use of CWs in different conflicts and terroristic attacks have been observed. Moreover, alleged use of CWs was noted during the period from 1925 to the present. It must be emphasized that the theoretical and practical basis for production, storage, and use of CWs still exists. Also, it must be clearly stated that CWs are applicable at any time, in any place, and in large quantities.

CWs consist of chemical warfare agents (CWAs) and the means to deliver to the target. They are characterized by high effectiveness for use against large targets and are known as area weapons or silent weapons. They are relatively low-cost and it is possible to achieve destruction of everything that is living while avoiding destruction of materials and buildings. They are also called the nuclear weapons of poor countries—"poor man's nuclear weapon." It should be pointed out that the use of CWs is connected with the use or release of toxic chemicals; thus, chemical warfare can be considered part of generally observed situations in which toxic chemicals are used or released and influence the environment and humankind.

A number of causal reasons for these events exist but, apart from accidents connected with the release of toxic chemicals from a natural source (e.g., volcanoes), the factors shown in Figure 3.1 or their combinations can be involved.

For military purposes a number of chemicals were tested, but only a few are contained in military arsenals. However, according to the definition in the CWC, any toxic chemical intended for military use must be considered a CW; in other words, the aim is to limit the designation of the compound in question for use as a CW. However, it is possible for terrorists to choose any chemicals with high toxicity.

BACKGROUND

The use of toxic chemicals against humankind is as old as any warfare conflict. The use of the poisoned arrow against humans—not animals—can be considered as the beginning of chemical warfare and is characterized as the intentional use of chemicals.

At the very beginning, chemical warfare was more closely connected with fire. "Greek fire" was an excellent naval weapon because it would float on water and set fire to the wooden ships. There are other examples from history; for example, toxic smoke was used in China in 2000 BC. In Thucydides' *History of the Peloponnesian War* (the fifth century BC war between Athens and Sparta), we find the first description of chemical warfare—the formation of toxic sulfur oxide by burning sulfur. In the year 184 BC, Hannibal of Carthage used baskets with poisonous snakes against his enemy. Both Socrates and Hamlet's father were poisoned with koniin. Aqua Toffana containing arsenic was also a known poison in ancient Italy. Leonardo da Vinci proposed a powder of arsenic sulfide in the fifteenth century. There are many more examples of the use of CWAs (Bajgar et al., 2007b).

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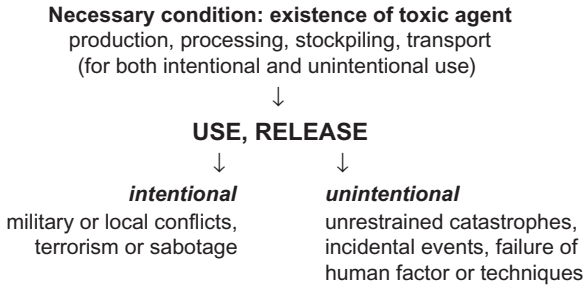

FIGURE 3.1 Possible reasons for release/use of toxic chemicals.

Modern history shows us that terrorists have used other chemicals, such as ricin (Bulgarian G. Markov was poisoned in 1978) or dioxin (the President of Ukraine Viktor Andriyovych Yushchenko was poisoned in 2004).

In a region of Bohemia, a "form" of CW was used as early as 600 years ago. It was in 1422 when the castle of Karlstein, the property of King Charles IV, was besieged and 1822 kegs containing the cesspools of the streets of Prague were hurled into the castle. Allegedly, the stench in the castle was unbearable. According to historical sources, the castle defenders were probably intoxicated with hydrogen sulfide released from the contents of the cesspools and therefore showed typical symptoms of poisoning (Bajgar, 2006).

There were some attempts to prohibit CWs by international agreement or law. Most of the early attempts were bilateral or unilateral agreements directed at the use of poisons. These included the 1675 agreement between France and Germany, signed in Strasbourg, to ban the use of poison bullets.

The first international attempt to control chemical and biological weapons took place in Brussels in 1874, when the International Declaration was signed and included a prohibition against poison and poisoned arms. Despite the first and second Brussels and Hague Conventions (1899 and 1907 signatories agreed not to use projectiles that could spread asphyxiating or deleterious gases), the world witnessed the application of chemicals during warfare to an unprecedented extent during World War I (WWI). A brief summarization of the events connected with the use/release of toxic chemicals is given in Table 3.1.

MILITARY USE OF CWS

The intentional use of CWs for military purposes can be found in both global and local conflicts. A typical example is the warning "Gas! Gas!" This was common in WWI and it is well-known from the E.M. Remarque novel *All Quiet on the Western Front* in which Remarque suggestively describes a chemical attack with chlorine.

During WWI, many chemicals were used, including mustard and asphyxiating and irritant agents. Approximately 45 types (27 more or less irritating and 18 lethal) of toxic chemicals were used. During the latter part of 1914, irritants were used by Germany and France; the effect was insubstantial. In late 1914, Nobel Prize winner Fritz Haber of the Kaiser Wilhelm Physical Institute in Berlin (chemical synthesis of ammonium in 1918) came up with the idea of creating chlorine, although this idea of using toxic chemicals in war was expressed by Admiral Dundonald as early as 1855. Chemical warfare really began in 1915, when German troops launched the first large-scale poison gas attack at Ypres, Belgium, on April 22, using 6000 cylinders to release 168 tons of chlorine gas, killing 5000 British, French, and Canadian soldiers. The date is recognized as "the birthday of modern chemical warfare," and thereafter the belligerent parties frequently used chemical gases against each other. Phosgene was introduced by Germany in late 1915. Soon after the first chlorine attack, the Allies used primitive emergency protective masks. In May 1916, the Germans started using diphosgene, while the French tried hydrogen cyanide 2 months later and cyanogen chloride the same year. The first time mustard gas was used by German troops was July 12, 1917. After its use near Ypres, it was also called yperit.

By the end of the WWI, approximately 124,200 tons of CWAs (chlorine, phosgene, mustard, etc.) had been released, causing at least 1.3 million casualties, of which more than 90,000 were fatal. The threat of the use of CWAs led to the development of protective means not only for humans but also for horses and dogs. The effectiveness of CWs in comparison with classic munition was evident: 1 ton of classic explosives caused 4.9 casualties; 1 ton of chemical munition caused 11.5 casualties; and 1 ton of yperite caused 36.4 casualties (Bajgar, 2006).

THE PERIOD BETWEEN WORLD WAR I AND WORLD WAR II

The terrible casualties of CWs used during WWI and the dangerous consequences for humans and the environment led to the signing of the "Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous and other Gases and Bacteriological Methods of Warfare" on June 17, 1925. This is recognized as one of the unique and famous international treaties on the prohibition of CWs. However, it neither comprises provisions for effective verification nor prohibits development, stockpiling, and transfer of CWs. Moreover, no definition of CWs was included. Despite the provisions of the Geneva Protocol, during 1935 to 1936 Italian troops used CWs during their invasion of Abyssinia (Ethiopia). This first major use of CWs after WWI came

TABLE 3.1 Some Milestones Related to the Use/Release of CWs and Toxic Chemicals

Year(s)	Event
2000 BC	Toxic smoke in China inducing sleep
Fourth century BC	Spartacus—toxic smoke
184 BC	Hannibal—baskets with poison snakes
1168	Fustat (Cairo) —use of "Greek fire"
1422	Bohemia region—cesspools (H_2S)
1456	Belgrade—rats with arsenic
Nineteenth century	Admiral Dundonald—proposed the use of chemicals in war
1914–1918	WWI—start of chemical war
1918–1939	Development of new CWs and protective means
June 17, 1925	Geneva Protocol
December 23, 1936	Lange and Kruger—synthesis of tabun
1940–1945	Concentration camps—cyanide
1943	Synthesis of sarin
1943	Hoffmann and Stoll—synthesis of LSD-25
1945	Kuhn—synthesis of soman
1950	V agents are begun
1961–1968	Production of VX
1961–1971	Vietnam War—herbicides (impurity dioxin)
1962	BZ was introduced into military arsenals
1970	Bicyclic phosphates considered as potential CWAs
1976	Seveso—release of dioxin
1980	Some rumors on intermediate volatility agent
1984	Bhopal incident—release of methylisocyanate
1985	Decision on production of binary CWs
1986, 1987	Demonstration of USA CWs (Tooele) and Soviet Union CWs (Shikhany) to the CD in Geneva
1987	Production of binary CWs
1988	Halabja—use of mustard
1980–1990	Rumors of new nerve agent Novichok
1989	Conference on chemical disarmament, Paris
1991	Persian Gulf War—veteran's syndrome
1992	BZ military stocks of the USA were destroyed
1992	Finalization of the rolling text of the CWC at the CD—Geneva
1993	Signing CWC in Paris
1993	Preparatory Commission on OPCW
1994	CWs of Iraq were destroyed
1994	Aum Shinrikyo—sarin attack in Matsumoto
1995	Aum Shinrikyo—sarin attack in Tokyo
April 29, 1997	CWC—entry into force; establishment of OPCW in The Hague
2000	Research on nonlethal weapons intensified
2002	Moscow theater—Fentanyl derivatives used against terrorists
April 29, 2012	CWs of the State Parties to the CWC will be destroyed but it was prolonged; this period varies from 2015 to 2023 years.
August 2013	Syria—use of sarin

after October 3, 1935, when Mussolini launched an invasion of this country. Despite the Geneva Protocol (Italy had ratified in 1928), the Italians used mustard gas with horrible effects. Later, CWs were used between Japan and China during 1937 to 1945. The Japanese attacked Chinese troops with mustard gas and lewisite. The Japanese, in addition to their biological program, had an extensive CWs program and were producing agent and munitions in large quantities by the late 1930s.

WORLD WAR II

Despite the storing and stockpiling of CWs by the great powers engaged in World War II (WWII), these fatal weapons were not practically used (except small examples) during WWII (probably because of the fear of massive retaliatory use of CWs). An example of intentional use, but not during military conflict, was the killing of prisoners in concentration camps in Nazi Germany. The agent first used in the camps was carbon monoxide, followed by the more "effective" hydrogen cyanide released from Zyklon B. Some experiments with aconitine-impregnated shells and some other toxic compounds including biological agents were tested on prisoners.

However, during WWII, an important step in the preparation of the most dangerous CWA was observed in Germany. In Schrader's group, organophosphates (OPs) were synthesized, primarily with the aim of obtaining more effective insecticides. Between 1934 and 1944, Schrader's team synthesized approximately 2,000 OPs, including two well-known OP compounds, parathion and paraoxon. As early as 1935, the government of Nazi Germany insisted that Schrader switch the primary aim from OP insecticides to CWAs. At present, OPs are widely used in agriculture, medicine (human and veterinary), and industry. These compounds also include nerve agents (the most toxic compounds of the OP group). Nerve agents such as sarin, tabun, soman, and VX are the main compounds of CWAs. The Germans were also the greatest producers of nitrogen mustard and produced approximately 2,000 tons of HN-3.

This part of history is well-known (Koelle, 1963, 1981; Bajgar, 2006; Tuorinsky, 2008; Klement et al., 2013). First synthesis of OP was described in the second half of the eighteenth century. For a long time the first OP (its toxicity was described later) was considered to be TEPP, which was synthesized by Clermont (1854–1855). Philippe de Clermont was a well-known chemist in Sorbonna. Charles Adolph Wurtz dedicated his work to the synthesis of esters of pyrophosphoric acid. These data were specified by Petroianu (2008), and thus he contributed to the discovery that the first synthesis of this OP—TEPP—was performed by Vladimir Moshnin of Moscau. These data are depicted in the work of Patočka (2010). New trends in the synthesis of nerve agents have been described by Halamek and Kobliha (2011).

Tabun was synthesized in 1936, followed by others (sarin at the end of WWII, followed by soman), and production of these agents for the military in large quantities and their stockpiling were recognized after WWII in Dyhernfurth, Poland (e.g., stocks of tabun and some quantities of sarin). The technology was subsequently transferred to Russia, and research and development of new OP nerve agents was continued. During this period, British and American scientists were evaluating the toxic properties of DFP.

THE PERIOD AFTER WORLD WAR II AND THE COLD WAR

At the end of WWII, many Allied nations seized the CWs. Most of the CW manufacturing plants in Germany were taken over and moved to new sites in Russia, such as the military area of Shikhany. This "takeover" prompted other states to begin even more research of CWs. Despite the Allies' own research into CWs, very important technologies and "know-how" were obtained from Nazi Germany for both the United States and the former Soviet Union.

The interest in CW technology was probably one reason for the change of the future border: according to Churchill's history of WWII, the proposed future boundary between Poland and Germany had been primarily agreed to consist, in part, of the Oder River flowing to the Baltic Sea, and its tributary, the Neisse River. Before their confluence, the Neisse consisted of two branches, the East Neisse and the West Neisse. The East Neisse should be the boundary, resulting in slightly more territory for Germany. Stalin held for the West Neisse and progress was delayed. No one knows why Stalin was so insistent in this matter. The reason was probably very simple: the small town of Dyhernfurth (now Brzeg Dolny), a few kilometers north of Breslau (Wroclaw) in the disputed territory, contained a factory for the production of nerve agents. It was estimated that when Dyhernfurth was captured it contained stockpiles of 12,000 tons of tabun, 600 tons of sarin, and an unknown amount of soman. Presumably, the factory was dismantled and, along with their stockpiles, transported to the Soviet Union (Koelle, 1981). It has been documented that the Soviets were ready to conduct a chemical attack and their research and development of CWs were intensified.

In the United States during the 1950s, the chemical corporations concentrated on the weaponization of sarin. At the same time, they became interested in developing CWs that incapacitated rather than killed the targets. Mescaline and its derivatives were studied

but without practical output. Five years later, the new project "Psychochemical Agents" (later K-agents) was established. The objective was to develop a nonlethal but potent incapacitant. Nonmilitary drugs like LSD-25 and tetrahydrocannabinol were also examined. None of these agents was found to be of military importance. The first and only incapacitant was BZ, developed in 1962; however, its stocks were destroyed in 1992, as declared by the US delegation to the Conference on Disarmament in Geneva (Document of CD, 1991). These agents, intended not to kill but to induce incapacity, are covered under the class of nonlethal weapons (Hess et al., 2005).

In the former Soviet Union as a whole during 1940–1945, approximately 110,000 tons of first-generation toxic chemicals were produced, and most of them were yperite, lewisite, and irritating agents. Second-generation CWs were composed of nerve agents such as sarin, soman, V agents, and, to a lesser degree, tabun. The development of new third-generation CWs comprised traditional and nontraditional CWs, for example, blister and irritant agents and nerve gases, including new types such as Novichok 5, whose exact chemical structure is unknown, although some assessments have been made (Bajgar, 2006). It could be a nerve agent having high toxicity, and its effects are difficult to treat using common antidotes.

An example of the unintentional use of CWs has also been observed. In March 1968, thousands of dead sheep were discovered in the Skull Valley area in Arizona in the United States. This area was adjacent to the US Army's Dugway open-air testing site for CWs. Nerve gas had drifted out of the test area during aerial spraying and killed the sheep. One year later, on July 8, 1969, the Army announced that 23 US soldiers and one civilian had been exposed to sarin in Okinawa during the clearing of sarin-filled bombs (Sidell and Franz, 1997).

There are a number of examples of localized conflicts during which CWs have been intentionally used but cannot be verified: from 1951 to 1952 during the Korean War; in 1963, the Egyptians used mustard bombs against Yemeni royalists in the Arabian peninsula; during the Indo-China War (see Vietnam War); in 1970, in Angola, antiplant agents were almost certainly used; and in former Yugoslavia, there were rumors of the use of psychotomimetic agents.

Iraq–Iran and Afghanistan War

On September 22, 1980, Iraq launched its invasion against Iran. There has been mention of the large-scale use of CWAs in the Iran–Iraq war. In November 1983, Iran informed the United Nations that Iraq was using CWs against Iranian troops. Soon after, the use of CWs was unleashed. In addition, mustard and tabun were used. It is well-known that the Iraqi Government used these agents against its own citizens, more conspicuously at Halabja in March 1988. The CWs attack was the largest against a civilian population in modern times. More than 100,000 Iranians were poisoned with CWAs; sulfur mustard was the most frequently used and has induced a number of delayed complications in Iranian veterans (pulmonary, dermal, ocular, immune system depression, reproduction, malignancy, etc.) (Afshari and Balali-Mood, 2006). Other localized conflicts involving alleged use of CWs are described in detail in an extensive review (Robinson, 1971).

The Soviet Union probably used mustard (and nerve gas) in Afghanistan. The Afghanistan war was considered the Soviet Union's "Vietnam." The use of CWs was described by Sidell and Franz (1997). The use of CWs by Soviet forces was also significant and has been confirmed against unprotected subjects. Despite the use of CWs, the withdrawal of Soviet troops from Afghanistan was realized at the beginning of 1989.

Vietnam War

After WWII, the main use of CWs was recorded during 1961 to 1972, when the US Army used defoliants. The herbicide Agent Orange was used during the Vietnam War and led to the injury of more than one million Vietnamese and Americans. Agent Orange (a mixture of 2,4-dichlorophenoxy acetic acid and 2,4,5-trichlorophenoxy acetic acid) contained the chemical contaminant dioxin as an impurity that caused many deaths on both sides. There were other herbicide mixtures such as Agent White (2,4-D and picloram) and Agent Blue (cacodylic acid). The biological effects of dioxin were described by Sofronov et al. (2001). The first major operation of this type was conducted over the Ca Mau peninsula during September–October 1962. The areas sprayed with defoliants were five-times larger and 10-times larger in 1965 and 1967, respectively. The scale of the use of defoliants was approximately in proportion to the overall involvement of US troops. In 1970, herbicides and defoliants were used in tens of tons, especially 2,4,5-T. The area sprayed enlarged from 23 km^2 in 1962 to 22,336 km^2 in 1969. The area exposed to spraying was assessed to be 58,000 km^2 and the number of people exposed was assessed to be more than one million; there were more than 1000 deaths. In addition to defoliants used to destroy vegetation concealing the North Vietnamese, the United States used tear gas for clearing tunnels and bunkers. The irritants CS, CN, and DM were reported to be used. The total CS procured was approximately 7,000 tons from 1963 to 1969.

Development of VX Agent

VX was synthesized in the 1960s on the basis of the results of Tammelin and Aquilonius (Tammelin, 1957;

Aquilonius et al., 1964). The manufacturing of VX began in the United States in 1961. Construction of the United States' VX agent production plant at Newport, Indiana, was completed in 1961, when the first agent was produced. The production facility only operated for 7 years and was placed on standby in 1968 (Smart, 1997).

During the same period, Soviet scientists developed the so-called Russian VX (VR, RVX, R 033) (Kassa et al., 2006; Kuca et al., 2006). The chemical structure of VX was unknown for a long time. Therefore, some attempts to resolve this question have been made (Bajgar, 1968). Because of these ambiguities and difficulties in synthesis, model V agent [EDMM, *O*-ethyl *S*-(2-dimethylaminoethyl) methylphosphonothioate] was initially used in the Eastern Block to study antidotal treatment. Another structural analog of VX known as Chinese VX (CVX) was also developed and studied (Eckert et al., 2006).

A very important step in the development in CWs has been the production of "binary munitions," in which the final stage of synthesis of the agent from precursors is performed in the munition (bomb, shell, or warhead) immediately before or during delivery to the target. In the 1950s, armed forces had begun looking at binary weapons. Until this time, CWs were unitary—the toxic agent was filled in the munition and then stored ready to be used. The binary concept—mixing or storing two less toxic chemicals and creating the nerve agent within the weapon—was safer during storage. The production of binary projectiles began on December 16, 1987, at the Pine Bluff Arsenal in Arkansas.

PERSIAN GULF WAR

On August 2, 1990, Saddam Hussein sent Iraqi troops into Kuwait, allegedly in support of Kuwaiti revolutionaries who had overthrown the emirate. Iraq was known to have a large stockpile of CWs during its conflict with Iran and confirmed that they would use CWs.

President George H.W. Bush ordered US forces to be sent to Saudi Arabia at the request of the Saudi Government (Operation Desert Shield); this was the build-up phase of the Persian Gulf War. As a consequence, in 1996, almost 60,000 veterans of the Persian Gulf War claimed certain medical problems related to their war activities. Some were caused by exposure to nerve agents (released after the bombing and destruction of the sarin production facility). Unexplained "Gulf War Syndrome" with low-dose exposure to CWAs was suggested as a possible cause. Extensive research failed to find any single case of the problem. However, some health effects, including alterations to the immune system 3 months after the exposure to low concentrations of sarin, were demonstrated (Kassa et al., 2001, 2003). In the desert, during the autumn and winter of 1990–1991, the threat of chemical warfare became very real to allied military personnel. It was demonstrated by the UN Commission that major Iraqi agents were mustard, tabun, sarin, and cyclosarin. Mustard agent was relatively pure, but nerve agents were a complex mixture of the agent and degradation products. During the period from June 1992 to June 1994, the Commission's Chemical Destruction Group destroyed 30 tons of tabun, 70 tons of sarin, and 600 tons of mustard, which were stored in bulk and in munitions.

Suddenly, it became clear to the whole world that there were countries with CWs and biological weapons, and there were other countries that might obtain or produce them.

SYRIA

The conflict in Syria has been the last conflict in which the use of CWs was confirmed by the UN Mission (UN, 2013). Nerve agent sarin was used in an attack on the Ghouta area of Damascus (August 21, 2013). It is not the intention of this chapter to evaluate political situations; however, it was not possible to decide exactly who used sarin (current government or FSA) against civilian victims. First complex reactions were published in October 2013 in the CBRNe World (Higgins, 2013; Johnson, 2013; Kaszeta, 2013; Winfield, 2013). For the Mission, there were not ideal conditions: difficult political situation, chaotic scene, and timing that was not ideal. However, the report was well-structured and conclusions were clear: sarin was present in some samples and rocket remains, and selected survivors showed symptoms supporting sarin exposure (Johnson, 2013).

There are different data regarding the number of victims, initially varying from hundreds to thousands. The Syrian Observatory for Human Rights reported more than 500 deaths and thousands of patients displaying "neurotoxic symptoms," including civilian people and children. Medicine Sans Frontiers said at least 3600 patients had these symptoms and, of those patients, 355 had died. UN Mission selected 36 from 80 survivors who met the criteria established by the Mission. Symptoms consistent with organophosphate intoxication were observed: decreased consciousness (78%), dyspnea (61%), blurred vision (42%), eye irritation or inflammation (22%), lacrimation (8%), miosis (14%), salivation (22%), vomiting (22%), and convulsion (19%). Johnson (2013) did not mention the postmortem samples or data regarding dead persons. Treatment of victims and the course of poisoning, including laboratory results, have not been specified. However, laboratory examinations would be useful, as in case of Tokyo victims (Polhuis et al., 1997). It would be possible to use other methods

of laboratory diagnoses of nerve agent intoxication, as described previously (Noort et al., 2009; Schans van der, 2009; Bajgar, 2013). Autopsies of victims were not conducted but would have been useful, as would postmortem examinations of dead animals. Regarding CWs of Syria, they will be destroyed under the supervision of Organization for Prohibition of Chemical Weapons (OPCW) (for Syria, CWC entered into force on October 14, 2013) and with international assistance.

UNINTENTIONAL USE OF TOXIC CHEMICALS

There are two main accidents connected with the release of toxic chemicals. In July 1976, in Seveso, Italy, more than 40,000 people were exposed to dioxin, a persistent and highly toxic chemical. The first signs were skin lesions appearing on children, and after some months there was evidence of chloracne. Health consequences have been observed from that time to the present. The Seveso accident was possibly the most systematically studied dioxin contamination incident. A similar contamination of one building of the Spolana company in Neratovice (a town in the former Czechoslovakia) was also observed (Bajgar et al., 2007a; Pelclová et al., 2011). Another example, the Bhopal accident, is probably the greatest industrial disaster in history. In 1984, on December 2 and 3, water inadvertently entered the methylisocyanate storage tank (containing approximately 40 tons of this chemical). As a result, methylisocyanate was released into the surrounding area. There was no warning. Many people who inhaled high concentrations of toxic gas asphyxiated because of extensive lung damage. Approximately 150,000 people were intoxicated (50,000 seriously poisoned) and more than 2500 people died (Bajgar, 2006).

TERRORIST USE OF CWS

Terrorists have expressed an interest in nerve agents and have deployed them in attacks on unprotected civilians (Rotenberg and Newmark, 2003). A Japanese religious cult, Aum Shinrikyo, independently manufactured numerous chemical and biological agents. The first such attack with sarin occurred in Matsumoto in 1994 and in the Tokyo subway in 1995. Thousands of people were affected and dozens of people died (Ohtomi et al., 1996; Nagao et al., 1997; Okomura et al., 1998; Yokoyama et al., 1998). In Matsumoto (1994), 600 people were poisoned and hospitalized and seven died (Morita et al., 1995; Nakajima et al., 1997; Yoshida, 1994). The attack in the Tokyo subway (1995) resulted in 5500 people seeking hospital evaluation and 12 deaths (Bajgar, 2006). An interesting terroristic act was described by Tsuchihashi et al. (2005)—a fatal intoxication with VX administered percutaneously.

Nerve agents belong to the group of OPs. These compounds in the form of pesticides are commercially available and are used in agriculture, which can lead to professional, suicidal, or accidental intoxication. The mechanism of action, diagnosis, and treatment of intoxication with OP pesticides and nerve agents are very hot topics at present. Moreover, some principles of the effects, diagnosis, and therapy are very similar for OP and highly toxic nerve agents; therefore, the principle of action and effective treatment can be applied in general for the civilian sector, too.

The use of these chemicals was observed in Moscow in 2002. The Moscow theater hostage crisis was the seizure of a crowded theater on October 23, 2002 by approximately 40 armed Chechen militants who claimed allegiance to the separatist movement in Chechnya. They took 850 hostages and demanded the withdrawal of Russians from Chechnya and an end to the Chechnya war. The leader of the terrorists was 22-year-old Movsar Baraev. After 2.5 days of waiting, Russian forces used an unknown gas pumped into the ventilation system. Officially, 39 terrorists and at least 129 of the hostages (nine of them foreigners) were killed. Some estimates have put the civilian death toll at more than 200. It was thought that the security services used an aerosol of a CWA, first assessed as BZ, but later it was specified that an aerosol anesthetic of the Fentanyl type was used (Bajgar and Fusek, 2006).

In the hospitals, the survivors were cut off from any communications with the outside and their relatives were not allowed to visit them. An incorrect list of hospitals for victims was released. The main problem was the lack of information about those dealing with the identification and characterization of the chemical used and the unavailability of known antidotes (e.g., naloxon) by medical staff treating the victims (Bajgar et al., 2007a). It appeared from this event that there were compounds not explicitly enumerated in the CWC and therefore not controlled by this Convention. Fentanyl can be considered as a nonlethal weapon (a group of so-called calmatives) and these chemicals can also be used to incapacitate animals; of course, its use against humans is not excluded (Bajgar, 2006; Hess et al., 2005).

NEGOTIATIONS

Although the Cold War was continuing, the political situation led to increased activities in international negotiations. At the Conference on Disarmament in Geneva, some attempts to negotiate a ban of CWs were begun, first as the ad hoc Working Group, and later as the ad hoc

Committee on Chemical Weapons with the mandate to negotiate the text of a convention banning CWs.

The discussions in Geneva were more intensive from 1987 and, in 1992, the elaboration of the so-called rolling text of future CWCs was finished. During these negotiations, the text of future Conventions ("rolling text") was enlarged: the final report (CD/342) of February 2, 1983 contained 23 pages; the same report of August 23, 1985 (CD/636) had 46 pages; and CD/952 of August 18, 1989 contained 134 pages. Simultaneously with the Geneva negotiations, in September 1989, the Memorandum of Understanding between the Governments of the United States and the USSR regarding a bilateral verification experiment and data exchange related to prohibition of CWs, otherwise known as the Wyoming Meeting, started negotiations between two main possessors of CWs. These countries also contributed to the negotiations in Geneva: they demonstrated their CWs to the Conference on Disarmament in the United States in November 1986 (Tooele) and in the USSR in October 1987 (Shikhany). The final document of the Convention is approximately 200 printed pages. The Convention was then agreed on in New York at the UN General Assembly and signed in Paris in 1993. The CWC (Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on their Destruction) entered into force on April 29, 1997, 180 days after the deposit of the 65th instrument of ratification of the Convention by Hungary. At this time, 87 countries ratified the CWC and became original States Parties to the Convention. Simultaneously, the OPCW in The Hague started its work of supervising the destruction of CW stocks and monitoring the world's chemical industry to prevent future misuse. There are many activities of the OPCW, for example, training of the inspectors for control of destruction of CWs including their medical protection, research, and supported activities, solving problems due to practical implementation of the CWC, control of chemical and military facilities, and other activities. Russia and the United States were unlikely to meet the final stockpile destruction deadline of April 29, 2012. By the middle of 2008, 183 signing States and 194 recognizing States had adhered to the Convention (Davey, 2008). However, there are still States that are nonsignatories to the Convention. CWs have a long and ancient history. A lack of CW use in WWII suggested that "gas warfare" had ended. However, further development and the utility of chemicals in Vietnam and in terrorist attacks have maintained a military interest in CWs.

Current information of OPCW provides the status of the destruction of CWs. April 29, 2012 was suggested to be the prolonged period for CW destruction. Seven State Parties declared they possessed CWs (Albania, India, Iraq, South Korea, Libya, Russia, and the United States). The stocks of Albania, India, and South Korea were destroyed. Until this date, 73.72% of all declared CWs (the sum of 71,195.086 tons) were destroyed (Streda, 2013). On the basis of the Conference of the State Parties (16th Session, December 2011), the destruction period was prolonged for Russia (2015), Libya (2016), and the United States (2023). Simultaneously, CW-producing facilities were also destroyed or dismantled—13 State Parties declared 70 of these facilities (Bosnya and Hercegovina, China, France, India, Iraq, Iran, Japan, South Korea, Libya, Russia, Serbia, Great Britain, and the United States) and 43 of these objects were destroyed and 21 were dismantled for peaceful purposes.

It is clear that the use (incidental or otherwise) of toxic chemicals has impacts on different spheres of human existence, such as state structures and infrastructure, economics, psychic and public behavior, and the environment. Toxic chemicals are a great consumer of natural sources, both renewable and nonrenewable. They also consume raw materials and energy and, as a consequence, cause pollution of the environment and lead to deficiency of raw materials throughout the world and therefore an unequal distribution of the world's natural sources. The impact on the psychology of humankind is also important, following either chemical wars (both global and local) or use of these chemicals by terrorists. The development of new technologies is equally important because they influence, positively and negatively, further human development. Research in this direction not only can contribute to "improvement" of chemicals to obtain more effective CWAs but also can improve our knowledge of basic sciences (toxicology, neuropharmacology, etc.) and allow us to better understand physiological functions in general. It is appropriate to recall the history of cholinesterases and their inhibitors. The existence of cholinesterases was predicted by H.H. Dale in 1914, 14 years before acetylcholine was demonstrated as a natural constituent of animal tissues. This research approach was changed during WWII and cholinesterases acquired a special significance in the context of chemical warfare and nerve agents (Silver, 1974). Another publication in this area (Koelle, 1963) can be considered as the first to deal with anticholinesterase agents including CWAs—nerve agents. One can only hope that in the future the only physiological and pharmacological research will be performed in a nonmilitary framework, but that may not be the case.

CONCLUDING REMARKS AND FUTURE DIRECTION

The threat of the use (either military or terroristic) of CWAs (and other toxic chemicals) still exists. The military use of these agents is limited, but their terroristic use is unlimited. The spectrum of these agents is very large and the ability to be prepared against the use of toxic chemicals is necessary.

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CHAPTER

4

The Tokyo Subway Sarin Attack: Acute and Delayed Health Effects in Survivors

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INTRODUCTION

The Tokyo subway sarin attack occurred in 1995. Prior to the disaster in Tokyo, Matsumoto sarin attack happened on June 27 in 1994 in Matsumoto city, Nagano Prefecture at the center of Japan main land. Sarin was dispersed into the open air using an electric heater fan to direct it to the target apartment. Eventually, eight people died and 660 were injured. In addition to these injured patients, one woman exposed to sarin died after 14 years hospitalization. This was the first terrorist attack using sarin on the general public in the world, an incident which served as a wake-up call for anti–nuclear, biological, and chemical (NBC) terrorism policy throughout the world. In the 10 years since the attack, efforts to combat NBC terrorism have focused on rapid and effective measures to respond to attacks employing nerve agents such as sarin.

SARIN TOXICITY AND MECHANISM OF ONSET

Sarin is an organophosphate compound. Within the context of chemical weapons, organophosphates are collectively referred to as "nerve agents," of which sarin, tabun, soman, and *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX) are examples. Organophosphates inhibit the enzyme acetylcholinesterase (AChE), which degrades acetylcholine (ACh), a neurotransmitter substance that acts locally on nerve synapses. Once organophosphates bind to the phosphorylate AChE to inhibit its activity, ACh accumulates at nerve terminals, resulting in enhanced ACh activity at receptor sites. ACh effects can be functionally classified based on their site of action and can have muscarinic, nicotinic, and central nervous system (CNS) effects. These effects cause the major symptoms associated with an acute organophosphate toxicity. Muscarinic effects increase parasympathetic nerve activity and cause miosis, visual disturbances (accommodation disorder), increased salivary and bronchial secretions, bronchospasm, bradycardia, and increased gastrointestinal peristaltic activity (e.g., abdominal pain, nausea, vomiting, and diarrhea). Nicotinic effects, due to hyperstimulation of neuromuscular junctions, cause fasciculations, muscle weakness, and respiratory paralysis, and increased sympathetic nerve activity leads to miosis, sweating, tachycardia, and hypertension. CNS effects due to ACh, when severe, include anxiety, headaches, excitement, ataxia, somnolence, disorientation, coma, and seizures.

Well-known symptoms of sarin toxicity include miosis, hypersecretions, bradycardia, and fasciculations. However, the mechanism of organophosphate toxicity seems to involve conflicting actions. For example, mydriasis or miosis, and bradycardia or tachycardia may occur. Acute respiratory insufficiency is the most important cause of immediate death. Early symptoms include (i) tachypnea due to increased airway secretions and bronchospasm (a muscarinic effect), (ii) peripheral respiratory muscle paralysis (a nicotinic effect), and (iii) inhibition of respiratory centers (a CNS effect), all of which lead to severe respiratory deficiency. If left untreated at this stage, death will result. Cardiovascular symptoms may include hypertension or hypotension. Various arrhythmias can also occur, and caution is required when the QT interval is prolonged. In particular, if hypoxemia is present, fatal arrhythmias may occur with intravenous administration of atropine

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sulfate, which means that this drug should be given intramuscularly to victims of sarin poisoning. Common gastrointestinal symptoms of this poisoning include nausea, vomiting, and diarrhea.

An intermediate syndrome lasting 1–4 days after sarin exposure appears to exist (De Bleecker, 1992). This is due to prolonged AChE inhibition, and it is associated with acute respiratory muscle paralysis, motor nerve paralysis, and cervical flexor and proximal muscle paralysis. Recumbent patients who have difficulty raising the head and neck require particular care. However, the intermediate syndrome has not been reported with nerve agent toxicity in animals or humans (Sidell, 1997), although this syndrome is well documented in humans following large exposure to organophosphate and carbamate pesticides (Gupta, 2005; Paul and Mannathukkaran, 2005; Gupta and Milatovic, 2012). The cause of the intermediate syndrome may be toxicity due to massive organophosphate exposure or inadequate treatment of such exposure (intestinal decontamination, antidote administration, and respiratory management). In organophosphate-induced delayed neuropathy (OPIDN), seen 2–3 weeks after exposure and characterized by distal muscle weakness without fasciculation, the pathophysiology is not well understood. OPIDN was first reported in the 1930s due to contamination of Jamaican ginger (nicknamed *Jake*) by organophosphates. This incident (so-called ginger jake paralysis) caused lower limb paralysis in about 20,000 victims. OPIDN symptoms have also recently been reported in Matsumoto and Tokyo subway sarin victims (Sekijima et al., 1997; Himuro et al., 1998). Inhibition and aging of neuropathy target esterase plays a role in OPIDN, but despite several basic research studies, the detailed pathophysiology has not yet been established, making OPIDN difficult to treat.

OVERVIEW OF THE TOKYO SUBWAY SARIN ATTACK

The attack took place during the morning rush hour, at about 8 a.m. on March 20, 1995, the day before the Spring Equinox holiday. The attack was carried out by members of a cult known as Aum Shinrikyo to distract police from carrying out a raid on the group's headquarters. The terrorist target was government buildings in Kasumigaseki in the heart of Tokyo. Most offices in Kasumigaseki open for business at 9:30 a.m., but the early-morning rush hour was unusually heavy because it was a Monday. Some believe that the time of 8 a.m. was chosen because some cult members had inside information about the government offices. Police suspected, based on an undercover investigation that they were conducting, that Aum Shinrikyo was manufacturing sarin for use in a terror attack, but few people, even within the police department, were aware of this fact. The police did not have personal protective equipment (PPE), which meant that they had to borrow PPE and receive training on use of the equipment from the Self-Defense Forces. Members of the Self-Defense Forces were alerted to some of Aum Shinrikyo's planned activities, but the general public, including healthcare providers and fire department personnel, knew nothing of these activities (Figure 4.1).

According to a subsequent police report, the terrorists placed sarin in five subway trains in the following way.

FIGURE 4.1 Scene from a sarin attack at Tsukiji station.

Approximately 600 g of sarin at a concentration of 33% was mixed with hexane and *N*, *N*-diethylaniline and placed in a nylon/polyethylene bag. Five terrorists then wrapped the bags in newspaper, punctured the bags with the tips of their umbrellas, and left the bags on the trains. In this way, the sarin seeped out of the bags and vaporized, but no other active means of dispersal were used. In this sense, as well as the relatively low number of deaths, the Tokyo subway sarin attack was not considered a full-scale attack.

Of the bags of sarin used in the attack, two bags were not punctured. These bags were returned to the police laboratory for analysis. At Kasumigaseki, one of the subway stations on the Chiyoda subway line, two station employees collapsed and died on the platform after they cleaned and removed one of the bags that didn't get punctured, even though they were wearing gloves. The number of victims of this attack varies depending on the source, but all known information confirms that 12 people died in the attack, and it is generally believed that at least 5,500 victims suffered mild to serious injuries. Firefighting agencies estimate 5,642 victims, and the police, 3,796 victims, while official figures released by the subway company put the total number of victims at 5,654. This includes the 12 who died (10 passengers, 2 employees), those hospitalized (960 passengers, 39 employees), and those treated for minor injuries (4,446 passengers, 197 employees).

Thus, the way in which we use the lessons learned from this attack will affect our ability to deal adequately with future terrorist attacks using sarin, which could be even greater and more serious with respect to the number of victims. Can we really assume that only 12 of the approximately 5,500 victims died because the Japanese medical system was particularly well prepared for such an eventuality? Probably not. It is more likely that the relatively small number of fatalities was due to the low concentration of sarin and the passive means of dispersing it. From this perspective, the Matsumoto sarin attack one year earlier was more aggressive than the Tokyo subway sarin attack. In a trial after the Matsumoto incident, it was revealed that a 70% concentration of sarin was actively volatilized using an electric heater and dispersed using an electric fan. A total of 7 victims died and 660 were injured and one victim died 14 years after sarin exposure. In other words, if the Tokyo subway sarin attack had been conducted using the same means as those employed in the Matsumoto sarin attack, the number of fatalities may have been 50 or 60. So humanity has not yet experienced the effects of a full-scale sarin attack in a major city.

Even if it did not rise to the level of a major attack, this incident was the first chemical terrorist attack in a large city. There were few first-responders who could even have conceived of such an attack, let alone be prepared to rapidly evacuate victims from the subway stations. Many passengers who had difficulty walking rushed out of the trains and onto the subway platform and fell down, which would have increased their exposure to the sarin permeating the stations. In addition, the site to which many of the victims were finally evacuated at ground level, where they could lie down, was close to an air exhaust vent from the subway below, so the exposure continued.

The first call for an ambulance came 9 minutes after the 8 a.m. attack, with the first report of a "victim with seizures at Kayabacho Station." By 8:15 a.m., the reports of victims started to increase. Around this time, the fire department received a report from Tsukiji Station stating that "an explosion occurred and several people were injured." Calls for ambulances eventually came from 19 subway stations, and after 8:30 a.m., victims began to pour into local clinics and hospitals. According to the Tokyo Fire Department, 5,493 people were treated at 267 medical institutions in Tokyo, and 17 people were treated at 11 medical institutions outside Tokyo. Among the victims, 53 were seriously injured (Ieki, 1997). Another source states that a total of 6,185 people were treated at 294 medical institutions (Chigusa, 1995). The discrepancy in the number of victims reported by different agencies attests to some of the confusion at the time. St. Luke's Hospital received the largest number of victims (640 on the day of the attack), probably because of its close proximity to the Hibiya line, where a large number of victims were located, and because of a report on television stating that "St. Luke's Hospital has the antidote for treatment."

EMERGENCY TREATMENT OF SARIN TOXICITY

The standard treatment for sarin toxicity includes (i) maintaining the airway, (ii) assisting breathing, and (iii) supporting circulation. In victims of the Tokyo subway sarin attack, endotracheal intubation was performed frequently. However, in the Matsumoto sarin attack, endotracheal intubation was more difficult to do in many victims because of airway hypersecretion and bronchospasm. This difference in symptoms is attributable to the 70% concentration and the active means by which the sarin was dispersed at Matsumoto, as opposed to the much lower 33% concentration and passive means of dispersal employed in Tokyo. Dr. Frederick Sidell, an expert on chemical terrorism in the United States, advocated decontamination, drugs, airway, breathing, and circulation (DDABC) as the basic treatment for nerve agent poisoning. Even if the advised emergency treatment is followed, initial efforts to achieve adequate ventilation may be in vain. Efforts to achieve adequate ventilation should be made after at least initial administration of atropine to control the buildup of airway secretions and bronchoconstriction (Sidell, 1997). If healthcare

professionals learn from the Matsumoto attack, they can better recognize early parasympathetic nervous symptoms, including miosis, hypersecretion, and rhinorrhea, as common indications of chemical terrorism due to nerve agents, and therefore be able to institute appropriate treatment with antidotes in time. In large-scale disasters with many victims, treatment is often deferred in those with cardiopulmonary arrest (CPA; so-called black tag). However, at St. Luke's Hospital, one in three persons with CPA and two patients with respiratory arrest made a full recovery and were discharged. This high rate of recovery and return to the community is unlike that seen in other types of disasters. Therefore, if medical resources are available, all victims of a sarin attack should be aggressively treated, including cardiopulmonary resuscitation (CPR) when necessary.

The global standard for the treatment of sarin toxicity is the administration of (i) atropine, (ii) an oxime agent like 2-PAM, and (iii) diazepam (Medical Letter, 2002).

Recommended doses of atropine are 2 mg in patients with mild symptoms that are primarily ocular, but without respiratory symptoms or seizures; 4 mg in patients with moderate symptoms, including respiratory symptoms such as dyspnea; and 6 mg in patients with severe symptoms, including seizures and respiratory arrest. The standard administration route should be intramuscular. As mentioned previously, intravenous administration of atropine in the treatment of severe symptoms such as hypoxemia can induce ventricular fibrillation; thus, intramuscular administration is advised. Oxime agents such as 2-pralidoxime methiodide (2-PAM), or 2-formyl-1-methylpyridinium iodide oxime should also be given. The recommended dose for 2-PAM in moderate and severe cases of inhalation, or for liquid exposure to a nerve agent, is 1 g by intravenous infusion over 20–30 min. Further continuous administration of 500 mg/h may also be required in severe cases. Since the rate of aging of the nerve agent–enzyme bond is correlated with time until 2-PAM is administered, if the aging half-life of sarin is 5 h, then 2-PAM must be administered before this time. The oxime of choice for sarin and VX is 2-PAM, but asoxime chloride (HI-6) should be used for soman and obidoxime for tabun. Seizures are treated with diazepam. This three-drug combination (atropine, 2-PAM, and diazepam) is the global recommendation for sarin toxicity, and autoinjectors are available in several countries (Vale et al., 2006) (Figure 4.2).

After the Tokyo subway sarin attack, St. Luke's Hospital, which treated 640 victims, used about 700 ampules of 2-PAM and 2,800 ampules of atropine (Okumura et al., 1998). This calculates out to 550 mg of 2-PAM and 2.2 mg of atropine per victim. The route of administration was intravenous in all cases, with a total dose of 1.5–9 mg of atropine in severe cases (Okumura et al., 1996); this range of dose reflects the low concentration and passive means of sarin dispersal used in the Tokyo attack.

However, in Tokyo, no one was saved by administration of 2-PAM; conversely, no one died because they did not receive it. In other words, if the victims' survival was the ultimate goal, there was no clinical evidence that 2-PAM was effective. The only reported finding was a more rapid return of plasma pseudocholinesterase levels to normal in patients who received 2-PAM, as compared to those who did not. But in terms of long-term prognosis, this does not rule out the effectiveness of oxime

FIGURE 4.2 Sarin victims at St. Luke's International Hospital.

therapy. Ideally, detailed studies are needed to evaluate the efficacy of 2-PAM, including for long-term prognoses. To date, however, there has been no sophisticated study of the Tokyo subway sarin attack in this vein.

One piece of evidence supporting the efficacy of 2-PAM to treat sarin toxicity has been the clinical benefit associated with it when treating toxicity due to organophosphorus pesticides. However, some experts now doubt whether such a benefit really exists. For example, Peter et al. (2006), using meta-analytic techniques, reevaluated the effects of oxime therapy in organophosphate poisoning. Not only did they find no beneficial effects, they reported possible adverse effects. The Cochrane Reviews for clinical evidence-based medicine (Buckley et al., 2005) reported no risk/benefit evidence supporting the use of oxime agents in organophosphate poisoning, but they did conclude that further detailed investigations are necessary.

According to reports about Iranian physicians who treated sarin toxicity during the Iran–Iraq war (Newmark, 2004), 2-PAM was not available on the front lines, and atropine alone was used for treatment. The doses of atropine used were considerably higher than those used in the Tokyo subway sarin attack, or that are generally recommended in the United States (Medical Letter, 2002). The Iranian protocol called for initial administration of 4 mg intravenously. If no atropine effects (improvement in dyspnea or decrease in airway secretions) were seen after 1–2 min, 5 mg was then administered intravenously over the next 5 min while the heart rate was monitored. A rise in heart rate of 20–30 beats per minute was diagnosed as an atropine effect. In severe cases, 20–200 mg was given. Regardless of the dose, the key to saving lives, according to this protocol, was how soon the atropine was administered.

Thus, treatment without the use of an oxime agent is possible. Of course, in countries where this is economically possible, treatment should use the combination of atropine, an oxime agent like 2-PAM, and diazepam. In addition, the use of autoinjectors for administration is also helpful. Unfortunately, terrorist attacks using sarin are also carried out in less economically developed countries; and even if the drugs are available, performance relative to cost needs to be considered. In this sense, preference should be given to the availability of atropine and diazepam. In other words, unless it is economically feasible, funds should be used to obtain atropine and diazepam rather than oxime agents, whose cost–benefit ratio is still inconclusive.

ACUTE AND CHRONIC SYMPTOMS OF SARIN TOXICITY

Based on data from 627 victims treated at St. Luke's Hospital (Okumura et al., 1998), the symptoms, listed in order of prevalence, were miosis (pupillary constriction; 90.5%), headache (50.4%), visual impairment (37.6%), eye pain (37.5%), dyspnea (29.2%), nausea (26.8%), cough (18.8%), throat pain (18.3%), and blurred vision (17.9%). Cases were categorized as severe if they involved seizures or respiratory arrest requiring mechanical ventilation, moderate for respiratory distress or fasciculations, and mild for eye symptoms only. Of 640 cases reported by St. Luke's Hospital, the degree of intoxication was severe in 5 victims, moderate in 107, and mild in 528, with nicotinic effects observed in those with moderate or severe symptoms.

In the Tokyo subway sarin attack, decontamination was not performed on site, and first-responders and healthcare workers initially did not wear PPE. As a result, of 1,364 firefighting personnel, 9.9% became secondary victims. At that time, TV, and newspapers reported on the sarin attack every day, and many people in Japan were scared of the sarin incident surrounding them. At St. Luke's Hospital, 23% of the hospital staff became secondary victims (Okumura et al., 1998). The percentage of secondary victims by hospital occupation was as follows: nursing assistants (39.3%), nurses (26.5%), volunteers (25.5%), doctors (21.8%), and clerks (18.2%). Thus, increased contact with a primary victim increased the risk of becoming a secondary victim. The percentage of secondary victims by hospital location were the chapel (45.8%), the intensive care unit (38.7%), the outpatient department (32.4%), the general ward (17.7%), and the emergency department (16.7%). The high rate of secondary victims in the chapel was attributed to poor ventilation and the large number of victims sheltered there. Because it was during the winter, victims entered the chapel fully clothed. When they removed their coats, and every time they moved thereafter, some of the sarin trapped inside the clothing probably escaped, causing secondary exposure. Fortunately, none of the secondary victims died. However, if a higher concentration of sarin and more effective means of dispersion had been employed in the Tokyo attack, as had been done in the Matsumoto incident, then it is likely that some of the secondary victims would have died.

Within the context of risk communication, the so-called worried-well, who were concerned about having been exposed to the nerve agent, and those complaining of symptoms even though actual exposure was unlikely, also flocked to hospitals seeking treatment (Bloch et al., 2007). As previously mentioned, among patients treated at St. Luke's Hospital on the day of the attack, 90.5% had miosis, an objective finding due to sarin exposure, but the remaining 9.5% were considered to be worried-well patients.

The reason or reasons for the small number of worried-well patients in the Tokyo subway sarin attack are unclear. Given the extensive coverage by the news media, who

mentioned that victims were crowding into St. Luke's Hospital, persons without definitive symptoms, or those who were unsure whether they had been exposed but who did not want to add to the confusion, likely avoided going to that hospital, which created a kind of natural selection process. Another contributing factor may have been that the target of the attack was the government buildings in Kasumigaseki in the heart of Tokyo, which meant that many of the victims were probably well educated. Conversely, unfamiliarity with sarin and toxic gases in general may also have contributed to the low number of such patients. In either case, these observations should be reviewed from the perspective of risk communication.

Only one victim from the Matsumoto and Tokyo subway sarin attacks has still not regained consciousness; that person remains in a vegetative state due to anoxic brain damage (Yanagisawa et al., 2006). Sarin victims treated at St. Luke's Hospital were regularly followed for the development of chronic symptoms. One year after the incident, a survey was conducted, and 303 of 660 victims responded (Ishimatsu et al., 1996). A total of 45% of the respondents reported that they still experienced symptoms. In terms of physical symptoms, 18.5% of the victims still complained of eye problems, 11.9% of easy fatigability, and 8.6% of headaches. Regarding psychological symptoms, 12.9% complained of fear of subways, and 11.6% still had fears related to escaping the attack. In another survey conducted after 3 years, 88% of the respondents reported several aftereffects (Okumura et al., 1999). Unfortunately, these surveys may lack objectivity. For example, the response rate may have been higher among victims still complaining of symptoms.

Murata et al. (1997) performed a controlled comparison study in victims 6 to 8 months after the attack, with evaluations of event-related and visual-evoked potentials (VEP), brainstem auditory evoked potentials, electrocardiographic R–R interval variability (CVRR), and scores on a posttraumatic stress disorder (PTSD) checklist. In the sarin victims, P300 and VEP (P100) latencies were significantly prolonged, and CVRR was abnormal, indicating depression of cardiac parasympathetic nervous activity. The findings suggested persistent effects of sarin in the higher and visual nervous systems. In another study, Yokoyama et al. (1998a) reported a delayed effect on the vestibulo-cerebellar system induced by acute sarin poisoning. Yokoyama et al. (1998b) also reported a chronic effect on psychomotor performance. In addition, Miyaki et al. (2005) described the chronic effects associated with psychomotor and memory function up to 7 years after exposure. As mentioned previously, two victims with OPIDN were reported (Sekijima et al., 1997; Himuro et al., 1998).

As part of a series of scientific studies sponsored by the Japanese Ministry of Health, Labor, and Welfare, Matsui et al. (2002) conducted two studies 7 years after the sarin attack. The first was a case control study comparing victims treated at St. Luke's Hospital with a non-sarin-exposed patient group. Statistical analysis showed significantly higher rates of chest pain, eye fatigue, presbyopia, eye discharge, nightmares, fear, anxiety, difficulty in concentrating, and forgetfulness in the victim group. Moreover, in the victim group, there were even significantly higher rates of visual blurring, myopia, problems with focal convergence, abnormal eye sensations, flashbacks, fear of returning to the attack site, and not wanting to watch news about the attacks. The rate of PTSD, as evaluated by several diagnostic criteria, was also higher in the victim group. The second research consisted of a cohort study comparing a group who required medical intervention after the attack with a group who did not. For lethargy, diarrhea, myopia, presbyopia, problems with focal convergence, eye discharge, and apathy, there were no significant differences between the groups; however, for other evaluated parameters, scores were significantly higher in the nonintervention group. Comparison of PTSD incidence, based on whether intervention was received or not, showed that the nonintervention group had a significantly higher rate of masked PTSD. There was a higher incidence of eye symptoms in the victim group than in the nonvictim group, but there was no difference between the intervention and nonintervention groups. Thus, eye symptoms are probably long-term physical aftereffects of sarin exposure. In some Matsumoto cases, persistent electroencephalogram changes without seizure activity have been reported up to 5 years later (Yanagisawa et al., 2006).

The results of these studies suggest some long-term effects of sarin toxicity and careful follow-up and observation are indicated in these victims.

LONG-LASTING INDEFINITE COMPLAINTS OF SARIN-EXPOSED VICTIMS

In 1996, the supporting group for sarin-attack victims was formed. The group members, who consist of medical doctors and volunteers, are committed to treating victims. In 2001, the group was reconstructed into the nonprofit Recovery Support Center (RSC). The RSC has followed up the long-lasting complaints of these victims up to 2012, looking at the aftereffects and indefinite complaints of the sarin victims. The psychiatric term "indefinite complaints" means one patient complains and many indefinite patients also complain without particular reasons.

As shown in Table 4.1, the AChE activity of the long-lasting victims has returned to normal levels. However, they continue to suffer from sustained "indefinite complaints" even 19 years after the disaster.

TABLE 4.1 The Sequelae of Indefinite Complaints of Sarin Victims

Complaints of the Sarin Victims	2008 (137) (%)	2010 (121) (%)	2012 (113) (%)
Sluggish	67.1	43.8	34.5
Fatigue	78.8	48.8	33.6
Easy to catch cold	30.6	22.3	12.4
Low grade fever	14.5	7.4	10.6
Dyspnea	20.4	16.5	21.2
Constricting chest pain	22.6	16.5	22.1
Palpitation	27.7	22.3	17.7
Nausea	8.0	9.1	0.7
Diarrhea	26.2	14.9	16.8
Abdominal pain	17.5	14.1	11.5
Appetite loss	5.8	10.7	0.4
Dizziness	45.9	29.8	23.9
Headache	61.3	33.1	24.8
Eye strain	83.9	66.9	46.0
Dim	76.6	60.3	43.4
Hard to see the distance	68.6	44.6	30.0
Hard to see neighborhood	55.4	45.4	32.7
Hard to match a focus out of focus	62.7	48.8	32.7
Eye mucus, discharge from the eye	29.1	35.5	21.2
Irritated eyes grittiness	35.7	23.1	16.8
Other symptoms of eyes	27.7	21.5	0.9
Sleeplessness	35.7	19.8	16.8
Nightmare	27.0	18.2	13.3
Recall the accident remember the case	24.0	19.8	16.8
Cannot approach the spot	24.0	17.4	15.0
Terror	29.4	11.6 fear	15.0
Irritate	40.1	28.1	17.7
Poor concentration	45.9	33.1	13.0
Avoid the accident	36.4	24.8	20.3
Acathexis	31.3	19.8 impassive	15.0
Forgetful	62.7	42.9	31.0
Depression	43.7	43.1	26.5
Be strained (frozen shoulder, sweat, etc.)	62.7	38.1	31.9
Numbness	49.6	26.4	32.7

Numbers in parenthesis indicate sarin victims who received medical examinations. (%) means the percent of victims who suffered from the complaints. Sarin victims who could not attend to consult medical examinations with similar numbers or more were taken separately by inquiries of questionnaire survey and their sequelae answered had similar tendency with respect of indefinite complaints.

Table 4.1 also shows a higher incidence of eye symptoms, including eye fatigue, eye discharge, chest pain, nightmare, fear, anxiety, and so on. Additionally, blurred vision, problems with focal convergence, abnormal eye sensations have been raised with higher rates compared to the non-exposed group.

In the medical examinations carried out on 2008, over 50% of the responding victims claimed a high incidence of symptoms such as sluggishness, fatigue, headache, eye strain, and difficulties seeing distant objects. These results suggest that victims still suffer from these indefinite complaints in their daily lives. Fortunately, comparing the data from 2010 and 2012 shows that these indefinite complaints tend to be going down each year. However, many sarin victims still live with discomfort. Thus, it is necessary to find therapeutic treatment methods to improve such long-lasting and intractable medical conditions.

LABORATORY FINDINGS IN SARIN TOXICITY

According to inpatient records from St. Luke's Hospital, the most common laboratory finding related to sarin toxicity was a decrease in plasma cholinesterase (ChE) levels in 74% of patients. In patients with more severe toxicity, plasma ChE levels tended to be lower, but a more accurate indication of ChE inhibition is the measurement of erythrocyte ChE, as erythrocyte acetylcholinesterase (AChE) is considered "true ChE" and plasma ChE is "pseudo-ChE." However, erythrocyte ChE is not routinely measured, whereas plasma ChE is included in many clinical chemistry panels; thus, it can be used as a simple index for ChE activity. In both the Matsumoto and Tokyo subway sarin attacks, plasma ChE served as a useful index of sarin exposure. In 92% of hospitalized patients, plasma ChE levels returned to normal on the following day. In addition, inpatient records from St. Luke's Hospital showed an elevated creatine phosphokinase and leukocytosis in 11% and 60% of patients, respectively. In severe cases such as the Matsumoto attack, hyperglycemia, ketonuria, and low serum triglycerides due to the toxic effects of sarin on the adrenal medulla were observed (Yanagisawa et al., 2006).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This chapter has discussed sarin toxicity based on experiences of the attacks in Matsumoto and the Tokyo subway, as well as the Iran–Iraq war. This section provides some conclusions drawn from the toxicological issues related to sarin.

Given the low concentration and poor means of dispersal, the Tokyo subway sarin attack can be referred to as a "passive" attack. The implication of such an assumption, therefore, is that no one has yet witnessed a full-scale sarin attack in any major city. While valuable information can be gained from the Tokyo subway sarin attack, the experience obtained from the more aggressive Matsumoto sarin attack and the Iran–Iraq war should also be considered when developing initiatives directed at dealing with a potential full-scale attack in the future, in which the effects would be more serious.

Importantly, reliable epidemiologic data is lacking regarding the long-term effects of sarin toxicity, the question of whether low exposure to sarin has any long-term effects, and specific effects on children, pregnant women, and fetuses (Sharp, 2006). The sporadic and limited epidemiological surveys undertaken to date suggest that some long-term effects are present. The RSC has clearly shown that sarin victims still suffer from persistent aftereffects of indefinite complaints, even 19 years later. Thus, well-designed international epidemiologic studies should be conducted in victims exposed to sarin in Japan, Iran, and during the Persian Gulf War.

Several issues regarding treatment need to be resolved. Before the Tokyo subway sarin attack in 1995, the treatment of chemical weapons victims was exclusively regarded as a military issue. However, since then, the deliberate release of nerve agents against the general public has become a serious public safety issue. Treatment of chemical weapon injuries in a military setting assumes that one is dealing with healthy males who have received basic and ongoing training, and who are wearing PPE. An attack on the general public, however, involves a heterogeneous population from many different backgrounds, and the victims are likely to include women (including those who are pregnant), children, and persons who are elderly, sick, and disabled. Furthermore, the public is defenseless against chemical weapons because of their lack of knowledge of dangerous chemical substances and their lack of experience with wearing PPE. Taken together, there is the potential for thousands of victims if nerve agents are deliberately released against ordinary citizens.

Therefore, the medical treatment required for responding to a chemical terrorist attack on the general public will require a different strategy than that employed for such attacks in a military setting. This is because even though numerous lessons can be learned from military experiences, there will be measures that are useful in those settings that may not be applicable to an attack on the general public. An important issue is the means by which appropriate drugs can be safely and reliably supplied to a large number of victims. In addition, it is unrealistic to expect that first-responders wearing PPE will be able to establish intravenous lines in large numbers of victims at the scene of a terrorist attack. Thus, the use of autoinjectors for intramuscular or intraosseous access is more realistic (Ben-Abraham et al., 2003). In this regard, what is needed are not the standardized autoinjectors issued to military personnel, but rather, a variety of autoinjectors that are uncomplicated and easy for victims in normal settings to use. Research on the drugs used to treat victims of chemical terrorism is being conducted in several countries in both military and private situations. However, unlike drugs that are designed for treating diseases, clinical trials cannot be performed in humans due to the fact that it is unethical to subject participants in experiments to poisonous substances. Conducting a randomized control study is also difficult because there is an insufficient number of cases of organophosphate poisoning to establish a reliable sarin toxicity model. A prime example is the oxime agent HI-6. It was developed and in existence for more than 10 years before its widespread use. From the standpoint of international security, collaborative research on drugs for treating chemical terrorism and a global agreement on standard treatment are needed. These are important issues in clinical toxicology that require international cooperation.

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CHAPTER

5

Early and Delayed Effects of Sulfur Mustard in Iranian Veterans After the Iraq–Iran Conflict

Mahdi Balali-Mood

INTRODUCTION

Brief Chemistry

Sulfur mustard (SM), which is bis(2-chloroethyl) sulfide and is also known as mustard gas, was first synthesized in 1822 by Despretz. SM is an oily liquid that is colorless if pure, but it normally ranges from pale yellow to dark brown. Iranian veterans have described it as having a slight garlic or horseradish odor. It has a density of 1.27 g/mL, a melting point of 14.4°C, and a boiling point of 217°C. SM is only 0.05% soluble in water (Kikilo et al., 2008; Pechura and Rall, 1993).

SM is generally regarded as a "persistent" chemical agent because of its low volatility. In cool weather there is little vapor; however, mustard's evaporation increases as the temperature increases. At higher temperatures, such as those in the Middle East during the hot season (38–49°C), mustard vapor becomes a major hazard (Pechura and Rall, 1993; Sidell et al., 1997; Kikilo et al., 2008).

SUMMARIZED HISTORICAL USES

SM has been the most widely used chemical warfare agent (CWA) of the past century. It was first used extensively in World War I (WWI) between 1914 and 1918. Despite the Geneva Protocol in 1925 on the prohibition of CWA, SM was used by Italian troops in Ethiopia (1935–1936) and by Egyptian forces in Yemen (1963–1967). The greatest military use of SM was by the Iraqi Army against the Iranian soldiers and even civilians in Sardasht and Halabjah between 1983 and 1988, resulting in more than 100,000 chemical casualties (Balali-Mood and Hefazi, 2005; Salem et al., 2008).

TYPES AND ROUTES OF EXPOSURE

Based on the situation involved, several types of exposure including single, multiple, secondary, subclinical, and chronic may occur. Most human cases of SM poisoning have occurred during armed conflicts and most accidents involved a single exposure (Pechura and Rall, 1993; Sidell et al., 1997; Balali-Mood and Hefazi, 2005; Kikilo et al., 2008; Salem et al., 2008). Multiple low SM exposures occurred occupationally, during WWI and in the Iran–Iraq conflict (Balali-Mood and Hefazi, 2005; Salem et al., 2008). First aid workers and nursing and medical staff who cared for SM casualties in the field clinics and hospitals during the Iraq–Iran War without proper personal physical protection have become intoxicated. Some of them (between 5% and 25%) are now suffering from the delayed toxic effects of SM and have disabilities (Balali-Mood and Hefazi, 2005).

Low-level SM exposure with or without symptoms but with delayed or long-term health effects has been described in detail (Mandel and Gibson, 1917; Hurst and Smith, 2001; Balali-Mood et al., 2005a,b; Balali-Mood and Hefazi, 2006). Subclinical exposure to SM in some Iranian combatants induced delayed toxic effects (Ghanei et al., 2004a–c). Chronic SM exposure is usually occupational. Some factory workers in Japan and in the United Kingdom were reported to have had SM

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poisoning and even malignancies attributable to SM (Brown, 1949; Nishimoto et al., 1970).

Inhalation is the major route of exposure that induces respiratory and systemic toxicity after absorption across the lung surface (Sidell et al., 1997; Balali-Mood and Hefazi, 2005; Hefazi et al., 2005). However, SM is a vesicant or blistering agent that has direct toxic effects on the skin, producing erythema, blistering, epidermolysis, and necrosis. It is a lipid-soluble compound and thus can be readily absorbed across the skin (Balali-Mood and Hefazi, 2005; Hefazi et al., 2006).

The eyes are the organs most sensitive to SM. This marked susceptibility is attributed to ocular features, including the aqueous–mucous surface of the cornea and conjunctiva as well as the high turnover rate and intense metabolic activity of the corneal epithelial cells (Pickard, 1919; Etezad-Razavi et al., 2006).

SM may also enter the body via oral ingestion. Some Iranian combatants were observed during the Iran–Iraq War who had ingested food contaminated by SM and subsequently became intoxicated. They experienced nausea, vomiting, hematemesis, abdominal pain, and dyspnea. SM may also be absorbed through the lower gastrointestinal (GI) tract (Balali-Mood and Hefazi, 2005). Injection is a very rare route of SM intoxication and has not been reported in humans.

HUMAN TOXICITY

Exposure to very high doses of SM in the field may induce convulsions and death in less than 1 h (WHO, 1970; Balali-Mood et al., 1986; Sidell et al., 1997; Balali-Mood and Hefazi, 2005). Such observations have not been reported during the Iran–Iraq War. Acute toxic effects generally appear after variable periods of latency depending on the dose, mode of exposure, the environmental temperature, and the individual (Anslow and Houk, 1946; WHO, 1970; Balali-Mood et al., 1986; Pechura and Rall, 1993; Balali-Mood and Hefazi, 2005).

Subacute exposure occurred during the Iran–Iraq War and in the workers in SM munitions factories. However, this type of exposure may present as mild acute SM intoxication, as a complication in the respiratory tract, or even as malignancy (Balali-Mood and Navaeian, 1986; Easton et al., 1988).

Delayed toxic effects of SM have been documented. The first report of delayed toxic effects in Iranian veterans was reported in 1986 (Balali-Mood, 1986). Several articles on the delayed toxic effects and complications of SM in Iranian veterans have since been published (WHO, 1970; Balali-Mood, 1986; Ghanei et al., 2004a–c; Balali-Mood et al., 2005a,b; Hefazi et al., 2005; Etezad-Razavi et al., 2006; Hefazi et al., 2006).

The workers who were chemically exposed to mustard agents in British and Japanese munitions factories developed chronic respiratory effects, including chronic bronchitis, bronchiectasis with progressive emphysema, and narrow attenuated bronchioles (Brown, 1949; Nishimoto et al., 1970; Easton et al., 1988).

MAIN MECHANISMS OF TOXICITY

The monofunctional mustards have one alkylating site and, therefore, can attack and break the DNA at specific nucleotides. Although SM reacts with RNA, proteins, and phospholipids, the general consensus is that a DNA alkylate plays an important role in delayed toxic effects (Walker, 1971; Ball and Robert, 1972). The major alkylating site of nucleic acids of mammalian origin is the nitrogen residue of guanine (Wheeler, 1962). Cell death from DNA cross-linking is delayed until the cell replicates its DNA or undergoes division. At higher cellular exposures, however, mechanisms other than DNA cross-linking become important and produce more rapid cell death. The acute damage to the cornea, mucous membranes, and skin seen after SM exposure is probably generated by one or more of these other mechanisms. One mechanism that may be involved in acute damage is nicotinamide adenine dinucleotide depletion. Other potential mechanisms of cell death are related to rapid inactivation of sulfhydryl containing proteins and peptides, such as glutathione. These sulfhydryl compounds are critical in maintaining the appropriate oxidation reduction state of cellular components. Glutathione is also thought to be critical in reducing reactive oxygen species in the cell and preventing peroxidation and loss of membrane integrity (Rankin et al., 1980; Eklow et al., 2004). Tumor necrosis factor-α is involved in SM-induced skin lesions (Worsmer et al., 2005).

TARGET ORGANS AND ACUTE CLINICAL FEATURES

Acute toxic effects of SM are mostly observed in the eyes, respiratory tract, and skin. These organs are thus known as the target organs, because their involvement is more prominent here than in the other organs. However, neuropsychiatric symptoms and signs and digestive and cardiovascular dysfunctions were also observed. Frequencies of clinical features of acute SM intoxications in 233 Iranian patients treated in Mashhad, Iran, are shown in Figure 5.1.

Eyes are the organs that are most sensitive to SM. The first symptoms of SM exposure usually occur in the eyes. The Iranian patients with SM exposure reported itching and burning sensation of the eyes, leading to acute

conjunctivitis, blepharospasm, and even keratitis. In the acute stage, the limbal region frequently presents a marbled appearance in which porcelain-like areas of ischemia are surrounded by blood vessels of irregular diameter.

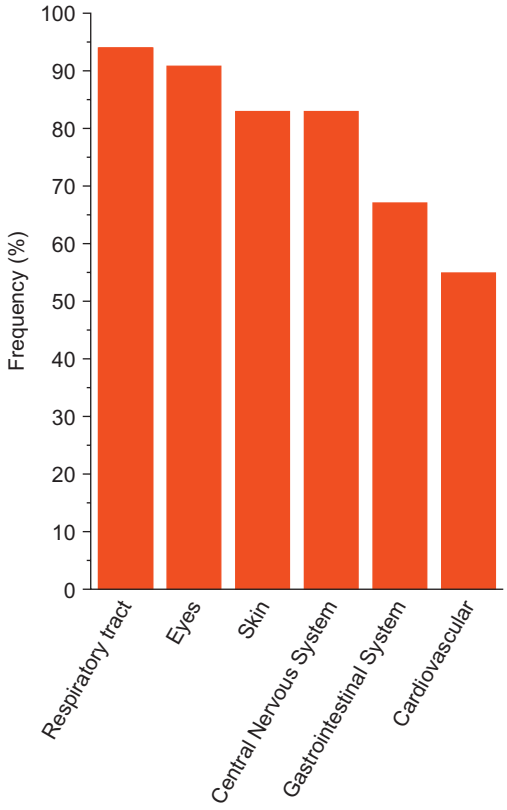

FIGURE 5.1 Frequency of clinical features of acute SM poisoning in different organs of 233 Iranian patients treated in Mashhad, Iran, a few days after exposure.

Later, the vascularized scars of the cornea often contain deposits of cholesterol, calcium, and fat (Giraud, 1917; WHO, 1970; Balali-Mood and Navaeian, 1986; Balali-Mood et al., 1986; Balali-Mood and Hefazi, 2005).

Respiratory effects occur in a dose-dependent manner from the nasal mucosa to the terminal bronchioles (WHO, 1970; Balali-Mood et al., 1986). Acute pulmonary effects commence with a tracheobronchitis, followed by bronchopneumonia, adult respiratory distress syndrome, and even pulmonary emboli in severely intoxicated patients, which may lead to death, mostly during the second week after SM exposure. Chest X-ray (CXR) of the Iranian SM-intoxicated patients revealed fewer abnormalities than the clinical manifestations. However, the severely intoxicated patients showed abnormal CXR results. CXRs of an Iranian patient with bronchopneumonia caused by acute SM poisoning before and after treatment in 1985 are shown in Figure 5.2.

Direct toxic effects of SM on the skin are the main apparent effects that lead to it being called a vesicant or a blistering agent. Erythema and blisters are the most common skin manifestations caused by SM exposure. A soldier who had a separate suit and face mask suffered from skin erythema and blisters on his neck 3 days after SM exposure, as shown in Figure 5.3.

A German and an Iranian medical toxicologist (first author) classified the cutaneous mustard gas lesions as described under the clinical manifestations (Helm and Balali-Mood, 1988).

GI effects after SM exposure have been documented in some studies. Destruction of the mucosa and shedding of the epithelial elements, however, begin days after exposure, resulting in the loss of large volumes of

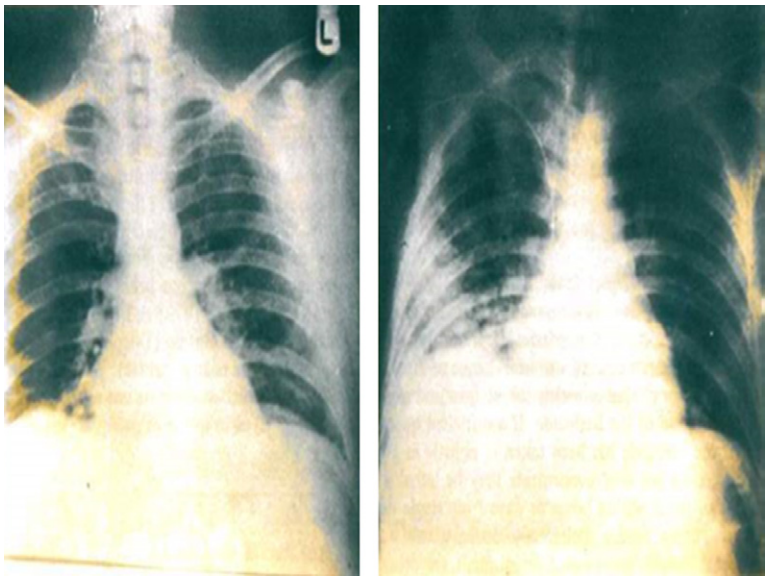

FIGURE 5.2 Chest X-rays of an Iranian patient with bronchopneumonia due to acute SM poisoning before (right) and after (left) treatment 8 days and 19 days after exposure in 1985.

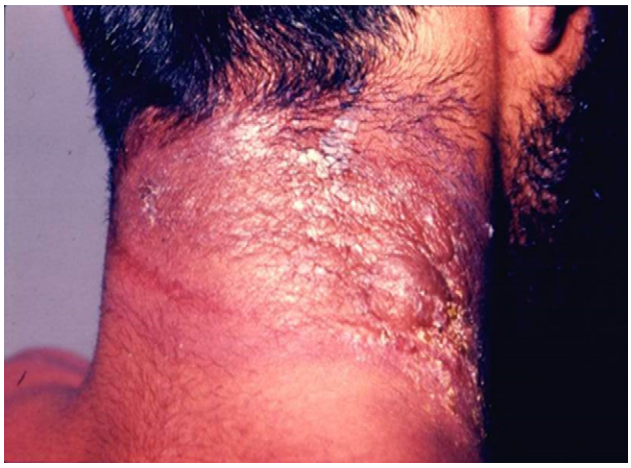

FIGURE 5.3 A soldier who had a separate suit and face mask revealed skin erythema and blisters on his neck 3 days after SM exposure in 1985.

fluid and electrolytes (Papirmeister et al., 1991). Acute gastroduodenitis with hemorrhagic erosions, acute desquamative enteritis, and severe hemorrhagic necrotic colitis were reported in WWI veterans (Canelli, 1918) but were not observed in the Iranian veterans.

Extremely heavy exposure to SM can cause central nervous system (CNS) excitation, leading to convulsions in animals (Anslow and Houk, 1946). Balali-Mood and Navaeian (1986) reported convulsions in six Iranian veterans who were hospitalized during the early stages of their intoxication (Balali-Mood and Navaeian, 1986). Most casualties from WWI and from the Iran–Iraq conflict, however, revealed mild and very nonspecific neurological effects, such as headache, anxiety, restlessness, confusion, and lethargy (Canelli, 1918; Balali-Mood and Navaeian, 1986). A frequent long-term complication in patients exposed to SM is delayed neuropathic toxicity, which was underrepresented in most previous studies (Thomsen et al., 1988).

HEMATOIMMUNOLOGICAL COMPLICATIONS

SM, as an alkylating agent, is particularly toxic to rapidly proliferating cells such as lymphoid and bone marrow cells. Leukocytosis is common within the first few days after exposure. White blood cell (WBC) counts then begin to decrease starting from the third or fourth day after exposure and reach their minimum level at approximately the ninth day. This leukopenia is followed by a decrease in megakaryocytes and, finally, in the erythropoietic series (Willems, 1989; Tabarestani et al., 1990; Balali-Mood et al., 1991). Bone marrow biopsies have shown hypocellular marrow and atrophy involving all elements (Tabarestani et al., 1990). If cytopenia is not marked and there are still remaining stem cells, then recovery will occur as the patient recovers (Krumbhaar and Krumbhaar, 1919; Tabarestani et al., 1988; Tabarestani et al., 1990; Balali-Mood et al., 1991; Mahmoudi et al., 2005). The bone marrow studies reveal a severe decrease in cellularity and fat replacement and also nuclear changes, such as budding, binuclear, and karyorrhexis in erythrocyte precursors. The toxic effects of SM on the hematopoietic system are dose-dependent, and it is concluded that SM causes aplastic or ineffective hematopoiesis (Tabarestani et al., 1988). Severe leukopenia, however, is an ominous sign, leading to secondary infections and higher mortality rates in these patients. SM victims with WBC counts of 200 cells/mL or less died during their initial admissions (Willems, 1989).

SM poisoning could result in the impairment of both humoral and cellular immune functions (Dayhimi et al., 1988; Zandieh et al., 1990; Ghotbi and Hassan, 2002). Along with the appearance of clinical disorders, both C3 and C4 titers showed an increase, followed by a gradual decrease, over 1 year. The majority of SM-exposed patients had increased levels of IgG and IgM during the first weeks and up to 6 months after exposure (Ghotbi and Hassan, 2002).

A decrease of cell-mediated immunity has been observed in Iranian veterans 1, 2, and 3 years after exposure (Zandieh et al., 1990). Natural killer (NK) cells, which are known to be one of the most important components of cellular immunity, have been found to be significantly lower in patients with severe respiratory complications 10 years after exposure (Ghotbi and Hassan, 2002).

Hematological and immunological complications of 40 patients 16–20 years after severe SM intoxication in comparison with 35 controls were reported (Balali-Mood et al., 2005a,b; Mahmoudi et al., 2005). Total WBC and red blood cell (RBC) counts and hematocrit (HCT) levels were significantly higher in the patients than in the control group. The percentages of monocytes and $CD3^+$ lymphocytes were significantly higher, and the percentage of CD16+ 56 positive lymphocytes (NK cells) was significantly lower in patients than in the control group. Other hematological and flow-cytometric parameters did not show any significant difference between the two groups. Serum IgM and C3 levels were significantly higher in the patients in comparison with the controls. Other immunoglobulins and complement factors did not show any significant difference between the two groups, as shown in Table 5.1.

DELAYED CLINICAL COMPLICATIONS

The main target of the long-term complication of SM poisoning is the respiratory system. In a study of incidence of common late complications of SM poisoning in

TABLE 5.1 Hematological and Immunological Changes in 40 Patients, 16–20 Years After Severe SM Intoxication Comparing with 35 Healthy Subjects

Parameters	Patient Mean ± SD	Control Mean ± SD	*P*-value
WBC (1000/mm^3)	7.24 ± 1.90	5.79 ± 1.12	0.025
RBC (million/mm^3)	5.46 ± 0.45	5.19 ± 0.28	0.035
Hb (mg/dL)	15.9 ± 0.7	15.6 ± 0.7	0.223
HCT (%)	48.3 ± 3.5	45.5 ± 1.9	0.047
PLT (1000/mL)	255 ± 99	238 ± 10.1	0.594
Lymphocyte (%)	31.5 ± 8.4	30.5 ± 10.8	0.651
Monocyte (%)	4.8 ± 1.6	3.9 ± 1.1	0.013
Polynuclear (%)	63.8 ± 8.7	65.4 ± 8.7	0.327
IgA (mg/dL)	302.6 ± 142.1	233.1 ± 59.3	0.154
IgM (mg/dL)	235.3 ± 84.8	136.8 ± 58.3	0.0001
IgG (mg/dL)	1438.6 ± 485.1	1140.0 ± 244.2	0.065
IgE (IU)	92.4 ± 112.1	86.5 ± 164.3	0.161
C3 (micg/dL)	109.8 ± 30.1	90.9 ± 14.8	0.030
CD3 (%)	71.1 ± 8.6	65.6 ± 10.7	0.037
CD16+ 5 (NK cells %)	11.6 ± 5.8	17.5 ± 9.6	0.006

TABLE 5.2 Frequency of Delayed Complications of SM in Different Organs of 40 Iranian Veterans in Mashhad 16–20 Years After Exposure

Organs	Number of Patients	Percentages
Respiratory tract	38	95
Peripheral neuromuscular	30	75
Skin	29	72.5
Eyes	27	67.5

lungs, eyes, and skin in 34,000 Iranians, the lungs were found to be the most affected organ (Khateri et al., 2003). In another study, the most common clinical complications of SM in different organs of 40 Iranian veterans 16–20 years after exposure showed that the respiratory tract was involved in 95% of the patients, as shown in Table 5.2 (Balali-Mood et al., 2005a,b).

RESPIRATORY TRACT

Respiratory complications are the greatest cause of long-term disability among people with SM exposure. A pulmonologist who investigated the pulmonary complications in Iranian veterans in 2007 named it mustard lung (Ghanei and Adibi, 2007).

A triad of cough, expectoration, and dyspnea has been found to be present in more than 80% of Iranian veterans 3 years after their initial exposure (Balali-Mood, 1992; Balali-Mood and Hefazi, 2006). Hemoptysis (mainly streaky), chest tightness, chest pain, and nocturnal dyspnea are also frequent. The main objective clinical findings are generalized wheezing (the most common sign), crackles, decreased lung sounds, clubbing, and cyanosis (Balali-Mood, 1992; Balali-Mood and Hefazi, 2006; Ghanei and Adibi, 2007).

Pulmonary function testing has revealed more obstructive patterns than restriction, and approximately half of these obstructive spirometric results are reversible in response to inhaled bronchodilators. FVC, FEV1, and FEV1/FVC (FEV1%) have all been found to be significantly lower in SM-intoxicated veterans in comparison with healthy nonexposed subjects and CWA survivors who had used a gas mask at the time of attack (Balali-Mood, 1992; Balali-Mood and Hefazi, 2005; Balali-Mood et al., 2005a,b; Ghanei and Adibi, 2007).

Abnormal spirometric findings in general and restrictive patterns in particular tend to increase over time (Balali-Mood and Hefazi, 2005; Balali-Mood et al., 2005a,b; Ghanei and Adibi, 2007). A study of 77 subjects who were present in a contaminated area and had no acute signs and symptoms at the time of exposure, but who now have respiratory disorders, indicates that subclinical exposure to SM can be responsible for the occurrence of delayed respiratory complications such as bronchiectasis and bronchiolitis obliterans (Ghanei et al., 2004a–c).

CXR findings in patients with late respiratory complications of SM have been described as increased bronchovascular markings, hyperinflation, bronchiectasis, pneumonic infiltration, and radiologic evidence of pulmonary hypertension (Balali-Mood et al., 2005a,b; Ghanei and Adibi, 2007). However, CXR is not sensitive enough for the detection of respiratory complications in these patients and high-resolution computed tomography of the chest may be required as the diagnostic imaging procedure of choice (Bagheri et al., 2003; Balali-Mood et al., 2005a,b; Bakhtavar et al., 2008). A study of 197 Iranian veterans 10 years after a single heavy exposure to SM revealed the development of a series of delayed destructive pulmonary sequelae, such as chronic bronchitis (58%), asthma (10%), bronchiectasis (8%), large airway narrowing (9%), and pulmonary fibrosis (12%). Each of these complications is described in more detail (Emad and Rezaian, 1997).

Chronic Bronchitis

Several studies have reported chronic bronchitis as the most common late complication of the respiratory system resulting from war exposure to mustard gas (Balali-Mood, 1992; Emad and Rezaian, 1997; Ghanei et al., 2004a–c, 2005, 2006a,b; Balali-Mood et al., 2005a,b).

Hypoxemia and hypercapnea are commonly observed in moderate to severe cases, leading to cor pulmonale and respiratory failure in the final stages of the disease (Emad and Rezaian, 1997; Balali-Mood and Hefazi, 2005; Balali-Mood et al., 2005a,b). Infection of the respiratory tract, resulting in bronchopneumonia, is also a common problem that is often complicated by septicemia (Balali-Mood et al., 1986, 2005a,b; Balali-Mood and Hefazi, 2005).

Asthma

Airway hypersensitivity, manifested as typical attacks of breathlessness, wheezing, and nocturnal cough, as well as a reversible obstructive pattern on pulmonary function tests, have been reported between 4 weeks and 20 years after SM inhalation. Patients with chronic bronchitis may also have some degree of bronchospasm, which does not respond to bronchodilators. Attacks of bronchospasm are characteristically triggered by respiratory infections, environmental allergens, and cold weather (Emad and Rezaian, 1997; Bijani and Moghadamnia, 2002; Ghanei et al., 2004a–c, 2005, 2006a,b; WHO, 2004; Balali-Mood et al., 2005a,b). New techniques, such as impulse oscillometry (IOS), have been used for evaluation of airway dysfunction. However, it was found to be less sensitive than spirometry in spotting small airways obstructions. IOS is a good diagnostic method in the detection of pulmonary involvement in uncooperative patients (Ghanei et al., 2004a–c).

Bronchiectasis

Direct effects of SM on the bronchial wall mucosa and more recurrent respiratory infections after inhalation of SM are known to be responsible for the development of bronchiectasis. Both the severity and frequency of bronchiectatic lesions tend to increase over the long-term, as evidenced by a study of 40 Iranian veterans with severe late complications of SM poisoning. These lesions usually begin bilaterally in the lower lobes and then progress toward the middle lobe and the lingula.

In severe cases with extensive bronchiectatic lesions, pulmonary hypertension and, ultimately, cor pulmonale may occur (Sohrabpour, 1992; Hosseini, 1998; Aslani, 2000; Ghanei et al., 2004a–c; Balali-Mood et al., 2005a,b).

Large Airway Narrowing

Airway narrowing, attributable to scarring or granulation tissue, is late sequelae of acute injuries to the trachea and large bronchi, usually developing 2 years after exposure (Aslani, 2000; Sohrabpour, 1992; Ghanei et al., 2004a–c). A study of 19 Iranian veterans with large airway narrowing caused by SM revealed stenosis in the trachea (seven cases), main bronchi (eight cases), and lobar bronchi (four cases) (Ghanei et al., 2004a–c). In contrast to stenosis caused by prolonged intubations, there is no predilection in the right main bronchus (Balali-Mood et al., 2005a,b). The major problem in these patients is the recurrence of the lesion, which usually occurs 6 months after treatment (Aslani, 2000).

Pulmonary Fibrosis

Late-onset pulmonary fibrosis has been reported in several Iranian veterans with combat exposure to SM (Aslani, 2000; Balali-Mood et al., 2005a,b). The analysis of bronchoalveolar lavage fluid from patients with mustard gas inhalation showed that these patients have an ongoing local inflammatory process of the lower respiratory tract, resulting in the development of pulmonary fibrosis years after the initial exposure. Histopathological examination of transbronchial lung biopsy (TBLB) samples of SM-exposed veterans revealed variegated fibrosis, diffuse fibrosis, and an absence of fibrosis in 86%, 4%, and 10% of the patients, respectively. Usual interstitial pneumonitis accounted for 97% of all cases of fibrosis (Emad and Rezaian, 1997). In another study, electron microscopic examination of seven TBLB specimens was performed in a WHO research center in Japan. Abnormal findings included: (i) proliferation, desquamation, and degeneration of the bronchial epithelial cells; (ii) interstitial fibrosis or fibrosing alveolitis; and (iii) increased type I and type II alveolar epithelial cells as well as hyperplasia of ciliated and goblet cells (Sohrabpour, 1992). Inflammation and fibrotic processes in the lung tissue of SM-exposed patients may be progressive (Ghanei et al., 2004a–c). Diffusing lung capacity could be used as an objective monitor of the degree of fibrosis and also as a good predictor for prognosis (Emad and Rezaian, 1997).

PERIPHERAL NEUROMUSCULAR COMPLICATIONS

Electromyography (EMG) and nerve conduction velocity (NCV) of 40 Iranian veterans with severe late manifestations of SM poisoning revealed abnormalities in the peripheral nervous system of 77.5% of the patients. NCV disturbances were more common in sensory nerves compared with motor nerves and were more prevalent in the lower extremities than in the upper extremities. EMG recordings revealed a normal pattern in 24 (60%) patients, incomplete interference with normal amplitude in 6 (15%) patients, and incomplete interference with low amplitude in 10 (25%) patients. NCV and EMG disturbances in both upper and lower extremities were

mostly symmetric (Ghanei et al., 2004a–c; Balali-Mood et al., 2005a,b).

DERMAL DELAYED EFFECTS

The occurrence and persistence of lesions after SM exposure are directly related to the duration and severity of exposure. Injury that results in erythema and edema without vesicle formation is almost always followed by complete healing and nonresidual effects (Chiesman, 1944; Balali-Mood and Hefazi, 2005). Blistering and necrotic wounds, however, cause permanent residual effects. The first report of delayed toxic effects of SM poisoning in 236 Iranian veterans 2 years after exposure revealed late skin effects such as hyperpigmentation (34%), hypopigmentation (16%), and dermal scarring (8%) (Balali-Mood and Navaeian, 1986). The most common skin symptom among these patients was itching, followed by a burning sensation and desquamation. These symptoms are basically caused by dryness of the skin and become worse in dry weather and after physical activity. A more recent study of 40 Iranian veterans who were heavily exposed to the gas 16–20 years previously revealed the most common cutaneous lesions to be hyperpigmentation, erythematous papular rash, dry skin, multiple cherry angiomas, atrophy, hypopigmentation, and hypertrophy. These lesions were found on the genital areas (48%), the back (48%), the front thorax and abdomen (44%), lower extremities (mainly inguinal) (44%), upper extremities (mainly auxiliary) (41%), and the head and neck (15%). Dry skin was more prominent in the extremities. Hyperpigmentation in some patients had the appearance of pigmented xerodermoid, which is a diffuse hyperpigmented area with superimposed macular hypopigmentations and hyperpigmentations (Balali-Mood et al., 2005a,b; Hefazi, et al., 2006).

In another study, the cutaneous lesions of 500 SM-exposed Iranian veterans were compared with 500 unexposed veterans. An association was found between SM exposure and late skin lesions such as severe dry skin, hyperpigmentation and hypopigmentation, localized hair loss, eczema, and chronic urticaria. There was also a higher incidence of vitiligo, psoriasis, and discoid lupus erythematosus among SM-poisoned patients. This could be attributable to the immunological basis of these disorders and to the fact that SM has adverse long-term effects on the immune system. Previously injured sites have been reported to be sensitive to subsequent mechanical stimulation and showed recurrent blistering after mild injury (Fekri and Janghorbani, 1992).

Histopathological examination of skin biopsy samples has revealed nonspecific findings including epidermal atrophy, keratosis, and basal membrane hyperpigmentation. Nonspecific fibrosis and melanophages have also been observed within the dermis (Fekri and Janghorbani, 1992; Balali-Mood et al., 2005a,b; Hefazi et al., 2006). Occupational exposure to SM has been demonstrated to cause a variety of skin changes, including pigmentary disorders, skin ulcers, and cutaneous cancers (Khehr, 1984).

OPHTHALMOLOGIC COMPLICATIONS

In less than 1% of patients with battlefield exposure to SM, a delayed type of ulcerative keratopathy may develop, leading to late-onset blindness (Hughes, 1942; Blodi, 1971; English and Benett, 1990; Pleyer et al., 1999; Javadi and Kazemi-Moghadam, 2000). The maximum delayed toxic effects usually occur 15–20 years after initial exposure, although latency periods as long as 40 years or as short as 6 years have also been reported (Solberg et al., 1997; Javadi and Kazemi-Moghadam, 2000; Etezad-Razavi et al., 2006). Patients are usually symptom-free for an indefinite number of years when delayed keratitis develops, characterized by photophobia, lacrimation, and failing vision (Pleyer et al., 1999). Vascularized scars of the cornea are covered with crystal and cholesterol deposits, leading to a worsening of the opacification, recurrent ulcerations, and sometimes corneal perforation. Opacification of the cornea is seen predominantly in the lower and central portions, whereas the upper part is often protected by the eyelid (Solberg et al., 1997; Pleyer et al., 1999). Surprisingly, lesions even recur after corneal transplantation (Javadi and Kazemi-Moghadam, 2000). The exact pathogenesis of this condition is unknown, but degenerative processes and immune reactions against corneal proteins (collagen–mustard compound) have been suggested as the cause of long-term damage (Solberg et al., 1997). Unfortunately, there has been no report of any long-term studies of mustard gas workers to determine their ocular status after prolonged occupational exposure.

PSYCHIATRIC COMPLICATIONS

Casualties from WWI and from the Iran–Iraq conflict were noted to be long-term mood and anxiety disorders, as well as posttraumatic stress disorder (Balali-Mood, 1986; Tabatabaee, 1988; Hashemian et al., 2006). Debility, loss of vitality, impaired concentration, sensory hypersensitivity, diminished libido, weakened potency, neuralgic symptoms, and disorders in autonomic regulation are common manifestations. Neuropsychiatric evaluation of 1,428 Iranian veterans 3–9 years after exposure to SM revealed anxiety (15%), depression (46%), personality disorders (31%), convulsions (6%), and psychosis (3%) (Tabarestani et al., 1988). Disorders of

consciousness (27%), attention (54%), emotion (98%), behavior (80%), thought process (14%), and memory (80%) were studied in 70 patients 3–5 years after SM exposure (Balali-Mood, 1986). Depression and posttraumatic stress in Iranian survivors of chemical warfare, mostly SM exposure, were also reported (Javadi and Kazemi-Moghadam, 2000). In another study, decreased libido and impotence were recorded in 52% and 9% of patients, respectively. Quite interestingly, 10% of the patients revealed an increased libido. Functional photophobia and aphonia and effort syndrome have also been reported (Balali-Mood et al., 2005a,b).

CARCINOGENICITY

SM is genotoxic because of its reactions with DNA, which is an important first step in carcinogenesis. Although most cells possess effective DNA repair mechanisms, these are not always effective in the case of SM damage. Alkylation of *O*6-guanine by SM seems to be critical. *O*6-ethylthioethylguanine is a poor substrate for the DNA repair enzyme *O*6-alkylguanine-DNA alkyltransferase (Ludlum et al., 1986). Therefore, this *O*6 lesion may be the most important mutagenic lesion. However, only limited data are available regarding the specific mutations produced by SM. Mutations in a tumor suppressor or an oncogene gene can favor a proliferate advantage of a clonal cell. Notably, alterations in the p53 tumor suppressor gene have been described in Japanese mustard gas workers (Takeshima et al., 1994). However, most of the lesions in this population were similar to smoking-related mutations. Mutations in lymphocytes at the hypoxanthine phosphoribosyltransferase (hprt) gene locus have also been reported (Yanagida et al., 1988).

REPRODUCTIVE

The effects of SM exposure during pregnancy are unknown. Data addressing the productive toxicities of SM in human models are both lacking and contradictory (Azizi et al., 1995).

CARDIOVASCULAR COMPLICATIONS

Cardiovascular complications such as myocardial perfusion abnormalities and coronary ectasis have recently been observed in some patients (Gholamrezanezhad et al., 2007). Further, more detailed studies of larger groups of veterans and controls are now being undertaken, especially in regard to cardiovascular complications.

RECENT ADVANCES IN SM POISONING AND ITS COMPLICATIONS

Because there are still several hundred Iranian veterans suffering from the long-term complications of SM poisoning, research of these patients is continuing. Attaran et al. (2010) have found a significant positive correlation between interleukin-6 and airflow limitation in SM-exposed patients with chronic obstructive pulmonary disease (Attaran et al., 2010). Another Iranian research group (Abolghasemi et al., 2010) has demonstrated a significant association between overall frequency of physical abnormalities in children and paternal SM exposure. They have concluded that SM exposure may have a lasting and important effect on generations to come (Abolghasemi et al., 2010).

Razavi et al. (2012) published a systematic review of the delayed complications of SM poisoning in Iranian veterans. They have reported numerous late complications among the victims and listed a wide range of respiratory, ocular, dermatological, psychological, hematological, immunological, GI, and endocrine complications, all which influence the quality of life of the exposed veterans. Although the mortality rate attributable to SM poisoning was 3%, morbidity was high, with variety and chronicity of toxic effects and complications (Razavi et al., 2012).

Karami et al. (2013) studied the effects of delayed complications of SM poisoning on the mental health of Iranian veterans 25 years after exposure and found a wide range of psychiatric complications, including neurotic and psychotic disorders. They have recommended that psychological state should be considered more often in SM-exposed veterans and that it is important to provide more mental health centers for these patients (Karami et al., 2013).

Unfortunately, there is no antidote or effective treatment for SM poisoning. Poursaleh et al. (2012) studied the treatment of SM poisoning in Iranian veterans and have found that mustard lung has an ongoing pathological process and is an active disorder even years after exposure. They concluded that there are no curative modalities for mustard lung; therefore, primary prevention and, if injury has occurred, secondary prevention for SM victims should be considered. They recommended studies that investigate underlying physiopathology and pharmacokinetics of drugs, as well as those that conduct more surveys and controlled clinical trials to obtain more effective treatments (Poursaleh et al., 2012).

Despite two centuries of research on SM in experimental animals and in humans, the animal studies are still ongoing. Mishra et al. (2010) showed that dermal SM exposure in euthymic hairless guinea pigs induced infiltration of both $CD4^+$ and $CD8^+$ T cells into the exposed skin. They also found strong upregulated expression of

pro-inflammatory cytokines and chemokines (TNF-α, IFN-γ, and IL-8) in distal tissues such as the lung and the lymph nodes. They claimed to report for the first time that SM induced a specific delayed-type hypersensitivity response that is associated with splenomegaly, lymphadenopathy, and proliferation of cells in these tissues. Their results suggest that dermal exposure to SM leads to immune activation, infiltration of T cells into the SM-exposed skin, delayed-type hypersensitivity response, and molecular imprints of inflammation in tissues distal from the site of SM exposure (Mishra et al., 2010). These immunological responses may contribute to the long-term sequelae of SM toxicity, as evident in many reports of the SM-exposed Iranian veterans (Krumbhaar and Krumbhaar, 1919; Dayhimi et al., 1988; Tabarestani et al., 1988; Zandieh et al., 1990; Ghotbi and Hassan, 2002; Mahmoudi et al., 2005).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A wide range of early and delayed toxic effects of SM can be categorized into two major groups: (i) direct toxic effects on the skin, eyes, and respiratory system with subsequent long-term complications such as chronic obstructive pulmonary disease (COPD), bronchiectasis, pulmonary fibrosis, large airway narrowing, hypopigmentation and hyperpigmentation of the skin, chronic conjunctivitis, and delayed keratitis and (ii) systemic toxicities, particularly the immunohematopoietic complications, that are believed to be responsible for the increased risk of infections and malignancies in these patients. However, there are still major gaps in SM literature and further studies of human subjects who have been exposed to the agent are required. Immunological and psychological dysfunctions, as well as the relationship between SM exposure and carcinogenesis and teratogenesis, are important fields that need further investigation. Recent findings of cardiovascular complications in some of these patients also require further investigations, particularly on a molecular basis to discover the mechanisms of early and delayed toxic effects of SM in humans. Future experimental and human studies may lead to a better understanding of SM poisoning and more effective treatment.

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CHAPTER

6

Epidemiology of Chemical Warfare Agents

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INTRODUCTION

While chemical warfare agents have been used in military conflict for decades, it is only in the last two decades that increased attention has been paid to the acute and chronic health effects associated with exposure to these agents. The subsequent reports of ill-defined illnesses in the veterans of the Gulf War of 1991, followed by the 1995 sarin terrorist attack in the Japanese subway system, focused attention on the capacity of deliberate or accidental exposure to chemical warfare agents, resulting in significant human death and subsequent disability.

Epidemiological studies of chemical warfare agents have suffered problems in determining exposure. Other than epidemiological investigations following the Japanese terrorist event, little objective epidemiological evidence is available. In this chapter, the major studies that have been conducted on populations exposed to the chemical warfare agents are discussed and methodological issues summarized.

PRE-WORLD WAR II

The first full-scale deployment of chemical agents in battle was during World War I in 1915, when the Germans used them against French, Canadian, and Algerian troops. Casualties were relatively heavy, though there were few deaths. A total of 50,965 tons of pulmonary, lachrymatory, and vesicant agents were deployed by both sides of the conflict, including chlorine, phosgene, and mustard gas. Official figures declare about 1,176,500 nonfatal casualties and 85,000 fatalities directly caused by chemical agents during the course of the war (Heller, 2005). In 1925, 16 of the world's major nations signed the Geneva Protocol, pledging never to use gas in warfare again; however, there were subsequent reports of its use. In 1935, Italy used mustard gas during the invasion of Ethiopia in the Second Italo-Abyssinian War with 15,000 chemical casualties reported. In this military conflict and subsequent wars in which chemical agents were used, no systematic attempt was made to accurately describe the epidemiology of the exposures, nor were any accurate data collected to monitor the health of exposed populations after the acute exposures.

Concern regarding the potential long-term effects of these exposures continued to be an issue, and in 1975, a longitudinal follow-up study of the mortality experience of three samples of World War I veterans was conducted to determine if a single exposure to mustard gas with respiratory injury was associated with increased risk of lung cancer in later life (Norman, 1975). Rosters of men born between 1889 and 1893 (2,718 exposed to mustard gas, 1,855 hospitalized with pneumonia in 1918, and 2,578 with wounds of the extremities (controls)) were traced via the Veterans Administration's death records. The 4,136 deaths reported were 95% of that expected. Observed deaths from lung cancer numbered 69 (2.5%) for the mustard gas group, as compared to 33 (1.8%) for the pneumonia group and 50 (1.9%) for the controls. The risk of death from lung cancer among men who were gassed relative to that for the controls was estimated as 1.3, with 95% confidence limits of 0.9–1.9.

WORLD WAR II

In 1938, the chemical structure of sarin nerve gas was discovered by the Germans, followed by the discovery

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of the nerve agent soman in the spring of 1944 (Schmaltz, 2006). However, chemical warfare agents were not extensively used by either side, due in part to fear of a devastating Allied retaliatory attack. There was one account of an exposure to mustard gas among Allied troops when several US ships were sunk by the Germans in 1943, including one carrying mustard gas intended for use in retaliation by the Allies if German forces initiated gas warfare. Because the presence of the gas was highly classified, authorities treating casualties ashore had no idea that they were seeing the effects of mustard gas; as a result, they prescribed improper treatment. This incident was not uncovered for many years, and military records indicate that 628 of the casualties hospitalized after the raid suffered from mustard gas exposure and 69 deaths were attributed in whole or in part to this cause (US Naval Historical Center, 1943). The due impact of the gas exposure to military and civilian populations was not accurately reported because of the high secrecy regarding the exposure and the difficulty discerning the effect of gas exposure from other types of injuries.

During the Holocaust, the Nazis used the insecticide Zyklon B containing hydrogen cyanide to kill several million people in extermination camps and reportedly used poison gases during the Warsaw ghetto uprising in 1943. In addition, experiments were conducted on concentration camp prisoners using mustard gas and phosgene.

In 1994, a US Senate report, entitled "Is Military Research Hazardous to Veterans' Health? Lessons Spanning a Half Century," stated that US military personnel were used as human subjects in the 1940s to test two chemical agents, mustard gas and a similar compound, Lewisite. This testing was done to determine how to best protect military troops from the effects of chemical warfare agents (Pechura and Rall, 1993).

During the war, the US military conducted a secret research program aimed at determining how best to protect military personnel against the effects of mustard gas and Lewisite (Pechura and Rall, 1993). Up to 4,000 men took part in the program, which required participants to wear gas masks and clothing that had been treated in an attempt to keep the gas from reaching the skin. Men were required to remain in the sealed test room from 1 to 4 h. Some men were tested in the field, where they were required to stay in an area that had been bombed with mustard gas anywhere from 1 h to 3 days. In 1992, the US Department of Veterans Affairs (VA) began to allow compensation for seven conditions that can result from mustard gas exposure: laryngitis, chronic bronchitis, emphysema, asthma, chronic conjunctivitis, chronic keratitis, and corneal opacities. Following publication of a report by the National Academy of Sciences (Pechura and Rall, 1993), the VA extended the list to include respiratory cancers (nasopharyngeal, laryngeal, and lung except for mesothelioma), skin cancer, chronic obstructive pulmonary disease, and acute nonlymphocytic leukemia.

Bullman and Kang (2000) conducted a 50-year mortality follow-up study of veterans exposed to low levels of mustard gas. The subjects were World War II Navy veterans who received low-level nonlethal exposures to mustard gas while participating in mustard gas chamber tests at Bainbridge, MD, between 1944 and 1945. These veterans were exposed to mustard gas while wearing protective clothing and masks. A control group consisted of 2,663 Navy veterans who served at the same location and time as the exposed people, but did not participate in chamber tests. The investigators found no excess of any cause-specific mortality associated with varying levels of mustard gas exposures that were sufficient to cause skin reactions. A significant strength of this study was that the length of time in the exposure chamber, the dose of exposure, and documentation of any observable acute effect were available for each of the exposed subjects so that a dose–response analysis could be done.

Schnurr et al. (2000) reported on the prevalence of current posttraumatic stress disorder (PTSD) associated with participation in these secret military tests of mustard gas exposure. Using the registry established by the VA, 363 male military veterans were randomly sampled and found to have a current prevalence of 32% for full PTSD and 10% for partial PTSD. Prevalence of PTSD varied as a function of risk and protective factors, including volunteering, physical symptoms during the tests, and prohibited disclosure. Veterans with full PTSD reported poorer physical health, a higher likelihood of several chronic illnesses and health-related disability, greater functional impairment, and higher likelihood of health-care use than those with no PTSD. Veterans with partial PTSD also had poorer outcomes than did veterans with no PTSD in a subset of these domains.

Schnurr et al. (1996) postulated that these exposures involved elements of "contamination stressors," in which information about the exposure or the lack of information serve as the stressors, rather than the actual exposure to the chemical. Lack of information during the time of exposure, and the notification decades later could have led to vague or diffuse fear with unknown consequences, contributing to the development of PTSD. The contamination stressor led to a future orientation; a worry about what problems will develop as a result of the previous exposure.

POST-WORLD WAR II

Development of other agents, such as the *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX) nerve agent continued during the 1950s, and in

1961, the United States was producing large amounts of VX and performing its own research into nerve agents. In 1952, the US Army patented a process for developing the powerful toxin ricin.

In 1969, 23 US servicemen and one US civilian stationed in Okinawa, Japan, were exposed to low levels of the nerve agent sarin while repainting the depot's buildings. When the exposure was publicized, the United States moved the weapons in 1971 to Johnston Atoll. Between 1951 and 1969, various chemical and biological agents were tested at the Dugway Proving Ground. From 1962 to 1973, more than 5,800 military personnel participated in a series of tests on the vulnerability of warships to biological and chemical attacks. Only some of the involved military personnel consented to these tests. Many of them used chemical warfare simulants, which were thought at the time to be harmless. The results of the tests were reported in classified documents (SHAD report). In 2000, the US Department of Defense (DoD) released the names of the participants and information about the testing that occurred. In 2002, the Institute of Medicine (IOM) agreed to undertake a scientific study of potential long-term health effects associated with these exposures. The IOM assembled a comparable control group and conducted a health survey by telephone. Mortality records were also examined. The primary outcomes of interest were mortality, general health, and medical conditions. The SHAD participants were divided into four groups:

- Group A consisted of 3,000 participants whose exposure was limited to either *Bacillus globigii* (BG) or methylacetoacetate (MAA).
- Group B consisted of 850 participants whose only potential exposure was to trioctyl phosphate (TEHP) and contained a large number of Marines.
- Group C consisted of 720 participants who were in tests where active chemical warfare agents were used.
- Group D consisted of 850 subjects potentially exposed to simulants who were not in group A, B, or C.

Control groups were assembled for each of the exposed groups. Of the nearly 12,500 Navy and Marine subjects, 9,600 were assumed alive (i.e., no evidence of death from available records sources) and were surveyed. The response rate for the SHAD participants was 60.8% and 46.6% for controls. No differences were observed in all-cause mortality between SHAD participants and controls, although the SHAD participants had a statistically significant higher risk of death due to heart disease. Lack of cardiovascular risk factor data makes this difference difficult to interpret. SHAD participants also reported statistically significantly worse health than controls, but no specific patterns of illness were found. Group C, the only group with potential exposure to active chemical or biological agents, reported the smallest differences in overall health compared to controls. Small differences in memory and attention as well as somatization were observed and SHAD participants had higher levels of neurodigenerative conditions. SHAD participants also reported higher rates of symptoms, thought to be related to reporting bias. There were no significant differences in self-reported hospitalizations.

This report was significant in that it was the first epidemiological investigation of a military population with documented exposure to chemical agents or stimulants. The survey was conducted, however, 30 years after the exposure and, with the exception of mortality records, was limited to self-reported measures of health. A mortality follow-up study of the SHAD cohort was published by Kang and Bullman (2009) in which the cause-specific mortality of 4,927 SHAD veterans was compared to 10,927 other Navy veterans of that era. The SHAD veterans had an increased risk of overall mortality, due primarily to heart disease; therefore, this heightened risk was not attributed to exposure to active chemical or biological warfare agents. This study was limited due to lack of information on other potential exposures, particularly exposure to Agent Orange, a defoliant used in Vietnam, and the possibility of error was introduced by exposure misclassification.

In 2012, the IOM was commissioned to conduct a second epidemiological study comparing the health status of the SHAD veterans with a comparison population. This research, currently underway, will build on knowledge gained from the first study and use veteran and Medicare data as well as death certificate data. The anticipated date for completion of the study is February 2015.

IRAN–IRAQ WAR

Saddam Hussein received chemical weapons from many countries, including the United States, West Germany, the Netherlands, the United Kingdom, France, and China (Lafayette, 2002). In 1980, Iraq attacked Iran, employing mustard gas and tabun, in a war that lasted 8 years. A total of 5% of all Iranian casualties were directly attributable to the use of these agents. Iran sustained approximately 387 chemical attacks during this war (Shemirani et al., 1993). About 100,000 Iranian soldiers, along with significant numbers of civilians, were chemical warfare victims. Nerve gas agents killed about 20,000 Iranian soldiers immediately. Shortly after the war ended in 1988, the Iraqi Kurdish village of Halabja was exposed to multiple chemical agents, resulting in the death of 10% of the 50,000 residents.

The extensive assault on military and civilian populations, terrible as it was, has resulted in some of the

best-designed epidemiological studies of the effects of exposure to chemical warfare agents in recent years. Hashemian et al. (2006) reported on the results of a cross-sectional, randomized survey of 153 civilians in three towns exposed to military conflict in northwestern Iran: Oshnaviveh (low-intensity conventional warfare), Rabat (high-intensity conventional warfare), and Sardasht (both high-intensity conventional warfare and chemical weapons). The surveys measured full or partial PTSD diagnosis and symptoms of anxiety and depression. Compared with individuals exposed to low-intensity warfare, those exposed to high-intensity warfare and chemical weapons were at a higher risk for lifetime PTSD (odds ratio (OR), 18.6; 95% confidence interval (CI), 5.8–59.4), current PTSD (OR, 27.4; 95% CI, 3.4–218.2), increased anxiety symptoms (OR, 14.6; 95% CI, 6.0–35.6), and increased depressive symptoms (OR, 7.2; 95% CI, 3.3–15.9). Exposure to high-intensity warfare but not to chemical weapons was also significantly associated with lifetime PTSD (OR, 5.4; 95% CI, 1.7–17.6), compared with those in the low-intensity warfare group. Further, compared with individuals exposed to high-intensity warfare alone, those exposed to both high-intensity warfare and chemical weapons were at higher risk for lifetime PTSD (OR, 3.4; 95% CI, 1.5–7.4), current PTSD (OR, 6.2; 95% CI, 2.0–20.1), increased anxiety symptoms (OR, 5.6; 95% CI, 2.5–12.6), and increased depressive symptoms (OR, 3.7; 95% CI, 1.8–7.2).

Sulfur mustard is rated by the International Agency for Research on Cancer (IARC) as a human carcinogen and is a known risk factor for occupational lung cancer (Nishimoto et al., 1987; Ghanei and Vosoghi, 2002). Zafarghandi et al. (2012) described the incidence of cancer in 7,570 Iranian sulfur mustard-exposed veterans compared to 7,595 unexposed subjects in a 25-year follow-up study. Cancer incidence was significantly increased with exposure to sulfur mustard. The incidence rate ratio for cancer was 1.81 (95% CI 1.15–2.34); however, no increased risk of site-specific cancers were found. The hazard ratio of cancer occurrence was 2.02 (95% CI 1.41–2.88).

The long-term clinical consequence of sulfur mustard exposure was looked at by the Sardasht-Iran Cohort Study (Ghazanfari et al., 2009), which included 372 individuals from Sardasht as the exposed group and 128 non-exposed individuals from Rabat. As part of this research, Ghanei et al. (2010) studied pulmonary complications and found that blistering at the time of exposure was associated with more respiratory symptoms and worse lung function, but not with air trapping, bronchiectasis, and mosaic parenchymal attenuation detected with computed tomography (CT) of the thorax. This cohort was also used to examine the long-term effects of sulfur mustard on civilian's mental health 20 years post exposure. There were significant differences in somatization, obsessive-compulsion, depression, anxiety, and hostility between the exposed and unexposed groups, suggesting significant long-term effects of sulfur mustard exposure.

Comparisons of the effects of sulfur mustard and nerve agents have also been conducted in this population. Emadi et al. (2012) compared late cutaneous complications between the two exposure groups of 154 sulfur mustard-exposed cases and 175 nerve agent exposed cases. Only 18.1% of the mustard-exposed group was asymptomatic, compared to 62.4% of the nerve agent exposures. A number of mustard-induced dermatologic lesions were reported, including scars, intertrigo, xerosis, cherry angioma, hyperpigmentation, pilar keratosis, poikiloderma, and malignant tumors.

1991 GULF WAR

Given the past use of chemical weapons of Iraq on its own citizens, there was much concern that Saddam Hussein would again employ these weapons during the conflict against coalition forces. The only known exposure to anticholinesterase chemical warfare agents during the Gulf War was during the destruction of munitions containing 8.5 metric tons of sarin/cyclosarin housed in Bunker 73 at Khamisyah, Iraq, on March 4, 1991, and additional destruction of sarin/cyclosarin rockets in a pit at Khamisyah on March 10, 1991. The US DoD reported that the exposure levels were too low to activate chemical alarms or to cause symptoms at the time of the detonation; however, several studies have been conducted to assess long-term health effects associated with this exposure. The DoD conducted modeling of the air plume that resulted from the detonation and estimated the extent of troops potentially exposed to the plume.

McCauley et al. (1999) conducted a computer-assisted telephone survey of 2,918 Gulf War veterans from Oregon, Washington, California, North Carolina, and Georgia to evaluate the prevalence of self-reported medical diagnoses and hospitalizations among this potentially exposed population and among comparison groups of veterans who were deployed and not deployed to the Southwest Asia theater of operations. Troops reported to be within 50 km of the Khamisyah site did not differ from other deployed troops in terms of reporting any medical conditions or hospitalizations in the 9 years following the Gulf War. Hospitalization rates among deployed and nondeployed troops did not differ. Deployed troops were significantly more likely than nondeployed troops to report diagnoses of high blood pressure (OR = 1.7), heart disease (OR = 2.5), slipped disk or pinched nerve (OR = 1.5), PTSD (OR = 14.9), hospitalization for depression (OR = 5.1), and periodontal disease (OR = 1.8). There was a trend for deployed veterans to report more diagnoses of any cancer (OR = 3.0).

Smith et al. (2003) investigated postwar morbidity for Gulf War veterans, contrasting those who may have been exposed to low levels of nerve agents at Khamisyah and those unlikely to have been exposed. Cox regression modeling was performed for hospitalizations from all causes and hospitalizations from diagnoses within 15 categories during the period March 10, 1991 through December 31, 2000, for the duration of active-duty status. Veterans possibly exposed to nerve agents released by the Khamisyah demolition were not found to be at increased risk for hospitalization from most chronic diseases nearly 10 years after the Gulf War. Only 2 of 37 models suggested that personnel possibly exposed to subclinical doses of nerve agents might be at increased risk for hospitalization from circulatory diseases, specifically cardiac dysrhythmias.

Bullman et al. (2005) reported the results of a mortality study of troops exposed to chemical warfare agents based on the air plume models that were developed after the detonation. The cause-specific mortality of 100,487 exposed veterans was compared with that of 224,480 unexposed US Army Gulf War veterans. The risks for most disease-related mortality were similar for exposed and unexposed veterans. However, exposed veterans had an increased risk of brain cancer deaths (relative risk = 1.94; 95% CI = 1.12, 3.34). The risk of brain cancer death was larger among those exposed 2 or more days than those exposed 1 day when both were compared separately to all unexposed veterans.

This same team of investigators also conducted a study to examine the association of exposure to the Khamisyah plume with subsequent self-reported morbidity (Page et al., 2005). The study sample included 1,056 deployed Army Gulf War veterans who responded to the National Health Survey of Gulf War Era Veterans in 1995 and who were resurveyed in 2000. Half of the subjects had been notified of potential exposure to chemical warfare agents. Comparing notified and nonnotified subjects, there were no statistically significant differences with respect to bed days, activity limitations, clinic visits, or hospital visits. Among 71 self-reported medical conditions and symptoms, there were five statistically significant differences, four of which were for lower rates of illness among notified subjects.

Page and colleagues also published a similar study undertaken to investigate whether possible chemical warfare exposure was associated with morbidity among Army Gulf War veterans using morbidity data for 5,555 Army veterans who were deployed to the Gulf region (Page et al., 2005). Responses to 86 self-assessed health measures, as reported in the 1995 National Health Survey of Gulf War Era Veterans, were evaluated. They found little association between potential exposure and health after adjusting for demographic variables. The investigators concluded that potential exposure to sarin or cyclosarin at Khamisyah did not seem to have adversely affected self-perceived health status, as evidenced by a wide range of health measures.

More recently, Heaton examined the association between modeled estimates of sarin/cyclosarin exposure levels and volumetric measurements of gross neuroanatomical structures in 1991 Gulf War veterans with varying degrees of possible low-level sarin/cyclosarin exposure (Heaton et al., 2007). A total of 26 veterans recruited from the Devens Cohort Study participated. Magnetic resonance images (MRIs) of the brain were acquired and analyzed using morphometric techniques, producing volumetric measurements of white matter, gray matter, right and left lateral ventricles, and cerebrospinal fluid. Volumetric data were analyzed using exposure estimates obtained from refined models of the presumed exposure hazard area in Khamisyah. No differences were observed in the 13 exposed veterans when compared to 13 nonexposed veterans in volumetric measurements of discrete brain tissues. However, linear trend analyses showed a significant association between higher levels of estimated sarin/cyclosarin exposure and both reduced white matter (adjusted parameter estimate = 4.64, $P < 0.0001$) and increased right lateral ventricle (adjusted parameter estimate = 0.11, $P = 0.0288$) and left lateral ventricle (adjusted parameter estimate = 0.13, $P < 0.0001$) volumes. These findings suggest subtle but persistent central nervous system pathology in Gulf War veterans potentially exposed to low levels of sarin/cyclosarin.

This investigative team also compared previous neurobehavioral performance results collected prior to notification of veterans who were potentially exposed during the Khamisyah detonation (Proctor et al., 2006). They hypothesized that the exposure to sarin and cyclosarin would be associated with poorer performances on objective neurobehavioral tasks in specific functional domains (particularly in visuospatial abilities and psychomotor functioning) in a dose-dependent manner. They found that sarin and cyclosarin exposure was significantly associated with less proficient neurobehavioral functioning on tasks involving fine psychomotor dexterity and visuospatial abilities 4–5 years after exposure. They concluded that the findings suggest a dose–response association between low-level exposure to sarin and cyclosarin and specific functional central nervous system effects 4–5 years after exposure.

To determine the generalizability of these findings in the Devens Cohort, investigators from the University of California, San Francisco studied a second cohort of Gulf War veterans with suspected sarin/cyclosarin exposure (Chao et al., 2010). They studied 40 Gulf War veterans categorized as exposed to sarin/cyclosarin at Khamisiyah in comparison to a control group of 40 nonexposed veterans. MRI data of the brain were analyzed, with

volumetric measurements of gray matter, white matter, cerebrospinal fluid and hippocampus. Exposed veterans had reduced total gray matter and hippocampal volumes compared to controls ($P < 0.01$). While no group differences were observed on measures of cognitive function or total white matter volume, there were significant positive correlations between total white matter volume and measures of executive function and visuospatial abilities in exposure sarin/cyclosarin. While limited in accurate exposure assessment and specific unit information, the authors argue that these findings in conjunction to those found by Heaton, Proctors, and colleagues point to the need for a follow-up study with more subjects and more sophisticated imaging technology.

TERRORISM

Two terrorist attacks with the nerve agent sarin affected populations in Matsumoto and Tokyo, Japan, in 1994 and 1995, killing 19 and injuring more than 6,000. Morita et al. (1995) described the acute effects, including instantaneous death by respiratory arrest in four victims in Matsumoto. In Tokyo, 2 people died in station yards, and another 10 victims died in hospitals within a few hours to 3 months after the poisoning. A total of six victims with serum cholinesterase (ChE) below 20% of the lowest normal levels were resuscitated from cardiopulmonary arrest (CPA) or coma with generalized convulsion. Of those, five recovered completely and 1 remained in a vegetative state due to anoxic brain damage. Electroencephalogram (EEG) abnormalities were observed for up to 5 years in certain victims. Miosis and copious secretions from the respiratory and gastrointestinal tracts (muscarinic effects) were common in severely to slightly affected victims. Weakness and twitches of muscles (nicotinic effects) appeared in severely affected victims. Neuropathy and ataxia were observed in a small number (less than 10%) of victims, in which findings disappeared between 3 days and 3 months. Leukocytosis and high serum creatine kinase (CK) levels were common. Hyperglycemia, ketonuria, low serum triglycerides, and hypokalemia were observed in severely affected victims, and these abnormalities were attributed to damage of the adrenal medulla.

The Matsumoto government assembled a committee of city government, local hospitals and physicians from Shinsu University to monitor the immediate and long-term effects of the exposure, resulting in the most comprehensive epidemiological studies of acute and residual effects of exposure to chemical warfare agents. Three weeks after the attack, community residents ($n = 2052$) residing in an area within 1,000–850 m of the attack were surveyed and categorized as severely affected if they were admitted to the hospital, moderately affected if treated in outpatient clinics, and slightly affected if they had symptoms but did not seek medical attention. At the time of this follow-up survey, 28% of the affected residents remained symptomatic (69% of the severely affected, 42% of the moderately affected, and 14% of the slightly affected). The most frequent persisting symptoms were fatigue, dysesthesia of extremities, and ocular pain. Visual problems continued in about 10% of severely affected victims (Yanagisawa et al., 2006).

In the Tokyo subway attack, 640 victims were seen within hours of the incident. Of these people, 5 were critically injured and required mechanical ventilation, and another 107 were moderately injured with systemic symptoms and signs of respiratory, digestive, or neurological damage, in addition to ocular problems. The large majority ($n = 528$) had only eye damage or symptoms and were released after several hours of observation (Yanagisawa et al., 2006).

There have been a number of investigations of the health of the survivors of the Tokyo subway attack. Yokoyama et al. (1998a) conducted a study of 18 victims 6–8 months after the attack. At that time, their mean plasma ChE was 72.1, lower than the normal range of 100–250 IU/L. In neurobehavioral testing at that time, sarin cases had significantly lower scores on the digit symbol test than the control group. Cases were more likely to have higher scores on both the General Health Questionnaire, an indication of psychological distress, and fatigue measures than controls, and PTSD scores were also increased. Postural balance was also different in victims, suggesting that integration of visual input might have been impaired (Yokoyama et al, 1998b). P300 and VEP (P100) latencies in the sarin cases were significantly prolonged in these victims compared with the matched controls (Murata et al., 1997). In the sarin cases, the CVRR (electrocardiographic R-R interval variability) was significantly related to serum ChE levels determined immediately after exposure; the PTSD score was not significantly associated with any neurophysiological data despite the high PTSD score in the sarin cases. These findings suggest that asymptomatic after effects to sarin exposure, rather than PTSD, persist in the higher and visual nervous systems beyond the turnover period of ChE.

The National Police Academy (1999) conducted a survey of 1,247 residents who reported to the police department that they had contact with sarin at the incident. More than half complained of physical symptoms, such as asthnopia and decrease in visual acuity; and 17% reported psychological trauma from the event, with 14% still unable to ride on subways 3 years after the incident.

There continue to be follow-up studies describing the residual effects of the attack. Ohtani et al. (2004) followed 34 victims 5 years after the attack. Not only PTSD, but also nonspecific mental symptoms, persisted in the victims at a high rate. A total of 11 victims were diagnosed with current

or lifetime PTSD. Victims with PTSD showed higher anxiety levels and more visual memory impairment.

Yamasue et al. (2007) conducted a 5 year follow-up study to identify persistent morphological changes subsequent to the attack. In the research, 38 victims of the sarin attack who had been treated in the emergency department for sarin intoxication and 76 control subjects underwent weighted and diffusion tensor MRIs. ChE values were compared to levels immediately after the attack. The voxel-based morphometry exhibited smaller than normal regional brain volumes in the insular cortex and neighboring white matter, as well as in the hippocampus, in the victims. The reduced regional white matter volume correlated with decreased serum cholinesterase levels and with the severity of chronic somatic complaints related to interoceptive awareness. Voxel-based analysis of diffusion tensor MRIs further demonstrated a significantly lower than normal fractional anisotropy in the victims. These findings suggest that sarin intoxication might be associated with structural changes in specific regions of the human brain.

Rescue and safety workers have also been studied. Nishiwaki et al. (2001) looked at 27 male rescue team staff and 30 police officers 3–45 months after the event. The subjects showed decreased performance on the digit span test; however, no effects on stabilometry and vibration perception threshold were found. Li et al. (2004) followed 27 male firefighters and 25 male police officers 3 years after the attack for genotoxic effects. They found an elevated frequency of sister chromatid exchanges in lymphocytes of the victims, which were related to the percentage of ChE inhibition observed just after the attack.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This chapter described the major epidemiological studies of populations who have been exposed to chemical warfare agents. Many of the studies of military populations have suffered from inaccurate exposure assessment and lack of clinical data. However studies in the past decade of terrorist attacks, as well as the Iran–Iraqi cohort studies, provide the most comprehensive data to date on the scope of health outcomes associated with these exposures. These reports point to the need for long-term follow-up studies of victims of such events. The data from the terrorist events and military assaults point to the prevalence of PTSD in populations with real or threatened exposure.

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CHAPTER

7

Chemical Weapons of Mass Destruction and Terrorism: A Threat Analysis

René Pita and Arturo Anadón

INTRODUCTION

Terrorism is a contested concept because there is not an international legal consensus regarding its definition. Barile defines terrorism as the threat or implementation of violent means to undermine, destabilize, inflict harm, or cause panic in a society (Barile, 2010).

After the September 11, 2001 (9/11) terrorist attacks in the United States, the high risk of possible attacks with chemical weapons (CWs), especially by groups linked to jihadist terrorism, has been perceived. Before 9/11, in 1994 and 1995, Aum Shinrikyo, a religious organization in Japan, used sarin (a nerve agent) in attacks in Matsumoto and on the Tokyo subway, causing a large number of casualties. These terrorist attacks had a big impact on the international chemical defense and intelligence communities but not on other circles, perhaps because a chemical attack by a religious organization in Japan seemed something far removed from the reality of the rest of the world. But this changed after 9/11, when the mailing of letters containing *Bacillus anthracis* spores accompanied by images of the attacks on New York City's World Trade Center towers increased the concern about weapons of mass destruction (WMD) attacks, including attacks by CWs.

In this chapter, information is analyzed from open sources regarding the possible use of CWs by terrorist groups, especially by those affiliated with or associated with the Al Qaeda terrorist network, as well as by supporters of the global jihad movement. As religious terrorist groups, Al Qaeda and the global jihad movement do not fit the assumption made by Brian Jenkins in 1975 that "terrorists want a lot of people watching and a lot of people listening, and not a lot of people dead" (Jenkins, 1975). This statement fits better with secular terrorist groups. But for religious terrorist groups like Al Qaeda, "divine duty" results in disappearance of moral restraints that would justify "a lot of people dead" in their terrorist attacks, such as the 9/11 attacks. And if CWs are part of the WMD concept because they can cause a large number of casualties, they could be very good tools for jihadist terrorist groups to achieve their goals.

CWs FOR TERRORIST ACTIONS

"Classical" Chemical Warfare Agents: Vesicants and Nerve Agents

The two main chemical warfare agents (CWAs) are vesicants and nerve agents (Table 7.1). The first chemicals used as weapons in World War I included toxic industrial chemicals (TICs) that were widely available in the chemical industry. These included lung-damaging agents like chlorine and phosgene, as well as cyanides that were named "blood agents." When these agents proved to be effective at the tactical level, research and development programs of CWAs started (Pita, 2008). These programs tried to obtain chemicals that would be effective in battle because of the physico-chemical and toxicological properties of the chemicals, although they had no use in the industrial field.

First, sulfur mustard, a vesicant warfare agent, was produced and used in World War I. Mustards alkylate a wide range of biologically important molecules producing cytostatic, mutagenic, and cytotoxic effects. In 1936, just before the start of World War II, tabun was synthesized and then produced as the first nerve CWA. Nerve agents inhibit acetylcholinesterase (AChE) throughout

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TABLE 7.1 Some Potential Chemical Agents for Terrorist Actions

Vesicants	Sulfur mustards
	Nitrogen mustards
	Lewisites
Nerve agents	Tabun (GA)
	Sarin (GB)
	Soman (GD)
	VX
Incapacitating agents	BZ
	Opioids
RCAs	CS
	CN
	CR
	Capsaicin
TICs	Chlorine
	Phosgene
	Cyanides
Toxins	Ricin

FIGURE 7.1 A jihadist manual dealing with CWs available on the Internet.

the body, causing an accumulation of acetylcholine (ACh), which produces overstimulation at muscarinic receptors, nicotinic receptors, and in the central nervous system (CNS).

Vesicant agents (sulfur mustards, nitrogen mustards, and lewisites) and nerve agents (tabun, sarin, soman, and VX, among others) are included in the Chemical Weapons Convention (CWC) Schedule 1 of chemicals for the application of verification measures (http://www.opcw.org, accessed February 21, 2014). This schedule features chemicals that have little or no use in the industry and have been developed, produced, stockpiled, or used as CWs.

Nerve agents can be considered as the most important CWAs because of their high toxicity and their high versatility for tactical use. This high versatility is attributable to the fact that although all nerve agents are liquid at room temperature, some can be considered persistent agents (e.g., VX) because of their low volatility, whereas others can be considered nonpersistent (e.g., sarin) because their volatility is higher. Persistent agents would be useful against targets with no occupational interest that would be contaminated for a certain period of time. On the contrary, nonpersistent agents would evaporate faster and would be the choice against targets that need to be occupied.

Toxicological and physico-chemical properties of nerve agents would also make this group the CWs of choice for terrorist groups. However, the synthesis process requires some level of expertise and is far more complex than the recipes featured in the "do it yourself" (DIY) manuals available on the Internet (Figure 7.1). Even Aum Shinrikyo—which had excellent financial resources and personnel with the required expertise and performed their attacks before the entry into force of the CWC—had some difficulties in synthesizing sarin (Tu, 2002).

The recent use of sarin on August 21, 2013, during the Syrian conflict was confirmed by the UN Mission, and it shows that nerve agents are still a real threat. Despite the limitations the UN Mission found while investigating the use of CWs in Syria, hopefully some interesting conclusions for the medical community will eventually emerge (Pita and Domingo, 2014).

Incapacitating Agents

Incapacitating agents are chemicals that produce a disabling condition that persists for hours to days after exposure has occurred. These were studied during the Cold War when it was assumed that incapacitating the enemy would cause damage not only because casualties would become unavailable for duty but also because they would need to be evacuated and would consume more logistical resources. Incapacitating agents include depressants and stimulants of the CNS. The main incapacitating agent is BZ (3-quinuclidinyl benzilate), a CNS depressant included in Schedule 2 of the CWC because it is produced in small commercial quantities for biomedical purposes.

Opioids have also been studied as incapacitating CWAs. In October 2002, a fentanyl derivative was used by Russian Special Forces to end a hostage crisis during

which Chechen terrorists had taken approximately 800 hostages at a Moscow theater (Wax et al., 2003). The dissemination of this opioid through the theater ventilation system killed approximately 130 people and injured more than 600. The fact that medical services did not have information about the identity of the chemical resulted in a lack of antidotal treatment of the poisoned hostages.

Riot Control Agents

Riot control agents (RCAs), popularly referred to as "tear gas" or "pepper spray," should not be confused with incapacitating agents. Unlike the latter, RCAs are local irritants with disabling effects that disappear within a short time after the exposure. Most important, RCAs do not act at the CNS level; therefore, they have lower toxicity and a wide margin of safety when compared with incapacitating agents. In fact, RCAs are not listed in the CWC schedules. Although the Convention prohibits its use as a method of warfare, it does not prohibit its use for law enforcement purposes.

Toxic Industrial Chemicals

TICs are of special interest in the chemical terrorism context because of the fact that they are widely available in large quantities, and even an attack against a chemical plant or transport vehicle may result in a release with potentially catastrophic consequences similar to the release of methyl isocyanate (a chemical used in the production of carbamate pesticides, as well as in the production of rubbers and adhesives) in Bhopal (India) in 1984.

The importance of TICs is reflected in the fact that the CWC features a schedule that includes chemicals that may be produced in large commercial quantities for industrial purposes, but that have been produced, stockpiled, or used as CWs. Schedule 3 includes phosgene, a lung-damaging agent, and some cyanides. Cyanides are commonly named "blood agents," something that may cause confusion, leading one to think that cyanides affect the oxygen transportation in the blood. The reason for calling cyanides blood agents was because chlorine and phosgene were toxic chemicals with local effects in the respiratory tract, whereas cyanides had to be absorbed in the lungs and passed to the blood to produce systemic effects. Although the toxicological mechanism of action of cyanides is well-known now, it is not rare to see them as blood agents when mentioned in the CWs or chemical terrorism context.

Toxins

Toxins are chemical substances of biological origin, although synthesis procedures for some nonprotein toxins that have been studied as weapons are widely available (Jacobi et al., 1984; Tanino et al., 1977). Toxins are included in the CWC because it covers toxic chemicals "regardless of their origin or of their method of production." Actually, two toxins, ricin and saxitoxin, are explicitly included in Schedule 1 of the CWC. However, toxins are also included in the Biological and Toxin Weapons Convention (http://www.opbw.org, accessed February 21, 2014). For these reasons, toxins can be considered CWs, biological weapons, or mid-spectrum agents.

EXTORTION ACTIVITIES WITH CWs

Throughout history, there have been extortion activities during which toxic chemicals have been used. For example, at the end of 1982, there were seven fatal cases of cyanide poisoning after the ingestion of acetaminophene-tampered capsules. McNeil Consumer Products had to destroy approximately 22 million units and changed the production from capsules to tablets that were more difficult to tamper with (Dunea, 1983; Wolnik et al., 1984). Those responsible were never identified, and some imitators in the United States started contaminating medicines and food products with toxic chemical substances.

In some cases blackmailers have even attacked governments. For example, on March 2, 1989, an anonymous person called the US and Japanese embassies in Santiago, Chile, alerting them that cyanide had been injected in fruit destined to be exported to those countries (Grigg and Modeland, 1989; Spiers, 2000; Wilkening, 1999). Although countermeasures were not taken initially, after a second call to the US embassy on March 17, the US Food and Drug Administration (FDA) decided to start inspections at the Philadelphia port, the port of entry of approximately 80% of the fruit from Chile. After an extensive search, only two grapes that had been injected with a small quantity of sodium cyanide were found. However, as a preventive measure, on March 13, all fruit coming from Chile was placed on quarantine. Subsequent searches did not find more tampered fruit and the quarantine ended on March 17. Although the event ended without any poisoning cases, it meant losses of approximately $300 million for Chile's fruit export market.

Since 2009, there have been suspected mass poisoning attacks on girls' schools in Afghanistan (Pita and Gunaratna, 2010). The victims have suffered nausea, vomiting, and unconsciousness, but there have been no deaths reported or confirmations that chemical attacks had taken place. The Taliban, which opposes female education, has denied responsibility.

STATE TERRORISM

CWs have also been used in the assassination of selective targets by Secret Services. The defection of KGB's Capt. Nikolai Khohhlov in February 1954 brought to light some of the devices created for this type of attack. In fact, Khohhlov defected in the middle of a mission to assassinate Georgi Okolovich, a Soviet dissident living in West Germany, by using a cigarette pack that concealed a hydrogen cyanide ampoule (Andrew and Gordievsky, 1990). Similar cases include the killing of two Ukrainian exiles in Munich in 1957 and 1959 by a KGB agent who used a special gun device to disperse hydrogen cyanide. According to Vadim Birstein, since the 1920s and at least until the late 1970s, different laboratories in the Soviet Union were in charge of the development of assassination devices for their Secret Services (Birstein, 2001). The 2004 dioxin poisoning of Viktor Yushchenko, former Ukrainian president, and the 2006 polonium-210 poisoning of Alexander Litvinenko show that these type of activities are not something of the past (le Polain de Waroux et al., 2011; Sorg et al., 2009).

One of the most bizarre stories of assassination is the murder of Georgi Markov, a Bulgarian journalist exiled in the United Kingdom, with an umbrella that delivered ricin (Crompton and Gall, 1980; Knight, 1979). The umbrella was allegedly modified to fire a small pellet filled with approximately 500 μg of ricin. On September 7, 1978, Markov was waiting for a bus on Waterloo Bridge when he felt a strong puncture in the back of his right thigh. When he turned around, a man with an umbrella apologized. The next day he was hospitalized with fever, vomiting, speaking difficulties, and a white cell count of 10,600/mm^3 that increased to 33,200/mm^3 3 days later. On September 11, Markov died of cardiac failure. The autopsy revealed the presence of a small spherical pellet with a diameter of 1.53 mm that had two holes of 0.34 mm. Days before Markov was attacked, on August 26, another Bulgarian exiled in Paris, Vladimir Kostov, also felt a puncture in his back and had to be hospitalized for 12 days, although he finally recovered. On September 26, a similar pellet—spherical, of 1.52 mm diameter, with two holes of 0.34 mm—was extracted from his back.

No public reports about the identification of ricin have been published, but the ricin link was made based on the (Birstein, 2001; Waller et al., 1966) differential diagnosis by the United Kingdom's Ministry of Defense scientists (they even administered ricin to a pig to compare the clinical signs of the poisoning with the ones observed in Markov), histopathological findings that were similar to those found in different *in vivo* tests with animal models, and intelligence regarding a ricin program in the Soviet Union and perhaps other Warsaw Pact nations.

Years later, former KGB members stated that the umbrella was provided by the KGB to the Bulgarian Secret Services, and previous attempts to kill Markov had been unsuccessful (Andrew and Gordievsky, 1990; Kalugin, 1994). Because Bulgarian documents about the Markov case seem to have been destroyed, only future KGB document releases would disclose more information about this murder (Carus, 2002). However, the former Warsaw Pact Secret Services were not the only ones to perform such programs, as seen during the appearances of CIA officials in the Senate Committee known as the Church Committee, which took place during September 16–18, 1975. Former CIA Director William Colby explained that the CIA had an incapacitating agents program (known as MKULTRA) and provided documents detailing the CIA stock of CWs at that time, which included small quantities of BZ, toxins, and cyanide pills, among others (Select Committee to Study Governmental Operations with Respect to Intelligence Activities, 1976).

NATIONALIST AND SEPARATIST TERRORIST GROUPS

Secular terrorist groups have not showed special interest in CWs, perhaps thinking that its use may be rejected by their own followers (although many of them have performed terrorist attacks with conventional explosives that produced a high number of casualties). There are some reported cases indicating that nationalist and independent groups have used chemical substances in sabotage actions. For example, in 1992 the Kurdistan Workers' Party (PKK) allegedly contaminated several water tanks with sodium cyanide in an air base near Istanbul. The attack was aborted when the recipients of 25 kg of potassium cyanide were found next to the tanks (Karasik, 2002).

Another example can be found in the Liberation Tigers of Tamil Eelam (LTTE) in Sri Lanka. Although the LTTE was a secular terrorist group, their members showed a level of veneration for their leader, Velupillai Prabhakaran, akin to that of a religious cult, and even used tactics used by jihadist terrorist groups. LTTE members were known to carry sodium cyanide capsules that had to be used to commit suicide in case of capture. There are also some reports about the use of sodium cyanide against Sri Lanka's economic interests (Carus, 2002; Wilkening, 1999). The first report is from 1986, which mentions attempts to sabotage tea exporting by informing different embassies that some lots were contaminated with cyanide. US authorities analyzed different lots but did not find anything strange. The second report is from December 1996, when potassium cyanide was

allegedly applied to stamps used by the Sri Lanka Army. But the most important LTTE chemical attack occurred in June 1990, using drums of chlorine against a military camp located at east Kiran, although there is no information about the number of casualties (Hoffman, 2009).

LEFT-WING TERRORIST GROUPS

In May 1975, the Baader-Meinhof Gang, also known as the Red Army Faction (RAF), a left-wing terrorist group based in West Germany, threatened to use mustard gas in Stuttgart and other cities in Germany if imprisoned members were not freed. However, in-depth studies of this incident have found no evidence of the access of the RAF to mustard gas (Claridge, 2000). The terrorist organization was taking advantage of media reports indicating that old mustard gas containers from World War I had disappeared from a CW destruction facility.

Colombian guerrillas (initially left-wing terrorist groups but today are considered narcoterrorism groups because of their association with organizations that deal with illicit traffic of drugs of abuse) have performed small attacks with CWs. On December 2, 2001, members of the Revolutionary Armed Forces of Colombia (FARC) attacked a police station in San Adolfo (Huila), probably with cyanogen chloride, killing four policemen. Since then, munitions charged with cyanide have been found in raids against FARC camps. In fact, in November 2007, Colombia's National Police showed their concern after finding a clandestine FARC laboratory near the frontier with Ecuador that was working on the filling of rockets with toxic chemicals (Pita, 2008). The chemicals included ammonia, chlorine, and cyanide compounds.

RIGHT-WING TERRORIST GROUPS AND LONE WOLVES

It is not uncommon to find criminal activities using CWs linked with right-wing groups or sympathizer lone wolves. The two most common agents used have been cyanides and ricin. Actually, hydrogen cyanide production and ricin extraction procedures are commonly found in "cookbooks," publications that are popular among members of white supremacist groups and in "amateur terrorist" circles. Some of these titles include *Assorted Nasties*, *The Preparatory Manual of Chemical Warfare Agents*, *The Poisoner's Handbook*, and *Silent Death*.

Although hydrogen cyanide gas (boiling point at 760 mmHg, 25.7°C; vapor pressure at 20°C, 600 mmHg) is easy to obtain by mixing the right cyanide salt and acid, transporting and mixing the reagents without being discovered constitutes one of the biggest hurdles in terrorist attacks. Regarding the ricin extracted with procedures included in cookbooks, it is not able to achieve a good product for causing a large number of casualties by any exposure route, mainly because of the low content of toxin of the final extracts (Pita et al., 2004).

In the mid 1980s, a group named the Covenant, the Sword, and the Arm of the Lord obtained potassium cyanide to contaminate water supplies of different US cities (Stern, 2000). In a raid that took place on one of their facilities, authorities found a drum with 30 gallons of potassium cyanide. When one of its members was told that the attack would kill not only those he considered enemies (Jews and "mud-people") but also other people, including members or sympathizers of the group, his reply was: "We felt that God would take care of this [and] that those who were meant to die would be poisoned" (Stern, 2000). And when he was told that 30 gallons were not enough to obtain a toxic concentration in the city's water reservoir he answered: "God would… make sure the poison got to the town" (Stern, 2000). He even explained that they had decided to act because there were signs of the arrival of the Armageddon: "You get tired of waiting for what you think God is planning" (Stern, 2000). These statements make it clear that terrorist groups with religious motivations most of the time have no moral restraints in performing attacks to cause a large number of casualties. In fact, Christian-identity right-wing groups and lone wolves have also carried out terrorist attacks that have caused a large number of casualties, like the April 19, 1995 bombing of the Alfred P. Murrah Federal building in Oklahoma that caused 168 fatalities and more than 500 nonlethal casualties.

In 1991, four members of the Minnesota Patriots Council, an anti-government right-wing group, obtained a kit called "Silent Tool of Justice" that contained approximately one dozen castor plant seeds and a toxin extraction procedure that one of the members followed. The plan was to mix the toxin with dimethyl sulfoxide (DMSO) and aloe vera gel and apply it on doorknobs of different individuals that were considered enemies of the group (Tucker and Pate, 2000). The four members were arrested in 1994.

Cases of lone wolves with intentions of using CWs are not rare. For example, in the mid 1970s, authorities found 25 pounds of sodium cyanide at the apartment of Muharem Kurbegovic, the "Alphabet Bomber" (Simon, 2000). Other chemicals and documents found showed a special interest in CWs. Kurbegovic had threatened to use nerve agents, but he did not have any when arrested. A good example of a lone wolf linked to right-wing groups is Thomas Leahy (Carus, 2002). In 1997, an enforcement officer in Wisconsin responded to a report of spousal abuse made by Leahy's wife. When the officer arrived, the wife opened the door wearing a mask and explained the reason to the officer: "Because my husband is in the basement making poison gas" (Gurr and Cole, 2000). Among

the items found in the laboratory were some cookbooks, ricin extracts, and three spray bottles with a mix of nicotine and DMSO. Also, in 2004, an arms trader with white supremacist group connections was sentenced to 11 years in prison (Kosal, 2006). In April 2003, authorities had raided his Texas warehouse arsenal and found that he was building an improvised chemical device (ICD) based on the mixing of sodium cyanide and an acid.

APOCALYPTIC CULTS: AUM SHINRIKYO

Although Aum Shinrikyo sarin attacks are detailed in Chapter 4, the Japanese cult is a clear example of how difficult it is to produce a CWA and use it as a weapon with an efficient dissemination system, especially taking into account that the cult had adequate financial resources and technological means. The cult also benefited from the 1951 Religious Corporation Law that grants tax exemptions to religious organizations in Japan and protection against possible interference of the state in their activities. This advantageous situation allowed Aum Shinrikyo, when it started its chemical, biological, and toxin programs, to enjoy a position that would be similar to that within a proliferating state—where there is no need to hide these activities from the security forces because the program is integrated within government activities—rather than a terrorist organization.

But sarin was not the only CW used by the cult, which even had a biological weapons program, although it was less successful than the chemical one. After the Tokyo sarin terrorist attacks in 1995, Aum Shinrikyo cult members tried an attack with hydrogen cyanide. On May 5, they used an ICD in Shinjuku's Tokyo subway station that consisted of two plastic bags, one with sodium cyanide suspended in 2L of water and another one with 1.5L of a sulfuric acid solution (Dolnik and Gunaratna, 2008; Tu, 2002). The activation system consisted of two condoms, one with sodium chlorate and another with sulfuric acid, so when the latter ate the latex the fire produced would break the plastic bags and allow the mixing of the cyanide salt and the acid, producing hydrogen cyanide. However, the quantities were not well-calculated and the fire destroyed the ICD. On July 4 and 5, another two attacks occurred in Tokyo, this time using an ICD in which the activation of an electrical device with blades would break the plastic bags. One ICD failed, while the other only produced one case of mild poisoning.

JIHADIST TERRORISM: AL QAEDA

Al Qaeda's WMD Intentions

There are three phases in the statements of Al Qaeda members related to WMD. In the first phase, Al Qaeda tended to justify the acquisition and possession of these weapons from the point of view of deterrence. This phase goes as far back as 1998, when Osama bin Laden had stated that acquiring WMD was a "religious duty" (Pita, 2007). This and similar statements were made by bin Laden in different interviews after the US attack on the Al Shifa Pharmaceutical Industries factory in Khartoum on August 20, 1998. This attack was part of Operation Infinite Reach in retaliation for the bombings of the US embassies in Kenya and Tanzania on August 7, 1998, for which the bin Laden terrorist network was blamed by US officials. The Al Shifa target was justified in the finding of *O*-ethyl methylphosphonothionate, a precursor of the nerve agent VX, in soil samples outside the factory, and in the financial contributions of bin Laden to the production of CWs. Soon after 9/11 and the mailings of envelopes with *B. anthracis* spores in the United States, bin Laden was interviewed again; when asked about reports claiming that he was trying to acquire WMD, he answered: "We have the weapons as deterrent" (Pita, 2007).

The second phase of statements of Al Qaeda members related to WMD began soon after the overthrow of the Taliban regime in Afghanistan. Al Qaeda's reasoning was that the Coalition Forces had used conventional weapons (e.g., missiles) that had caused a large number of casualties and destruction, and for this reason these weapons could be considered WMD. This interpretation justified the use of chemical, biological, radiological, and nuclear (CBRN) weapons as retaliation for similar attacks. The most well-known statement in this second phase was made by Suleiman Abu Gheith, Al Qaeda's spokesman, who wrote in his 2002 electronic article "In the Shadow of the Lances" that based on this reasoning they had the "right to kill four million Americans—two million of them children—and to exile twice as many and wound and cripple hundreds of thousands" (Pita, 2007).

The third phase started in May 2003, when Shaykh Naser bin Hamad Al Fahd, a Saudi cleric who supports the global jihad movement, issued a fatwa justifying and authorizing the use of WMD (Paz, 2005). Al Fahd used arguments based on reciprocity, stating that the United States had used weapons that caused a large number of casualties and mass destruction. But what was new in this fatwa was that Al Fahd's arguments were also based on Islamic texts that supposedly justify that it is permissible to use WMD if those engaged in jihad decide there is benefit in using them. And this is the case of Al Qaeda's influential strategist Mustafa Setmarian Nasar, better known as Abu Musab Al Suri, who posted a letter on the Internet in December 2004 stating that the use of WMD was "a necessity."

Al Qaeda's interest in WMD may also be based on their important psychological effects. An objective of using these weapons in a military scenario is not only to

cause physical casualties but also to demoralize troops. Similarly, in a terrorist attack on civilians, one of the primary goals is to create a general sense of panic and fear, resulting in psychological trauma and disruption of economic and social activities (Zanders, 2003). For these reasons, WMD can also be regarded as "weapons of mass *disruption*." For instance, it is frequently asserted that the Aum Shinrikyo sarin subway attack caused more than 5,000 casualties, but actually only approximately 1,000 patients had clinical signs of sarin exposure (Woodall, 1997). That means that approximately 4,000 people who sought attention in medical facilities were, in fact, mainly psychological casualties with psychogenic symptoms. Based on a book by Abu Walid Al Misri, editor of a magazine for the Taliban, quoted in Peter Bergen's book *The Osama bin Laden I Know* (2006), Al Qaeda has been aware of the psychological effects of WMD since they first thought about acquiring them.

Al Qaeda's CWs Capabilities

The first information of Al Qaeda's CWs programs came from Jamal Ahmed Al Fadl (an Al Qaeda member who defected and became a US government informer in 1996), who claimed that in the early 1990s he and other Al Qaeda members discussed the start of a manufacturing program of CWs with a Sudanese army officer (Bergen, 2001; Clarke, 2004; Tucker, 2006). In fact, the Al Shifa facility would have been part of this program.

Since October 2001, reporters and military forces in Afghanistan found written and electronic documents with rudimentary procedures for the production and use of toxic chemicals (Pita, 2007). These procedures are similar and, in some cases, word-for-word translations from the ones included in the cookbook publications mentioned previously. Actually, two well-known cookbooks were found in Afghanistan, *Assorted Nasties* and *The Poisoner's Handbook*. This material came mostly from the Abu Khabab camp located in the Darunta training camp complex that specialized in explosives and toxic chemicals training (Gunaratna and Acharya, 2006). This camp was named after the man who ran it, the Egyptian Midhat Mursi, commonly known as Abu Khabab, who was killed in a US air strike on the Pakistan–Afghan border in July 2008.

Ahmed Ressam, an Algerian arrested by US authorities for carrying explosives that he intended to use in a bombing against the Los Angeles International Airport, explained in court in July 2001 that he had been trained in the Darunta training camp complex in 1998 to prepare hydrogen cyanide by mixing a cyanide salt and sulfuric acid (Pita, 2007). He was told to release it near the air intake vents of buildings and even participated in live training exercises using dogs. In August 2002, Cable News Network aired several Al Qaeda videotapes obtained in Afghanistan that revealed experiments with chemical agents on dogs. One of those tapes showed several men—apparently after having mixed several chemical reagents—rushing out of an enclosure, inside which was a tied-up dog. Soon, a white vapor appeared and a few moments later the dog started showing the first clinical signs of exposure. The quality of the images was not good enough to identify a toxidrome, but the videotape was reminiscent of what Ahmed Ressam said about his training with hydrogen cyanide in Afghan camps. These experiments were allegedly recorded by Abu Khabab in the Darunta training camp complex. In November 2006, a book published by Nasiri (2006), the pseudonym of an alleged informer of the British and French intelligence services, explained his participation in experiments with animals using cyanide at the Khalden training camp. Nasiri describes the failed attempts to develop mustard gas munitions in Khalden. He states that after many trials, finally the camp members celebrated when the shell produced a "thick cloud of smoke." However, this does not mean that the munition was effective in disseminating mustard gas.

One of the most relevant discoveries in Afghanistan regarding the chemical and biological weapons program was made by *Wall Street Journal* reporter Alan Cullison, who obtained two computers from a looter who allegedly stole them from Al Qaeda's central office in Kabul on November 12, 2001 (Cullison, 2004; Cullison and Higgins, 2001). The looter told Cullison he had found the computers in the office of Al Qaeda's military commander Muhammad Atef (aka, Abu Hafs), a strong supporter of Al Qaeda's acquisition of WMD, who was killed in a US air strike that same month. Computer files included information of Al Qaeda's effort to start a chemical and biological weapons program code-named "Al Zabadi" ("yogurt") in May 1999 with an initial budget of only $2,000–4,000. Based on Cullison's analysis of the computer files, Ayman Al Zawahiri—Al Qaeda's second-in-command at that time—and Abu Hafs (assisted by Abu Khabab) started the program after studying different books and articles from biomedical journals.

According to former CIA Director George Tenet, Al Qaeda became interested in chemical and biological weapons after Aum Shinrikyo's 1995 sarin attack on the Tokyo subway (Tenet and Harlow, 2007). But an electronic message sent by Al Zawahiri to Hafs in 1999 stated that it was "the enemy" who brought these weapons to his attention, possibly US Secretary of Defense William Cohen (Leitenberg, 2004, 2005). In November 1997, Cohen appeared on television showing a 5-pound sugar package and saying that if it were to contain spores of *B. anthracis* and were spread over Washington, DC, half the city's population would die. A photograph of Cohen holding the 5-pound sugar package was allegedly also found in Afghanistan (Leitenberg, 2004).

No public report of sophisticated CBRN means or production facilities found in Afghanistan has yet been made. Only a centrifuge and an "oven" found near Kandahar have been presented by the US Department of Defense as the equipment Al Qaeda intended to use to produce chemical and biological weapons (Miller, 2002). This material was part of a laboratory that was being built for the production of *B. anthracis* spores (Pita and Gunaratna, 2009).

Based on intelligence assembled from collected documents, detainee interviews, and reconnaissance of Al Qaeda facilities during Operation Enduring Freedom, the Commission on the Intelligence Capabilities of the United States Regarding Weapons of Mass Destruction (US WMD Commission) concluded in its unclassified report that Al Qaeda did not have large-scale chemical and biological weapons capability (US WMD Commission, 2005). Still, past and current chemical and biological programs are said to be not fully understood, especially because of difficulties in penetrating the terrorist network and, therefore, in collecting human intelligence (HUMINT).

After the disappearance of the Afghan training camps, the Internet and jihadist websites have acquired more relevance. Al Suri's book, *The Global Islamic Resistance Call*, posted on the Internet in 2005, suggests an asymmetric approach that includes the use of WMD as well as a decentralized and diffused global jihad in which autonomous cells play an important role. Autonomous cells were to be self-sufficient, including having training capabilities. For these reasons, jihadist websites are important tools providing autonomous cells with training manuals as well as lessons learned from attacks by other cells. These electronic training manuals include information and procedures about toxic chemicals similar to those found in Afghanistan, that is, similar to the information included in cookbooks. Some of these websites offer scanned copies of these cookbooks.

Al Qaeda Plots with CWs

A detailed study of incidents with CWs linked to Al Qaeda shows that cyanides, ricin, and TICs have been the main choices of jihadist terrorists (Pita, 2007). Nerve agents also seem to be of interest, especially because of their toxicological and physico-chemical properties, which make them ideal for tactical use in terrorist attacks. However—and as previously mentioned—the synthesis process requires some level of expertise and is far more complex than the recipes featured in jihadist manuals.

Nerve Agents

Open sources have reported jihadist cells planning terrorist attacks with nerve agents and showing interest in these CWAs; in any case, they had the chemical agent or proved that they had acquired the capability to produce it.

The *Daily Telegraph* (London) reported that members of an Al Qaeda cell based in the United Kingdom were arrested while planning to release sarin in the European Parliament building in Strasbourg in February 2001, although no actual evidence of sarin was found (Bamber et al., 2001). Also, in February 2001, the *Daily Telegraph* reported that British police had foiled a plot involving sarin against the London underground (Hastings and Bamber, 2001). Once again there was no evidence of the actual presence of the nerve agent. Both incidents seemed to be linked and related to a transnational network of Al Qaeda cells in the United Kingdom, Germany, Italy, Belgium, France, and Spain. What these cells had in common was that their key members had been trained in Afghan camps in the mid to late 1990s. The network was planning terrorist attacks with conventional weapons and chemical substances. In a conversation on March 13, 2001 that was bugged, a member of the Italian cell mentioned "an extremely efficient liquid that suffocates people," suggesting it could be placed in tomato cans, exposing people when the cans were opened (Finn and Delaney, 2001). Referring to the German cell, he mentioned, "They arrested them while they were preparing the gas." The chemicals this cell possessed were acquired in 48 separate purchases at pharmacies but were, in fact, intended to be used in improvised explosive devices (IEDs), not in nerve agent production (Finn, 2002; Finn and Delaney, 2001; Pita, 2007).

On November 14, 2003, the following headline appeared in the Spanish newspaper *La Razón* (Madrid): "The FBI believes that the Salafists intended to mix napalm with sarin gas" (Arnuero, 2003). This was in reference to the chemical substances found in possession of a supposed Al Qaeda–related cell whose members were arrested in Barcelona in January 2003. However, sarin was not present among the chemicals, nor were any of its precursors. Later, it was made public that the Spanish cell, helped by other cells based in France and the United Kingdom, was planning a chemical attack against a naval base in Rota (Spain), used jointly by Spain and the United States (Martínez and Muñoz, 2005).

Based on Iraq Survey Group investigations, the special advisor to the director of central intelligence on Iraq's WMD reported on September 30, 2004, that the Al Abud network (a network of Iraqi insurgents) attempted to produce tabun, nitrogen mustard, and ricin from late 2003 to mid 2004 (Duelfer, 2009; Iraq Survey Group, 2004). These attempts failed, but they did fill nine mortar rounds with malathion, an organophosphate insecticide.

Based on an internal British police document and on information disclosed by a UK senior officer, the *Sunday Times* (London) reported in August 2005 that Scotland

Yard had thwarted an Al Qaeda attack on the House of Commons with "chemicals, a dirty bomb, and sarin gas" (Leppard and Winnett, 2005). The report mentioned that the plot was discovered after decoding encrypted e-mail messages in 2004, but there was no mention of the actual presence of the agents. This report was presumably linked to the arrest of eight men in August 2004 in the United Kingdom who were charged with conspiracy to commit public nuisance by the use of "radioactive materials, toxic gases, chemicals, and/or explosives" (Pita, 2007). No chemicals were found, although one of the arrested men, Dhiren Barot, had two notebooks with information of explosives and toxic chemicals as well as reconnaissance information of financial facilities in the United States. Later, Barot was reported to have been a trainer in Afghanistan camps in the late 1990s.

More recently, in June 2013, the Iraqi Defense Ministry said that they foiled a plot by an Al Qaeda cell to use remote-controlled toy planes to disseminate CWs, including sarin and mustard gas (Roggio, 2013). The cell was allegedly planning to execute the attacks in the Middle East, Europe, and North America. However, when the five cell members were arrested they had not yet been able to produce any CWA.

Cyanides

The first case of an Al Qaeda terrorist attack linked to chemical terrorism is the February 1993 World Trade Center bombing, as it is commonly believed that the explosives were mixed with a cyanide compound. The reason is that during the trial the judge stated: "You had sodium cyanide around, and I'm sure it was in the bomb. Thank God the sodium cyanide burned instead of vaporizing. If the sodium cyanide had vaporized, it is clear what would have happened is the cyanide gas would have been sucked into the north tower and everybody in the north tower would have been killed. That to my mind is exactly what was intended" (Parachini, 2000).

This was based on the idea that the terrorists considered the tactic of using a toxic chemical in the attack and on the fact that the FBI found a small bottle of a sodium cyanide solution in a storage shed. However, hydrogen cyanide was never detected in the attack and even a detailed case study of this event concludes that no toxic chemical was mixed with the explosives (Parachini, 2000).

On February 19, 2002, four Moroccans, members of the Salafi Group for Preaching and Combat (GSPC, now Al Qaeda in the Islamic Maghreb) were arrested in Rome (Pita, 2007). They had approximately 4 kg of potassium ferrocyanide that they intended to use to contaminate the water supplies near the US embassy. Nevertheless, that particular substance is widely used as a food additive (E 536) and, because of its toxicological properties, was probably not the best choice to use in a chemical attack.

Based on information in Ron Suskind's *The One Percent Doctrine*, an Al Qaeda cell based in Saudi Arabia planned an attack on the New York City subway with Al Mubtakkar devices (Suskind, 2006). Basically, the Al Mubtakkar device is an ICD supposed to work as a crude binary munition (a binary chemical munition is one in which chemical substances held in separate containers react when mixed or combined as a result of being fired, launched, or otherwise initiated to produce a CW). It produces hydrogen cyanide when a barrier that separates the cyanide salt (potassium cyanide) and the acid (hydrochloric acid) is broken. Potassium permanganate is also included for the device to potentially produce a mix of hydrogen cyanide, cyanogen chloride, and chlorine. The device can be activated manually or by using the explosive triacetone triperoxide (commonly known as TATP) and a detonator. The detonator can be activated remotely or with a temporizer, allowing terrorists to escape; however, the probability is high that, if not well-regulated, the explosion will inactivate the chemical reagents and obviate production of the cyanogen agent.

Suskind (2006) states that the cell members had traveled to New York City from North Africa in the autumn of 2002, and had even carried out reconnaissance missions to identify targets. Surprisingly, when Al Qaeda's leader in Saudi Arabia, Sheikh Yousef Al Ayiri, told Al Zawahiri about the plot in January 2003, the latter decided to cancel the operation. Suskind's book claims that this decision was made just 45 days before the intended attack.

Ricin

Ricin was reported to have been detected in January 2003 in an apartment in the north of London where North African Al Qaeda sympathizers were living. The raid took place when the United Kingdom received a tip-off from the Algerian intelligence services indicating that "poison" had been prepared there. This ricin finding turned out to be a false positive, because subsequent analyses of samples by the British reference laboratory did not identify ricin (Leitenberg, 2005). However, 20 *Ricinus communis* seeds and a written ricin extraction procedure—copied from a cookbook and downloaded from the Internet—were found (Stenersen and Lia, 2007). The alleged plot was to use ricin on door handles (perhaps with DMSO). The fingerprints on the material and the handwriting analysis identified Kamal Bourgass, an Algerian refugee who had applied for asylum in the United Kingdom in 2000. It seems (but is not clear) that he attended training camps in Afghanistan. Of all the arrested men, only Bourgass was convicted, on April 8, 2005, of conspiring to commit a public nuisance by the use of poisons and/or explosives. At an earlier trial in 2004, he had been convicted of killing a police officer

during his capture in an apartment in Manchester on January 14, 2003.

Intelligence reports from 2010 mentioned that Al Qaeda in the Arabian Peninsula was trying to acquire large quantities of *R. communis* seeds in Yemen to produce ricin (Schmitt and Shanker, 2011). No further information about this has been made public.

Toxic Industrial Chemicals

On March 30, 2004, anti-terrorism police in the United Kingdom arrested eight alleged sympathizers of Al Qaeda who were supposedly planning to use osmium tetroxide against Gatwick Airport, the London subway, and other enclosed high-traffic areas, although they did not have the chemical when arrested (Baker and Kosal, 2004). Osmium tetroxide is often used as a stain in biology laboratories. One month later, Jordanian authorities announced that they had defeated an Al Qaeda plot to use explosives and large quantities of TICs such as sulfuric acid, cyanide salts, and pesticides against the US embassy in Amman, the Jordanian prime minister's office, and the headquarters of the Jordanian General Intelligence Department (Levitt and Sawyer, 2004).

From October 2006 until mid 2007, suicide terrorists in Iraq detonated vehicle-borne IEDs carrying chlorine cylinders (Pita, 2012). This new tactic clearly shows that the use of TICs is an option that may yield better results than following the crude and rudimentary procedures of Al Qaeda–related publications for the production of CWs. However, the chlorine attacks in Iraq were still rudimentary in their means of delivery. In most of the attacks casualties were not caused by chlorine exposure, but rather because of the mechanical and thermal effects of the explosion. Also, in some attacks chlorine was not released because the low mechanical effect of the explosion was not enough to break the cylinders.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Nerve agents are the most important CWs because of their high toxicity and versatility in tactical use. However, the production difficulty seems to be their most important drawback. Amateur production of CWAs or extraction of toxins by using cookbooks and Al Qaeda–related manuals has commonly been overrated, leading to supposedly catastrophic consequences of terrorist attacks. In fact, CWs are frequently described as "the poor man's atomic bomb," but this description was used at the end of World War II to explain that for a state actor tying to acquire WMD capability, chemical and biological weapons would be a more feasible option than nuclear weapons, which require more complex technical resources.

An additional problem for terrorists is the need to have a reliable delivery system. Effective dissemination may be even more difficult than obtaining the agent, especially if the objective is to cause a large number of casualties. The "art" of CWs includes the research and development of special munitions that, among other things, do not inactivate the agent by the thermal effect of the explosion. Aerosolization dispersal systems are another option. Luckily, another gap in the information in the Al Qaeda–related publications and cookbooks is the often inaccurate information regarding delivery systems.

We should also take into account advances in science and technology, because some of the drawbacks in the production of CWs and dissemination systems may disappear in the future, making re-evaluation of threat assessments necessary. The lack of adequate procedures and programs for the production of CWs may explain why, until now, jihadist terrorists have not been capable of achieving an effective chemical attack. But it is clear that they are both interested in CW capability and are actively trying to obtain it. For this reason, it should not be dismissed that options other than self-production may also be attempted, for example, resorting to sponsoring states or black-market arms trafficking networks. In fact, the possibility that Syrian CWs could have fallen into the hands of terrorist groups during the ongoing civil war should be seriously considered.

The ready access to TICs in the chemicals industry makes them attractive to nonstate actors also. The threat of insiders who work with toxic chemicals and whose integrity may be compromised by terrorist groups is one of the main concerns of intelligence services today. Insiders who decided to participate in chemical terrorism could have a variety of motivations, from ideological to financial. Advances in science and technology have also increased the number of people working with toxic "dual-use" chemicals and with the explicit and tacit knowledge that could be used for terrorist purposes.

In conclusion, analysis of the chemical terrorism threat shows how important it is that medical personnel consider CWs poisoning in the differential diagnosis in cases of suspected chemical terrorist attacks and have knowledge of CWAs, including their effects and medical treatment.

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SECTION II

AGENTS THAT CAN BE USED AS WEAPONS OF MASS DESTRUCTION

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CHAPTER

8

Mustards and Vesicants

Robert A. Young and Cheryl B. Bast

INTRODUCTION

In the simplest terms, vesicants are chemicals that cause tissue blistering. Their toxic activity is not limited to the skin, however, and their mode of action is complex. These cytotoxic alkylating agents were initially developed as chemical weapons used to induce ocular, dermal, and respiratory damage that results in immediate casualties, reduction in fighting efficiency, and demoralization. Depending on the exposure, injury may be local or systemic. This chapter will focus on sulfur mustards, nitrogen mustards, and lewisite. (Although occasionally classified as a vesicant due to its action as a skin, eye, and respiratory tract irritant, phosgene oxime (CX) is more appropriately considered an urticant, or nettle agent, and is not discussed here.) Extensive information regarding the chemistry and toxicology of vesicants is available in several publications (Papirmeister et al., 1991; Somani, 1992; USACHPPM, 1996; ATSDR, 2003; Romano et al., 2008). In response to a request by the U.S. Department of Veterans Affairs (VA), a panel of experts extensively reviewed and evaluated the medical and scientific literature on mustard agents and lewisite, as well as the military testing programs relative to these agents (IOM, 1993).

The term *sulfur mustard* may refer to distilled mustard (bis(2-chloroethyl)sulfide; HD, sesqui mustard (SM)), Levenstein mustard (H), agent HT (a mixture of HD and bis(2-chloroethylthioethyl)ether), or a sulfur mustard–lewisite mixture (HL). Distilled mustard (HD) is relatively pure (97%) bis(2-chloroethyl)sulfide and results from the vacuum distillation of HD. Generic references to sulfur mustard usually mean HD. Levenstein mustard (H) is a mixture of HD and sulfur impurities (generally in a 70:30 ratio), and as its name implies, it was produced by the Levenstein process, which involves reacting ethylene with sulfur chloride. Agent H may contain sulfur impurities imparting a yellowish color and sweet, garliclike odor.

Agent HT is generally a mixture of 60% HD and 40% bis(2-chloroethylthioethyl)ether (T), although this ratio may vary. Agent HL is a mixture of sulfur mustard (HD) and lewisite (L) that was developed for cold weather or high-altitude use due to its lower freezing point. SM is 1,2-bis(2-chloroethylthio) ethane and is considered a more potent vesicant than HD, but its very low vapor pressure limits its effectiveness as a warfare agent. That condition is remedied by combining it with HD to form agent HQ. The removal of one chlorine from sulfur mustard results in "half-mustard" (2-chloroethyl ethyl sulfide, or CEES), a monofunctional sulfur mustard analog. Although retaining some alkylating properties, half-mustard is not as highly regulated as sulfur mustard and is frequently used in sulfur mustard research. Most of the discussion of sulfur mustards in this chapter will be about distilled sulfur mustard (HD).

Nitrogen mustards include HN1 [bis(2-chloroethyl) ethylamine], HN2 (2,2′-dichloro-N-methyldiethylamine), and HN3 [tris(2-chloroethyl)amine hydrochloride]. As will be discussed later in this chapter, some of these were used in therapeutic arenas rather than in warfare.

Lewisite [L or L-1; dichloro(2-chlorovinyl) arsine] is an arsenical vesicant developed early in the twentieth century. Lewisite occurs as cis- and trans-isomers, the typical ratio being 10:90. Several impurities, including bis(2-chlorovinyl)chloroarsine (L-2) and tris(2-chlorovinyl)arsine (L-3), are typically present. The chemical and physical properties of cis- and trans-isomers are similar.

Sulfur Mustards

Sulfur mustards are chemical vesicants capable of causing severe skin and eye damage at very low concentrations. Table 8.1 shows the chemical name, synonyms, identification codes, chemical formula, and structural formula for sulfur mustard.

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TABLE 8.1 Nomenclature, Chemical Formulae, and Chemical Structure of Sulfur Mustard

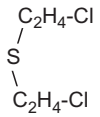
Sulfur Mustard (HD)	
Synonyms	Bis(2-chloroethyl)sulfide;1,1′-thiobis(2-chlorethane); 1-chloro-2-(2-chloroethylthio)ethane; SM; distilled mustard; agent HD; mustard gas; yperite; yellow cross
CAS No.	505-60-2
Chemical formula	$C_4H_8C_{l2}S$
Chemical structure	

TABLE 8.2 Selected Physical and Chemical Properties of Sulfur Mustard

	Value	References
Physical state	Oily liquid	MacNaughton and Brewer (1994)
Molecular weight	HD: 159.08	DA (1996)
Density	5.4	DA (1996)
Boiling point	HD: 215–217°C	Budavari et al. (1989); DA (1996)
Freezing point	HD: 14.5°C	DA (1996)
Vapor pressure (mmHg)	HD: 0.072 mmHg at 20°C; 0.11 mmHg at 25°C	DA (1996)
Water solubility (g/L)	Sparingly soluble in water; soluble in organic solvents	Budavari et al. (1989); DA (1996)

Although frequently referred to as "mustard gas," the chemical is a liquid at normal ambient temperatures. Due to its oily consistency and low aqueous solubility, sulfur mustard is persistent in the environment. Information on the half-life of HD in air is unavailable. As previously noted, impurities may impart a garliclike odor to sulfur mustard. Odor thresholds ranging from 0.15 to 0.6 mg/m^3 have been reported for sulfur mustard (Dudley and Wells, 1938; Bowden, 1943; Fuhr and Krakow, 1945; Ruth, 1986).

Watson and Griffin (1992) have summarized information on the distribution of unitary chemical weapon stockpiles in the United States. The chemical and physical properties of sulfur mustard (agent HD) are shown in Table 8.2.

The water solubility of sulfur mustard has been reported as 0.092 g/100 g water at 22°C (DA, 1974), and 5×10^{-3} M at room temperature (MacNaughton and Brewer, 1994). In dilute aqueous solutions, sulfur mustard hydrolyzes almost completely to thiodiglycol and hydrochloric acid (Papirmeister et al., 1991). For dissolved HD, the hydrolysis half-life ranges from about 4 to 15 min for temperatures of 20–25°C. Bulk HD may persist in water for up to several years (Small, 1984). It has been estimated that it takes 15 days for the mass of a 1-cm droplet of HD in quiescent water to decrease by half (Small, 1984). The Henry's law constant for HD has been estimated to be 2.1×10^{-5} atm m^3/mol (MacNaughton and Brewer, 1994), indicating a moderate potential for evaporation from water.

The persistence of sulfur mustard in soil depends on the soil type, pH, moisture content, and whether the agent is at the soil surface or buried. Small (1984) reported that HD applied to the soil surface volatilized and would likely be the main route of HD loss (with a half-life of about 30 min). In wet soil, however, hydrolysis would be the primary loss pathway. When sprayed onto soil, a vesicant action may persist for about 2 weeks, but when the agent continually leaks into the soil, vesicant action may be present after 3 years (DA, 1974). Rosenblatt et al. (1995) state that the persistence of sulfur mustard in soil is due to the formation of oligomeric degradation products that coat the surface of the mustard agent and that are resistant to hydrolysis. This may greatly enhance the environmental persistence of sulfur mustard. Sulfur mustard has a log K_{ow} of 1.37 and a K_{oc} of 133, indicating that binding to soil organics would limit transport through soil to groundwater (MacNaughton and Brewer, 1994). MacNaughton and Brewer (1994) also calculated a leaching index of 7.2 for HD (i.e., the number of leachings required to reduce the HD soil concentration to one-tenth of the original amount, assuming that for each leaching, 1 kg of soil is in equilibrium with 1 L of water).

Nitrogen Mustards

Nitrogen mustards are tertiary bis(2-chloroethyl) amines with vesicant activity (NDRC, 1946). Tables 8.3 and 8.4 summarize the nomenclature and chemical and physical properties of HN1, HN2, and HN3. Due to their toxicity and various physical–chemical properties, initial interest in these chemicals as warfare agents developed shortly before and during World War II. Although HN2 and HN3 were specifically developed as military agents, HN1 was originally developed as a pharmaceutical. HN2 (mechlorethamine) later found use as an antineoplastic agent. Nitrogen mustards and derivatives such as melphalan, chlorambucil, and cyclophosphamide are alkylating agents used as cancer therapeutic agents (Somani, 1992).

TABLE 8.3 Nomenclature, Chemical Formulae, and Chemical Structures of Nitrogen Mustards

HN-1	
Synonyms	Ethyl-bis(2-chloroethyl)amine; bis-(2-chloroethyl)ethylamine
CAS No.	538-078
Chemical formula	$(ClCH_2CH_2)_2NC_2H_5$
HN-2	
Synonyms	Methyl-bis(-chloroethyl)amine; 2,2′-dichloro-N-methyldiethylamine; "S"; mechlorethamine
CAS No.	51-75-2
Chemical formula	$(ClCH_2CH_2)_2NCH_3$
HN-3	
Synonyms	Tris(-chloroethyl)amine; [tris(2-chloroethyl)amine hydrochloride]
CAS No.	555-77-1
Chemical formula	$N(CH_2CH_2Cl)_3$

TABLE 8.4 Physical and Chemical Properties of Nitrogen Mustards

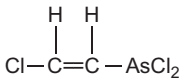
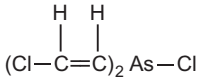
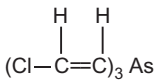
	Value	References
Physical state	HN-1: oily liquid HN-2: oily liquid HN-3: oily liquid	USACHPPM (1996)
Molecular weight	HN-1: 170.08 HN-2: 156.07 HN-3: 204.54	USACHPPM (1996)
Boiling point[a]/ freezing point	HN-1: 194°C/ − 34°C HN-2: 75°C/ − 60°C HN-3: 256°C/ − 3.7°C	USACHPPM (1996)
Vapor pressure (mmHg)	HN-1: 0.25 mm @ 25°C HN-2: 0.43 mm @ 25°C HN-3: 0.01 mm @ 25°C	USACHPPM (1996)
Water solubility (g/L)	HN-1: limited; miscible with organic solvents HN-2: limited; miscible with organic solvents HN-3: limited; miscible with organic solvents	USACHPPM (1996)

[a]*Decomposes prior to reaching boiling point.*

Lewisite

Lewisite is composed of cis- and trans-isomers in the ratio of 10:90 and several impurities including bis(2-chlorovinyl)chloroarsine (L-2) and tris(2-chlorovinyl) arsine (L-3) (Rosenblatt et al., 1975). The chemical and physical properties of the cis- and trans-isomers are similar. The nomenclature and chemical and physical properties of L-1, L-2, and L-3 are presented in Tables 8.5 and 8.6, respectively. In pure form, lewisite is colorless and odorless, but it usually occurs as a brown oily liquid with a distinct geraniumlike odor. Gates et al. (1946) reported an odor threshold of 14–23 mg/m^3 for lewisite.

TABLE 8.5 Nomenclature, Chemical Formulae, and Chemical Structure of Lewisites

Lewisite (L)	
Synonyms	2-Chlorovinyldichloroarsine; (2-chlorovinyl)arsenous dichloride; beta-chlorovinyldichloroarsine; dichloro(2-chlorovinyl) arsine; chlorovinylarsine dichloride; EA 1034
CAS No.	541-25-3
Chemical formula	$ClCH{=}CHAsCl_2$
Chemical structure	
L-2	
Synonym	Lewisite-2
CAS No.	40334-69-8
Chemical formula	$(ClCH{=}CH)_2AsCl$
Chemical structure	
L-3	
Synonym	Lewisite-3
CAS No.	40334-70-1
Chemical formula	$(ClCH{=}CH)_3As$
Chemical structure	

Information regarding the atmospheric transformation of lewisite is limited. MacNaughton and Brewer (1994) reported that some photodegradation may take place and that hydrolysis may also occur in the gas phase. Lewisite is only sparingly soluble in water; 0.5 g/L (Rosenblatt et al., 1975). Hydrolysis of lewisite results in the formation of lewisite oxide and HCl and this may occur rapidly. The hydrolysis of lewisite is complex and includes several reversible reactions (Epstein, 1956; Rosenblatt et al., 1975; Clark, 1989; MacNaughton and Brewer, 1994). Under slightly acidic conditions, lewisite initially undergoes rapid and reversible conversion to dihydroxy arsine, 2-chlorovinyl arsine oxide, and two equivalents of hydrogen chloride:

TABLE 8.6 Physical and Chemical Properties of Lewisite

	Value	References
LEWISITE		
Physical state	Oily, amber brown liquid	Lindberg et al. (1997)
Molecular weight	170.08	USACHPPM (1996)
Boiling point	190°C	Trammell (1992)
Freezing point	−18°C; varies depending on purity	Watson and Griffin (1992)
Vapor pressure (mmHg)	0.34 mmHg at 25°C; 0.22 mmHg at 20°C	USACHPPM (1996)
Water solubility (g/L)	0.5 g/L in water; soluble in most organic solvents	USACHPPM (1996)

$$Cl—CH{=}CH—AsCl_2 + 2H_2O \rightarrow Cl—CH{=}CH—AsCl_2 + 2HCl$$

The production of two equivalents of chloride occurs within 3 min at 20°C; at 5°C, the reaction is 90% complete within 2 min, and the completion of the reaction requires several hours. The hydrolysis rate constant is reported as $1\,min^{-1}$ at 20°C. Hydrolysis of 2-chlorovinyl arsine oxide is slower, resulting in lewisite oxide (chlorovinyl arsenous oxide) and polymerized lewisite oxide:

$$Cl—CH{=}CH—As(OH)_2 + H_2O \rightarrow Cl—CH{=}CH—AsO + (Cl—CH{=}CH—AsO)_n$$

The forward reaction is favored because lewisite oxide and polymerized lewisite oxide are insoluble. In a basic solution, the trans-lewisite isomer is cleaved by the hydroxyl ion to give acetylene and sodium arsenite; this reaction may occur even at low temperatures (Rosenblatt et al., 1975; Clark, 1989). Cis-lewisite heated to over 40°C reacts with sodium hydroxide to yield vinyl chloride, sodium arsenite, and acetylene (Rosenblatt et al., 1975). In aqueous solution, the cis-isomer undergoes a photoconversion to the trans-isomer (Rosenblatt et al., 1975). Epstein (1956) reported that the toxic trivalent arsenic of lewisite oxide in standing water is converted to the less toxic pentavalent arsenic.

Lewisite in soil may rapidly volatilize or may be converted to lewisite oxide due to moisture in the soil (Rosenblatt et al., 1975). The low water solubility suggests intermediate persistence in moist soil (Watson and Griffin, 1992). Both lewisite and lewisite oxide may be slowly oxidized to 2-chlorovinylarsonic acid (Rosenblatt et al., 1975). Possible pathways of microbial degradation in soil include epoxidation of the C=C bond and reductive dehalogenation and dehydrohalogenation (Morrill et al., 1985). Due to the epoxy bond and arsine group, toxic metabolites may result. Additionally, residual hydrolysis may result in arsenic compounds. Lewisite is not likely to bioaccumulate. However, the arsenic degradation products may bioaccumulate (Rosenblatt et al., 1975).

HISTORY AND BACKGROUND

Sulfur Mustards

Sulfur mustard was synthesized and its biological activities first characterized in the 1800s (Guthrie, 1860; Niemann, 1860; Despretz, 1992). By the early 1900s, its synthesis was further refined and its use as a warfare agent had been established when used as a warfare agent (as Levenstein mustard) during World War I. More recent use occurred during conflicts in the Middle East. Its oily nature makes it persistent on surfaces it makes contact with. Because sulfur mustard exerts toxic effects following dermal, ocular, and inhalation exposure, its use necessitated full body protection which, in turn, required the development of protective clothing and significant changes in warfare operations.

Minute quantities of sulfur mustard are used by various military and contract laboratories for defense research purposes, and for verification of Chemical Weapons Convention compliance. Bulk quantities of sulfur mustard are no longer manufactured in the United States. Military stockpiles of sulfur mustard are awaiting destruction or are in the process of being destroyed. Some sulfur mustard may also be found buried or abandoned at former defense sites. Sulfur mustard was frequently loaded into artillery shells and aerial bombs (often with lewisite). Various quantities of sulfur mustard also exist in other countries. Large amounts of sulfur mustard have been disposed of at sea.

Outside of military conflicts, exposure to sulfur mustard has occurred or may occur in work environments that involve chemical weapon materials (e.g., storage depots, demilitarization facilities, and research laboratories), during emergency response operations or remediation and decontamination activities, or during treaty verification activities in support of the Chemical Weapons Convention. Chemical weapons such as vesicants are still considered potential military threats and terrorist targets. The most likely route of exposure to sulfur mustard is via aerosol/vapor exposure of the skin, eyes, and respiratory tract.

Nitrogen Mustards

Due to the toxicity and various physical–chemical properties of nitrogen mustards (which are structurally similar to sulfur mustard), initial interest in these chemicals as warfare agents began shortly before and during World War II. Like sulfur mustard, all are alkylating agents. This chapter only discusses the nitrogen mustards

referred to as HN1, HN2, and HN3; selected chemical and physical properties of these substances are summarized in Tables 8.3 and 8.4. Although HN2 and HN3 were initially investigated as military agents, HN1 was originally developed as a pharmaceutical. HN2 (mechlorethamine) later found application as a pharmaceutical. Nitrogen mustards and derivatives such as melphalan, chlorambucil, and cyclophosphamide are alkylating agents used as cancer therapeutic agents (Somani, 1992). HN1 and HN3 are among the chemical agents found in Chemical Agent Identification Sets (CAIS), which are considered a component of nonstockpiled material. Generally, nitrogen mustards have not had the interest or high profile that sulfur mustard and lewisite have.

Lewisite

Lewisite, an organoarsenic compound, was developed in an attempt to create a more effective blister agent than sulfur mustard. Its development is generally credited to Winford Lewis at the Catholic University, Washington DC, and is based upon a thesis by Julius Nieuwland, who described the synthesis of lewisite from arsenic trichloride, acetylene, hydrochloric acid, and mercuric chloride. Early on, the compound was frequently referred to by the vividly descriptive term "Dew of Death." Like sulfur mustard, it is both a vesicant and a systemic poison with target tissues not limited to the skin. In an attempt to develop an antidote to lewisite, British anti-lewisite (BAL), also known as dimercaprol, was developed (Peters et al., 1945) which later became invaluable in the treatment of arsenic poisoning. Late in World War I and into World War II, large quantities of lewisite were manufactured by Germany, the United States, Italy, the Soviet Union, and Japan (reviewed by Trammell, 1992). Large amounts of lewisite were manufactured (up to 2 tons/day by Japan) and stored prior to and during World War II (Tanaka, 1988; Trammell, 1992). Lewisite was frequently a component (often mixed with sulfur mustard) of artillery shells and aerial bombs (often mixed with sulfur mustard). Like sulfur mustard, there are reports of large amounts of the compound being disposed of at sea (Spiers, 1968). With the possible exception of its use against Iranian soldiers during the Iran–Iraq conflict (Perera, 1985), there has been little or no use of lewisite in battle. Goldman and Dacre (1989) have reviewed the chemistry and toxicology of lewisite.

TOXICOKINETICS

Sulfur Mustards

With its high lipophilicity, toxicologically relevant amounts of sulfur mustard are absorbed in epithelial tissue (Papirmeister et al., 1991). Dermal absorption depends on the thickness of the epidermis and on the presence of moisture, which enhances penetration. Absorption tends to be greater at the base of hair shafts and in the hair follicle, where the epithelial tissue is thinner than the surrounding surface area (Papirmeister et al., 1991). Approximately 20% of sulfur mustard applied to skin may be rapidly absorbed, while 12–50% of this may react and remain at the application site (Somani and Babu, 1989). About 12% of the absorbed material remains at the contact site; the rest enters the circulation (Renshaw, 1947). Renshaw (1947) noted that the rate of penetration is 1–4 μg/cm^2/min at 75°F.

For dermal exposure, penetration rates over 2–8 h ranged from 2.9 to 6.7% and rates of absorption ranged from 1.2 to 4.0% following application of 400 μg of radiolabeled sulfur mustard per cm^2 of isolated perfused porcine skin (Riviere et al., 1995). The average total recovery of the radiolabel was 9.3% (3.8–17.7%), suggesting substantial loss due to volatilization.

Relative to dermal absorption, little is known about absorption in the respiratory tract. Cameron et al. (1946) calculated the absorption of sulfur mustard vapor in the noses of rabbits and rhesus monkeys. The concentration of the agent in the nasal passages was 10–30% of the chamber concentrations (40, 100, and 500 mg/m^3), implying an absorption of approximately 70–90%.

Several studies using radiolabeled sulfur mustard have shown that sulfur mustard and its metabolites may be widely distributed in the body after percutaneous or intravenous exposure. Maximum levels of radioactivity were detected in the kidneys, lungs, and livers of rabbits following intravenous administration (Boursnell et al., 1946). At 15 min following percutaneous exposure of rats, sulfur mustard–derived radioactivity was found in all examined tissues except the eyes (Young et al., 1944). Similarly, Clemedson et al. (1963) noted uniform distribution of radioactivity in mice after either percutaneous or intravenous exposures, with most radioactivity occurring in the nasal region, kidneys, liver, and intestine. Hambrook et al. (1993) reported on the uptake and distribution of radiolabeled sulfur mustard in the skin and blood of rats after cutaneous application. It was found that much of the agent entering the blood binds to hemoglobin and, to some extent, with glutathione (GSH). Results of studies with rabbits showed that sulfur mustard was concentrated in the cornea and, to a lesser extent, in the iris, lens, and conjunctiva within 5 min after application (Axelrod and Hamilton, 1947).

The biotransformation of sulfur mustard after intravenous or intraperitoneal injection of radiolabeled compound in rats has been examined. Following intravenous injection, the major urinary metabolite was GSH-bis-chloroethyl sulfide conjugates (45% of total urinary radioactivity) and smaller amounts of sulfone conjugates

(7%) and thiodiglycol and its conjugates (14.4%) (Davison et al., 1961). Roberts and Warwick (1963) found the major urinary product of cysteine-bis-(β-chloroethyl)sulfone after intraperitoneal injection of sulfur mustard in rats. Papirmeister et al. (1991) concluded that hydrolysis to thiodiglycol and reaction with GSH are the most important routes of detoxification. This is supported by human data showing that thiodiglycol is present in the urine for one week or more after exposure (Wils, 1987).

Nitrogen Mustards

The effects of time, temperature, and humidity on the vapor penetration of HN1 and HN3 into the forearm skin of human male volunteers were reported by NDRC (1945). Results of this research showed similar effects of temperature and humidity as observed for sulfur mustard (e.g., greater absorption with increased temperature and humidity). The penetration of HN1 and HN3 was found to be linear with time (5–20 min for HN1 and 30–60 min for HN3). At 71–72°F and 50–52% relative humidity, the HN1 penetration rate was 2.8 μg/cm^2/min; and for HN3, it was 0.18 μg/cm^2/min at 72–73°F and 45–48% relative humidity. At 86–87°F and 47–49% relative humidity, the HN1 penetration rate increased to 5.2 μg/cm^2/min, and the HN3 penetration rate increased to 0.3 μg/cm^2/min at 85°F and 47–48% relative humidity. Excretion via the urine is likely a major route of elimination, especially due to the water solubility of the immonium ion (see "Mechanism of Action" section).

Lewisite

Little information is available regarding the toxicokinetics of lewisite. It is readily absorbed by mucous membranes and, because of its lipophilicity, dermal absorption is significant (HSDB, 2004). Dermal absorption reportedly occurs faster for lewisite than for sulfur mustard (Hurst and Smith, 2008). Axelrod and Hamilton (1947) reported that radiolabeled (^{74}As) lewisite applied to a 0.43 cm^2 area of human skin was primarily fixed on the epidermis and that very little was found in the dermis; most was detected in hair and hair follicles. In experiments with guinea pigs, histological examination revealed that lewisite applied to skin entered the epidermis within 2 min and penetrated the dermis within 10 min (Ferguson and Silver, 1947). Only trace amounts were detectable in the dermis at 24 h post-application.

MECHANISM OF ACTION

Sulfur Mustards

Sulfur mustard is a bifunctional alkylating agent. The mechanism of action of sulfur mustard is multifaceted and complex, and has been reviewed in some detail by Papirmeister et al. (1991); Hurst and Smith (2008); Smith et al. (2008); Shakarjian et al. (2010), and Ghanei and Harandi (2011). Efforts to understand the mechanisms of sulfur mustard toxicity are ongoing. Basically, sulfur mustard disrupts the interface of the epidermis and basement membrane causing blistering between the epidermis and dermis. Both immediate (immediate cell membrane damage) and delayed phases (secondary effects resulting from inflammatory responses, deoxyribonucleic acid (DNA) damage, and vascular leakage) have been described for sulfur mustard-induced dermal effects (Somani and Babu, 1989). Sulfur mustard may initiate intramolecular and intermolecular crosslinks that provide many targets, including proteins, nucleic acids, and lipids. Many of the toxic effects of sulfur mustard also appear to be attributed to oxidative stress resulting from the disruption of normal cellular metabolism.

Among the most studied mechanisms of sulfur mustard toxicity are thiol depletion, resulting in intracellular calcium imbalance and subsequent cell death; alkylation of DNA and other cellular macromolecules; lipid peroxidation resulting from sulfur mustard-induced gluthathione depletion; and induction of an inflammatory response. The overall mechanism of sulfur mustard toxicity likely involves an interlinking of the aforementioned processes which are briefly described below.

A key component of sulfur mustard toxicity is the formation of a sulfonium ion and resulting episulfonium intermediate that may react with sulfhydryl-containing macromolecules. Damage may include Ca^{2+} translocases (Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase), which depend on thiol groups to maintain cellular Ca^{2+} homeostasis, and microfilamentous proteins. The resulting increase in intracellular calcium levels ultimately causes a decrease in cellular integrity and induction of apoptosis. Oxidative stress in sulfur mustard toxicity has been reviewed by Smith et al. (2008).

The role of DNA alkylation and the poly(ADP-ribose) polymerase (PARP) hypothesis theory for sulfur mustard toxicity has been reviewed by Papirmeister et al. (1991). In this mechanism, DNA is the initial target of the mustard agent. Alkylated DNA purines are enzymatically depurinated, creating apurinic sites that are cleaved by apurinic endonucleases, resulting in DNA strand breaks. The accumulation of DNA breaks leads to activation of the chromosomal enzyme PARP, which utilizes NAD^+, causing a severe reduction in cellular NAD^+. Depletion of NAD^+ results in the inhibition of glycolysis, and stimulation of the nicotinamide adenine dinucleotide phosphate ($NADP^+$)–dependent hexose monophosphate shunt, ultimately resulting in the induction and secretion of proteases and subsequent cellular changes. Kehe et al. (2009) has reviewed dermal toxicity

mechanisms of sulfur mustard. Sulfur mustard-induced apoptosis and activation of inflammatory mediators such as various interleukins (ILs) such as IL-1alpha, IL-1beta, IL-6, IL-8, as well as tumor necrosis factors, also appear to be involved in the toxic response to sulfur mustard.

In the review by Papirmeister et al. (1991), it was noted that sulfur mustard-induced cytotoxicity is dose-dependent and that DNA appeared to be more sensitive to mustard-induced alkylation than are other cellular constituents. The low-dose effects of sulfur mustard are characterized by genotoxicity and inhibition of mitosis. The loss of cellular reproduction may be due to bifunctional alkylation that ultimately prevents normal DNA replication. It was hypothesized that monofunctional DNA damage might be responsible for low-dose mutagenic and possibly carcinogenic effects.

Sulfur mustard-induced lipid peroxidation is a function of GSH depletion. For this mechanism, depletion of GSH results in an accumulation of reactive oxygen species via hydrogen peroxide–dependent processes (Miccadei et al., 1988). The oxygen radicals react with membrane phospholipids, forming lipid peroxides that alter membrane structure, causing it to break down.

Additional work has focused on the identification of possible biomarkers of sulfur mustard exposure and injury (Buxton et al., 2000, 2001; Danne et al., 2000). More recently, the role of metalloproteinases and collagen degradation (Gerecke et al., 2005), platelet-activating factor (Clark et al., 2005, 2006), and interaction with cytochrome P450 processes (Brimfield and Hodgson, 2005; Brimfield et al., 2006; Mancheco and Brimfield, 2006) is being investigated relative to the mechanism of action of sulfur mustard.

Nitrogen Mustards

A key component of nitrogen mustard toxicity is analogous to that of sulfur mustard: the formation of a cyclic onium cation. This occurs in the presence of polar solvents such as water (Somani, 1992). The immonium ion may react with nucleophiles (such as nitrogen) in the base components of nucleic acids and with sulfhydryl groups in proteins and peptides. The precise mechanism of nitrogen mustard activity is unclear, but several have been proposed: DNA/RNA alkylation and resultant effects, effects on GSH, membrane effects (protein cross-linking, ion transport effects), and cytoplasmic effects (release of lysosomal enzymes). The possible mechanisms of nitrogen mustard have been reviewed by Gray (1989). Results of preliminary work by Elsayed and Omaye (2006) in mice given HN2 intraperitoneally showed pulmonary alterations indicative of oxidative stress and impaired detoxification processes that are consistent with the aforementioned mechanisms.

Lewisite

Dermal or intravenous exposure to lewisite leads to local skin edema and pulmonary edema due to increased capillary permeability. The increased capillary permeability results in blood plasma loss and resultant physiological responses collectively referred to as *lewisite shock*. Lewisite shock may be likened to shock observed in severe burn cases. It has been hypothesized that functional changes in the lungs, kidneys, respiratory tract, and cardiovascular and lymphatic systems may be the result of a disturbance of osmotic equilibrium (Goldman and Dacre, 1989).

Lewisite-induced vesicant and systemic toxicity are likely due in part to interactions with thiol groups (Goldman and Dacre, 1989). The interaction with enzyme sulfhydryl groups may cause inhibition of enzyme by the formation of stable cyclic structures with arsenic. These thiol interactions result in energy depletion, leading to cell death (Young, 1999).

TOXICITY

Sulfur Mustard

The toxic effects of sulfur mustard in humans and animals have been extensively reviewed by Sidell and Hurst (1992); Somani (1992); Watson and Griffin (1992); IOM (1993); ATSDR (2003); NRC (2003); Romano et al. (2008); and Ghanei and Harandi (2011). Sulfur mustard affects the skin, respiratory tract, and eyes. The acute effects include edema, ulceration, and necrosis of epithelial tissue. Systemic toxicity may also occur and is characterized by nausea and vomiting, fever, and malaise. There is evidence of systemic toxicity (gastrointestinal tract) following dermal exposure only (Dacre and Goldman, 1996). Delayed effects include conjunctivitis and blindness following ocular exposure and chronic bronchitis following inhalation exposure. Affected tissues may have an increased susceptibility to secondary infections, and possibility of carcinogenicity of the skin and respiratory tract. However, the often-observed latency between acute exposure and late-onset tissue damage is not fully understood. Of the approximately 100,000 casualties of sulfur mustard exposure during the Iran–Iraq war (1983–1988), approximately 45,000 are still experiencing long-term effects of exposure over 20 years later (Ghanei et al., 2010).

Ambient temperature and humidity govern the degree of toxicity of sulfur mustard; in hot and humid conditions, lower mustard concentrations are required to produce debilitating effects. The severity of sulfur mustard effects is also greater in areas of the body with greater moisture (e.g., the axilla, groin, and eyes). Information regarding the toxic effects of long-term exposure to low

levels of sulfur mustard that are not acutely toxic is limited. Ghanei et al. (2010) have reviewed the acute and long-term cutaneous effects of sulfur mustard.

Available data suggest that the location and severity of damage resulting from exposure to sulfur mustard are concentration-dependent and a function of the highly reactive nature of sulfur mustard (Papirmeister et al., 1991). The eyes are generally considered to be the most sensitive and rapidly responding target (Reed, 1918; Reed et al., 1918; Anderson, 1942). For low exposures, sulfur mustard-induced injury appears to be limited to the upper respiratory tract (Eisenmenger et al., 1991) and eyes (Reed, 1918; Reed et al., 1918; Guild et al., 1941; Anderson, 1942). In work with informed volunteer subjects, Anderson (1942) reported that Ct values of 60–75 mg-min/m^3 would result in conjunctivitis, photophobia, and ocular irritation, while Ct values of 75–90 mg-min/m^3 would cause a high proportion of casualties, as determined by more severe ocular damage requiring several weeks of treatment. A chronic ocular injury known as *mustard gas keratopathy (MGK)* has been identified in some individuals following moderate- to high-level exposure. A weak positive correlation between severity of ocular and pulmonary involvement was found in a study of 292 Iran–Iraq conflict veterans (Ghasemi et al., 2012). McNutt et al. (2013) provided insight into the progression and pathophysiological correlates for this injury.

At exposure concentrations higher than those causing ocular damage, pulmonary effects would be expected (Eisenmenger et al., 1991). Regardless of the target tissue, there is a latency period between initial exposure and development of effects. The eyes and respiratory tract appear to have the shortest latency period, with effects appearing within hours depending on the exposure level. Later-onset lung injury is often referred to as mustard lung (ML) because the injury differs somewhat from more typical chronic obstructive pulmonary disease. In a review of the mechanisms of sulfur mustard-induced lung injury, Ghanei and Harandi (2011) found that oxidative stress and apoptosis may be more relevant mechanisms regarding ML than for other chronic obstructive pulmonary diseases.

In addition to the acute toxic effects on the eyes, skin, and respiratory tract, both acute and longer-term neuropsychiatric effects (e.g., depression, anxiety, neurasthenia, insomnia, and posttraumatic stress syndrome/PTSD) have been documented for individuals exposed to sulfur mustard (Romano et al., 2008). Many of these effects have been documented for individuals exposed during noncombat conditions (e.g., those of munitions plant workers) and are not always the result of high-level exposure that result in serious overt effects. Longer-term effects, such as chronic bronchitis, have been associated with occupational exposures that included episodes of acute toxicity, and delayed or recurrent keratitis may occur 8–40 years after a severe vapor exposure. Sulfur mustard-induced immunosuppression resulting in greater susceptibility to infections has also been reported.

TABLE 8.7 Acute Lethality of Sulfur Mustard in Laboratory Species Following Inhalation Exposure

Species	Lethality Value	Concentration (mg/m3) and Exposure Duration (min)	References
Rat	2 min LCt_{50}: 1512 mg-min/m^3	756 mg/m^3 (2 min)	Fuhr and Krakow (1945) (not verified)
	30 min LCt_{50}: 990 mg-min/m^3	33 mg/m^3 (30 min)	
	60 min LCt_{50}: 840 mg-min/m^3	14 mg/m^3 (60 min)	
Mouse	2 min LCt_{50}: 4140 mg-min/m^3	2070 mg/m^3 (2 min)	Fuhr and Krakow (1945) (not verified)
	30 min LCt_{50}: 1320 mg-min/m^3	44 mg/m^3 (30 min)	
	60 min LCt50: 860 mg-min/m^3	14.3 mg/m^3 (60 min)	
Mouse	60 min LCt_{50}: 42.5 mg/m^3	42.5 mg/m^3 (60 min)	Vijayaraghavan (1997)
Monkey	10 min LCt_{50}: 800 mg-min/m^3	80 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Dog	10 min LCt_{50}: 600 mg-min/m^3	60 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Cat	10 min LCt_{50}: 700 mg-min/m^3	70 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Goat	10 min LCt_{50}: 1900 mg-min/m	190 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Guinea pig	5 min LCt_{50}: 800 mg-min/m^3	160 mg/m^3 (5 min)	Langenberg et al. (1998)
	10 min LCt_{50}: 1700 mg-min/m^3	170 mg/m^3 (10 min)	Rosenblatt et al. (1975)

Acute lethality data in animals are summarized in Table 8.7. Based upon the animal data, interspecies variability in the lethal response to sulfur mustard vapor is less than an order of magnitude. For nonlethal effects, the animal data suggest that test species exhibit signs of toxicity that are qualitatively similar to humans when acutely exposed to sulfur mustard vapor. Ocular and respiratory tract irritations are clearly evident in studies using dogs, rats, mice, rabbits, and guinea pigs.

Effects of orally administered sulfur mustard in rats were studied by Sasser et al. (1996a). Repeated gavage administration of sulfur mustard in sesame oil produced epithelial hyperplasia of the forestomach at the highest dose tested, but no deaths and no other treatment-related

pathological lesions or changes in clinical chemistry or hematological parameters.

Results of a multigeneration study in rats given sulfur mustard by gavage showed no significant adverse effects on reproductive parameters at any dose level, but revealed dose-related lesions of the squamous epithelium of the forestomach (acanthosis and hyperplasia) (Sasser et al., 1996b). It is likely that the forestomach lesions were a function of the treatment regimen, whereby the bolus dose in an oil vehicle (sesame seed oil) would enhance the effects of sulfur mustard having direct contact with the forestomach tissue. Studies by Hackett et al. (1987) in which rabbits were gavage-dosed with sulfur mustard were equivocal regarding reproductive/developmental effects, due in part to the dose regimen and overt maternal toxicity.

Studies in animals have shown that sulfur mustard may induce developmental and reproductive effects (reviewed in NRC, 1999, 2003). Acute exposures resulting in systemic uptake may have effects on reproductive organs, including inhibition of spermatogenesis. Fetal anomalies were observed in tests with rats given sulfur mustard during gestation, but only at maternally toxic doses.

The genotoxicity of sulfur mustard is well documented. The chemical is known to produce DNA cross-links, mutations following replication or repair errors, chromosomal breaks, and chromosomal aberrations. Occupational exposures have been associated with increased frequencies of somatic cell mutations, sister chromatid exchanges, and chromosome abnormalities. Studies with rats indicate that subchronic inhalation or oral exposures can produce dominant lethal effects.

The carcinogenicity of sulfur mustard in animals has been reviewed in IARC (1975); Watson et al. (1989); IOM (1993); NRC (1999) and USACHPPM (2000). McNamara et al. (1975) studied the potential carcinogenicity of sulfur mustard in rats, mice, rabbits, guinea pigs, and dogs exposed via inhalation for up to one year. No tumors were detected in the mice, rabbits, guinea pigs, or dogs, but skin tumors (i.e., basal and squamous cell carcinomas, trichoepitheliomas, and keratoacanthomas) were associated with sulfur mustard exposure at the highest exposure tested (0.1 mg sulfur mustard/m^3 for 6.5 h, followed by 0.0025 mg sulfur mustard/m^3 for 17.5 h/day, 5 days/week). An increased incidence of pulmonary tumors in Strain A mice was observed following intravenous injections (4 doses over 2 days) of sulfur mustard (Heston, 1950), and an increase in injection site tumors in mice given subcutaneous injections of sulfur mustard over a 6-week period (Heston, 1953).

In a cohort study reported by Easton et al. (1988), there was a significant excess of laryngeal, pharyngeal, upper respiratory tract, and lung cancers in workers at a sulfur mustard manufacturing facility during World War II. A study of the Iranian military veterans exposed to sulfur mustard under battlefield conditions during the Iran–Iraq conflict at levels sufficient to cause severe signs of toxicity indicated a potential increased incidence of chronic myelocytic leukemia (CML). In several earlier studies on World War I veterans who were exposed to sulfur mustard, leukemia was not identified as a possible effect, although it is unclear if examination for CML had ever occurred in those populations. Confounders, such as exposure to benzene or radiation which complicate the analysis, have not yet been ruled out in the ongoing epidemiologic study of Iranian veterans. Two cases of CML were reported for Japanese workers exposed to sulfur mustard (Shakil et al., 1993) but the incidences of CML in the entire population of sulfur mustard–exposed workers and in an unexposed control population were not reported. Studies in animals provide supporting evidence for the carcinogenicity of sulfur mustard, although the results of some studies are compromised by insufficient exposure durations and injuries resulting from caging situations.

Nitrogen Mustards

Information regarding the toxicity of nitrogen mustards is less extensive as that for sulfur mustard. Limited lethality data in animals are summarized in Table 8.8. Like sulfur mustard, exposure to nitrogen mustards may cause skin blistering, as well as respiratory tract injury and ocular damage. Response data from tests with informed human volunteer subjects (NDRC, 1944) suggested a relative potency of HN3 > HN1 > HN2 for vesicant effects, although the differences were minor. Like sulfur mustard, dermal effects were enhanced by moisture (as from sweating). Estimated thresholds for skin blistering and ocular injury are summarized in Table 8.9. Ocular injury (irritation resulting in compromised operational effectiveness of military personnel) was detected at exposures much lower than those causing dermal effects. All of the toxic effects of nitrogen mustard appear to involve a latency period of several hours for ocular responses and several days for dermal blistering. Nitrogen mustards are alkylating agents with known mutagenicity, but there are no animal cancer bioassays and no human carcinogenicity data.

Nitrogen mustard and its hydrochloride salt have been shown to be teratogenic in mice and rats. Intraperitoneal administration of HN2-hydrochloride/g to mice during gestation resulted in serious teratogenic effects (Danforth and Center, 1954). Haskin (1948) and Murphy et al. (1958) reported similar findings in rats given HN2-hydrochloride/kg subcutaneously during gestation.

Nitrogen mustards are bifunctional alkylating agents that produce a carcinogenic response (primarily lung tumors and lymphomas) in mice following subcutaneous,

TABLE 8.8 Lethality of Nitrogen Mustard (HN2)

Route	Species	Dose (mg/kg)	Exposure Time	Effect	References
Oral	Rat	10–85	–	LD_{50}	NDRC (1946)
	Mouse	10–20	–	LD_{50}	Fox and Scott (1980)
Percutaneous	Rat	14	–	LD_{50}	NDRC (1946)
		12	96 h	LD_{50}	Vojvodić et al. (1985)
	Mouse	29–35	–	LD_{50}	NDRC (1946)
	Monkey	<50	–	LD_{50}	NDRC (1946)
Subcutaneous	Rat	6	–	LD_{50}	Vojvodić et al. (1985)
	Mouse	1.4	–	LD_{50}	Fox and Scott (1980)
		2.6–4.5	–	LD_{50}	NDRC (1946)
Intraperitoneal	Rat	1.8–2.5	–	LD_{50}	Fox and Scott (1980)
	Mouse	4.4	–	LD_{50}	Fox and Scott (1980)
Intravenous	Rat	1.1	–	LD_{50}	NDRC (1946); Fox and Scott (1980)
	Mouse	~2	–	LD_{50}	NDRC (1946)

TABLE 8.9 Estimated Effects Thresholds in Humans Exposed to Nitrogen Mustard Vapors

HN1	HN2	HN3	Effect
–	0.012 mg-min/m^3	–	No observable effect level during therapeutic use of HN2 (Van Vloten et al., 1993)
90 mg-min/m^3	70 mg-min/m^3	42 mg-min/m^3	Moderate but reversible ocular effects (Porton Report, 1942a,b, 1943a–d; U.S. Army Med. Div., 1945a,b; NDRC, 1946)
>21,170 mg-min/m^3	5,800 mg-min/m^3	1,800 mg-min/m^3	Median blistering Ct (10 or 20 min exposure) for normal skin
		31,300 mg-min/m^3	Median blistering Ct (20 min exposure) for sweating skin (NDRC, 1944)

intraperitoneal, and intravenous administration, as well as by skin painting (IARC, 1987). Intravenous administration of nitrogen mustard to rats produced tumors in multiple organs (IARC, 1987). Information in humans is limited to reports of squamous cell carcinomas of the skin following therapeutic application of nitrogen mustard in the treatment of mycosis, fungoides, and psoriasis (IARC, 1987).

Lewisite

The toxicology of lewisite has been reviewed by Goldman and Dacre (1989); Trammell (1992); Watson and Griffin (1992); Hurst and Smith (2008) and NRC (2013). Because lewisite L-2 and L-3 are of lower volatility than L-1, occur in lesser amounts, and are toxicologically similar to L-1, the discussion of toxicity is limited to lewisite (NRC, 2013). Its characteristic geraniumlike odor is detectable at 14–23 mg/m^3 (Gates et al., 1946). Lewisite may be lethal in humans following inhalation, or dermal, or oral exposure. It is reportedly immediately highly irritating at estimated concentrations of 6–8 mg/m^3. Gates et al. (1946) estimated an LC_{50} of 3,300 mg/m^3 for 30 min for lewisite vapor absorption through the bare skin and an inhalation LC_{50} of 120 mg/m^3 for 10 min and 50 mg/m^3 for 30 min. Inhalation of 10 mg/m^3 lewisite for 30 min may result in severe intoxication and incapacitation lasting for several weeks, and inhalation of 10 mg/m^3 for 15 min caused inflammation of the eyes and swelling of the eyelids (Franke, 1977). Like sulfur mustard, moist tissues are particularly sensitive to lewisite. The eyes exhibit the greatest sensitivity (IOM, 1993).

The vesicant properties of lewisite result from direct contact with the skin. Signs of dermal toxicity (pain, inflammation) may be experienced within a minute after exposure. Acute lethality is usually the result of pulmonary injury. Ocular exposure may result in corneal necrosis. Due to its lipophilicity, percutaneous absorption of lewisite is rapid and, at a sufficient exposure, may be associated with systemic toxicity characterized by pulmonary edema, diarrhea, agitation, weakness, hypothermia, and hypotension (IOM, 1993). The threshold for severe systemic toxicity in humans following dermal exposure to lewisite has been estimated at 10 mg/kg (9.1–13.4 mg/kg) (Sollman, 1957).

Eldridge (1923) conducted tests on human volunteers to assess the effects of dermal exposure to lewisite vapor.

The arms of men (1–7 men with previously determined average sensitivity to lewisite) were exposed to varying concentrations of lewisite vapor for periods ranging from 10 min to 3 h for the purpose of determining the concentration of lewisite required to create blistering. The resulting dermal responses ranged from reddish discoloration to a clear, watery blister over the entire burned area, accompanied by reddening, swelling, and hardening of the surrounding skin. The burns reached maximum severity in 36–48 h, and healing was complete in 6 days to 2 weeks. The men also reported that the healed skin remained sensitive for several weeks after the healing was complete.

It has been hypothesized that fatalities following dermal exposure to lewisite may be due to blood plasma loss resulting from extensive capillary damage (lewisite shock; Cameron et al., 1946). Sollman (1957) estimated that an oral dose of as little as 2 mL in an adult human (equivalent to 37.6 mg/kg) may be fatal within several hours. The target tissues and organs for systemic toxicity of lewisite include the liver, gallbladder, urinary bladder, lung, and kidneys (Cameron et al., 1946; Snider et al., 1990). Generally, there is a data deficiency regarding definitive exposure–response data for lewisite.

In studies with rats, Silver and McGrath (1943) found little difference in the acute lethality of cis- or trans-lewisite exposed for 10 min. Ten-minute mouse LC_{50} values for the cis- and trans-isomers were 190 and 200 mg/m^3, respectively. All mice exposed to 240 mg/m^3 lewisite for 10 min died. Clinical signs in dogs exposed for 7.5 or 15 min included immediate continual eye blinking, followed by excessive nasal secretion, lacrimation, and sneezing (Armstrong, 1923). Ocular inflammation and vomiting also occurred in some dogs by the end of the 7.5- and 15-min exposures. Dogs exposed for 30 min or longer exhibited frequent retching, vomiting, extreme salivation, labored breathing, and inflammation of the entire respiratory tract. Necropsy revealed a thick membrane in the nostrils, larynx, and trachea, which was accompanied by purulent bronchitis, hemorrhage, pneumonia, edema, and congestion of the lungs.

Similar to the work on sulfur mustard, Sasser et al. (1989a) conducted experiments in rats given lewisite by gastric intubation (in sesame oil) at doses of 0.01, 0.1, 0.5, 1.0, or 2.0 mg/kg, 5 days/week for 13 weeks. A dose-related response was observed for lethality (deaths in the three highest dose groups) and frequency and severity of forestomach lesions. The forestomach lesion incidence and severity were due, at least in part, to the bolus of dosing and the sesame oil vehicle.

Multigeneration reproductive studies in rats (Sasser et al., 1989b) and teratology studies in rats and rabbits (Hackett et al., 1987) given lewisite by gastric intubation were negative or compromised by concurrent maternal toxicity.

The carcinogenic potential of lewisite is not well understood. In a long-term follow-up study, Krause and Grussendorf (1978) reported the formation of a malignant lesion at the site of contact 8 years following a single, acute dermal exposure of a German soldier accidentally exposed to liquid lewisite on his lower right leg in 1940. The lesion was diagnosed as malignant 8 years later, and 38 years after exposure, the area of contact remained ulcerated and diagnosed as Bowen's disease (an intradermal squamous cell carcinoma). Bowen's disease was also diagnosed in workers at a Japanese lewisite production facility (Inada et al., 1978). Findings in these workers were not conclusive due to concurrent exposure to diphenylcyanoarsine and sulfur mustard. Furthermore, no quantitative estimates of dose or exposure rates were available (Inada et al., 1978).

Increased incidences of cancer mortality (respiratory tract: 14%; digestive tract: 9.6%) in workers from the Okuno-Jima poison gas factory were reported by Wada et al. (1968). When cancer rates were correlated with job classifications, the frequency of respiratory and gastrointestinal tract neoplasms was highest in workers who were involved in the production of sulfur mustard or lewisite, followed by those who worked indirectly with sulfur mustard or lewisite. The lowest frequency occurred in the group having no direct contact with the vesicant agents (Yamakido et al., 1985). Similar to the Bowen's disease findings, the cancer incidences were confounded by the fact that workers were also exposed to sulfur mustard, hydrocyanic acid, diphenylcyanoarsine, chloroacetophenone, and phosgene.

RISK ASSESSMENT

Sulfur Mustards

Noncancer

Various standards and guidelines have been developed for sulfur mustard. These values are applicable to occupational exposures, emergency planning and response efforts, and remediation efforts. Airborne exposure limits (AELs) and health-based environmental screening levels (HBESLs) for sulfur mustard have been developed by the U.S. Army (USACHPPM, 1999, 2000). Most health-based criteria for sulfur mustard vapor exposure are based upon protection of the eyes and respiratory tract, which are the most sensitive targets.

Acute exposure guideline levels (AEGLs) for sulfur mustard have been developed for emergency planning and emergency response applications (NRC, 2003; US EPA, 2012). The AEGLs represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min

TABLE 8.10 Inhalation Standards and Guidelines for Sulfur Mustard

	Exposure Duration				
Guideline	**10 min**	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	0.06 ppm	0.02 ppm	0.01 ppm	0.003 ppm	0.001 ppm
AEGL-2[a]	0.09 ppm	0.03 ppm	0.02 ppm	0.004 ppm	0.002 ppm
AEGL-3[a]	0.59 ppm	0.41 ppm	0.32 ppm	0.08 ppm	0.04 ppm
Dept. of the Army/Civilian Occupational WPL[b]					0.0005 ppm (24-h)
Dept. of the Army/Civilian GPL[c]					$1.5 \times 10 \times 10^{-5}$ ppm (24-h)
CDC-CSEPP[d]					0.3 ppm

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. The AEGL-1 values for sulfur mustard are based upon minor ocular irritation in informed human volunteers (Anderson, 1942). AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. The AEGL-2 values for sulfur mustard are based on marked conjunctivitis, edema, photophobia, and eye irritation in informed human volunteers (Anderson, 1942). AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 values for sulfur mustard are based on a lethality threshold estimated in mice (Kumar and Vijayaraghavan, 1998).

[a]*AEGL (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels—AEGL-1, AEGL-2, and AEGL-3—are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects.*

[b]*Worker population exposure limit (DA, 1991, 1997; DHHS, 1988), 8-h TWA, 5 days/week.*

[c]*General population limit (no observable effects), 24-h TWA, 5 days/week.*

[d]*Recommended acute effect levels for determining emergency evacuation distances in the Chemical Stockpile Emergency Preparedness Program (CSEPP); no specified exposure duration.*

to 8 h (Table 8.10). The AEGLs for nitrogen mustards are interim status values that have not undergone final review and publication by the National Research Council/National Academy of Sciences. Provisional Advisory Levels (PALs) are developed by the U.S. Environmental Protection Agency (EPA) as public emergency exposure limits for three severity levels (PAL 1, PAL 2, and PAL 3) and for exposure durations of 24 h, 30 days, 90 days, and 2 years for both oral and inhalation exposure (Young et al., 2009). Oral PALs for sulfur mustard are not recommended due to insufficient data and because of the chemical nature of sulfur mustard (Bast et al., 2013). Inhalation PAL 1 values for sulfur mustard are 0.00083 mg/m^3 for 24 h and 0.00010 mg/m^3 for 30 days, 90 days, and 2 years. Inhalation PAL 2 values are 0.0042 mg/m^3 for 24 h, 0.0029 mg/m^3 for 30 days, and 0.00097 mg/m^3 for 90 days and 2 years. PAL 3 values are 0.088 mg/m^3 for 24 h, but are not recommended for longer durations (Bast et al., 2013).

An interim reference dose (RfDi), an estimate of a daily dose to humans that is likely to be without appreciable risk of deleterious health effects during a lifetime, of 7×10^{-2} mg/kg/day has been developed for sulfur mustard (NRC, 1999).

Inhalation standards and guidelines for sulfur mustard are summarized in Table 8.10. Other standards and guidelines for sulfur mustard have been summarized by ATSDR (2003).

Cancer

The International Agency for Research on Cancer (IARC) classified sulfur mustard as a Group 1 carcinogen (carcinogenic to humans) (IARC, 1987). The National Toxicology Program (NTP) considers mustard gas to be a substance "known to be a human carcinogen" (DHHS, 1988). These assessments are based upon human and animal data.

Studies of occupational exposures to sulfur mustard indicate an elevated risk of respiratory tract and skin tumors following long-term exposure to acutely toxic concentrations. Overall, several factors are important regarding the assessment of the carcinogenicity of sulfur mustard. Increased cancer incidence in humans appears to be associated only with exposures that caused severe acute effects, and occupational exposures tended to involve repeated exposures and repeated injury of the same tissues. Although numerous individuals suffered sulfur mustard-induced injuries in various conflicts over the years, incidences of skin cancer are rarely reported (Firooz et al., 2011). Evidence for lung cancers resulting from sulfur mustard exposure are well documented with most cases associated with long-term exposures, although there is emerging evidence for the involvement of acute and short-term exposure (Ghanei and Harandi, 2010). Because the therapeutic use of the sulfur mustard analog nitrogen mustard is associated with an increased incidence of CML, the reports of CML in HD-exposed individuals appear to be relevant to the carcinogenicity of sulfur mustard.

TABLE 8.11 AEGLs for Nitrogen Mustards (HN-1, HN-2, HN-3)

	Exposure Duration				
Guideline	**10 min**	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	NR	NR	NR	NR	NR
AEGL-2[a]	0.13 mg/m^3	0.044 mg/m^3	0.022 mg/m^3	0.0056 mg/m^3	0.0028 mg/m^3
AEGL-3[a]	2.2 mg/m^3	0.74 mg/m^3	0.37 mg/m^3	0.093 mg/m^3	0.047 mg/m^3

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. No AEGL-1 values for nitrogen mustards are currently recommended due to insufficient data. AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. A threshold for ocular irritation in informed human volunteers is the basis for the AEGL-2 values for nitrogen mustards. AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 values for nitrogen mustards are based on an estimated lethality threshold in rats.
[a]AEGL (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels—AEGL-1, AEGL-2, and AEGL-3—are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects.

Cancer slope factors and unit risk values for sulfur mustard have been summarized by ATSDR (2003).

Nitrogen Mustards

Noncancer

Very few standards and guidelines are available for nitrogen mustards. AEGL values for the nitrogen mustards (HN1, HN2, and HN3) have been developed and are based upon ocular irritation in human volunteers (AEGL-2) and lethality in rodents (AEGL-3), as shown in Table 8.11. Data were insufficient for derivation of level AEGL-1 values. The AEGL values are currently awaiting finalization. The U.S. army (USACHPPM, 1996, 2004) has developed worker population limit (WPL) values and general population limit (GPL) values for nitrogen mustard (USACHPPM, 1996, 2004).

Cancer

Data are not available with which to quantitatively assess the cancer risk from nitrogen mustards. However, IARC (1987) considers nitrogen mustard a Group 2A carcinogen based upon limited evidence in humans and sufficient evidence in animals.

Lewisite

Noncancer

The U.S. Army has developed HBESLs for lewisite (USACHPPM, 1999). Additionally, a chronic oral reference dose (RfD) of 1×10^{-4} mg/kg/day is available (NRC, 1999; USACHPPM, 1999), as are inhalation and dermal RfDs (USACHPPM, 1999). Acute Exposure Guidelines Levels (AEGLs) have been developed for lewisite (NRC, 2013), and are presented in Table 8.12. Inhalation PAL 1 values are not recommended for lewisite. Inhalation PAL 2 and 3 values for 24-h exposure durations are 0.01 mg/m^3 and 0.037 mg/m^3, respectively (Bast et al., 2013). Exposure values for longer PAL-specific durations (30 days, 90 days, and 2 years) are not recommended.

Cancer

Data regarding the potential carcinogenicity of lewisite are anecdotal and insufficient for a quantitative assessment. Although quantitative data are lacking, the position maintained by the Centers for Disease Control and Prevention (CDC) (DHHS, 1988) is that some evidence suggests that lewisite may be a carcinogen. For environmental exposure and remediation concerns, the arsenic component and/or arsenic-containing degradation products, however, are relevant.

Although the carcinogenicity of lewisite is equivocal and a quantitative assessment is not feasible, several lewisite degradation products are known carcinogens. Combustion products of lewisite include the inorganic arsenicals, arsenic trichloride, arsenic trioxide, and vinyl chloride. Inorganic arsenic is carcinogenic in humans and animals and is classified as a Group A carcinogen by U.S. EPA (2008). Arsenic trioxide and vinyl chloride are both considered Group A carcinogens by U.S. EPA (1984) and Group 1 carcinogens by IARC (1987).

TREATMENT

Sulfur Mustard

Medical management of sulfur mustard exposure begins with preventing exposure. As previously noted in

TABLE 8.12 AEGLs for Lewisite

	Exposure Duration				
Guideline	**10 min**	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	NR	NR	NR	NR	NR
AEGL-2[a]	1.3 mg/m^3	0.47 mg/m^3	0.25 mg/m^3	0.070 mg/m^3	0.037 mg/m^3
AEGL-3[a]	3.9 mg/m^3	1.4 mg/m^3	0.74 mg/m^3	0.21 mg/m^3	0.11 mg/m^3

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. No AEGL-1 values for lewisite are currently recommended due to insufficient data. AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. A 3-fold reduction of the AEGL-3 values for lewisite were considered an appropriate and defensible estimate for the AEGL-2 values (NRC, 2013). AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 values for lewisite were based upon an estimated LC_{01} in dogs (Armstrong, 1923).

[a]*AEGL (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels—AEGL-1, AEGL-2, and AEGL-3—are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects.*

this chapter, the military use of sulfur mustard necessitated full body protection. As a result, considerable effort has been expended in the development and evaluation of protective clothing and equipment (Schier and Hoffman, 2005). In general, these include air-purifying and atmosphere-supplying respirators and chemical-protective clothing (e.g., chemical and vapor impermeable coverings and clothing treated with adsorbing or neutralizing chemicals). Following exposure, rapid decontamination is essential and may include removal of contaminated clothing and removal or neutralization of the agent. Ocular exposure will require rapid removal of the agent from the eyes by flushing with water. Vapor exposure may necessitate respiratory support. Because there are no antidotes for sulfur mustard poisoning, medical management must rely on prevention, decontamination, and palliative treatment of signs and symptoms. The use of possible antidotes (e.g., antioxidants) has been reviewed by Smith et al. (2008) and Laskin et al. (2010). Polyurethane sponges containing detoxification additives are being developed and evaluated for decontamination and detoxification (Gordon et al., 2006). The medical management of sulfur mustard (and other vesicant agents) has been reviewed by Munro et al. (1990); Keyes et al. (2005) and Laskin et al. (2010).

Nitrogen Mustards

Medical management of nitrogen mustard exposure is similar to that for sulfur mustard. It involves preventing exposure and, where exposure has occurred, decontamination and support therapy. The use of antioxidants in the treatment of nitrogen mustard toxicity is under investigation (Hardej and Billack, 2006).

Lewisite

Similar to the mustard agents, exposure prevention is the first line of defense against lewisite. Rapid decontamination is especially relevant to lewisite exposure due to the rapid development of pain (1–2 min) associated with lewisite exposure. Unlike other vesicants, an effective antidote for lewisite toxicity exists in the form of BAL (2,3-dimercaptopropanol), which binds with arsenicals, thereby countering the lewisite-induced damage. Such chelation therapy is associated with notable side effects (e.g., renal effects) and requires careful medical management. More effective analogs of BAL have been developed that have less significant side effects.

Mouret et al. (2013) reported that for the SKH-1 hairless mouse model, topical application of dimercapto-chelating agents such as BAL and meso-2,3-dimercaptosuccinic acid (DMSA) were more effective than subcutaneous administration in the attenuation of lewisite vapor-induced injury. Although both agents reduced neutrophil infiltration, wound size, and necrosis of the skin barrier, BAL was found to be more effective than DMSA.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

As chemical warfare agents, vesicants have received considerable attention over the last two decades due to concerns regarding destruction of agent stockpiles, remediation of contaminated sites, the documented and speculated use of these agents in regional conflicts, and possible use in subversive/terrorist activity. This elevated interest profile has resulted in summaries of

older toxicological data, generation of new data, and a greater understanding of the effects of these agents on biological systems. Application of these data has been invaluable in the development of various health-based criteria, standards, and guidelines for use in remediation efforts, risk planning, and emergency response activities.

In accordance with the Chemical Weapons Convention, the United States is destroying (via chemical neutralization followed by biotreatment or supercritical water oxidation) the remaining sulfur mustard stored at the Blue Grass and Pueblo Army Depots (PEO ACWA, 2014). The sulfur mustard occurs in artillery projectiles and mortar rounds. Destruction is scheduled for completion by 2019 at Pueblo Army Depot and 2023 at Blue Grass Army Depot.

Future directions appear to focus on acquiring additional in-depth understanding of the mechanism of action of these agents through the development of experimental models for vesicant-induced injury, as well as an application of this information in the development of therapeutic measures for the prevention and treatment of vesicant-induced injury.

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CHAPTER

9

Organophosphate Nerve Agents

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INTRODUCTION

The chemical warfare (CW) nerve agents primarily addressed in this chapter include the anticholinesterase nerve agents tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and VX, all of which are, or have been, part of the US domestic munitions inventories (Carnes, 1989; Opresko et al., 1998; NRC, 1999). Russian VX (often represented as VR) is evaluated in Chapter 10 by Rembovskiy et al. (2015). Other less well-characterized nerve agents such as compound GE, VG (Amiton™), or V_x are evaluated as data allow.

These agents are potent anticholinesterase compounds deliberately formulated to induce debilitating effects or death during wartime hostilities and have been used by military authorities of several nations to develop munitions (e.g., Germany during the Nazi era, the United States, the former Soviet Union). US military stockpiles of CW munitions manufactured as a Cold War deterrent decades ago await demilitarization at designated stockpile sites and have been the subject of extensive emergency preparedness and response planning. Additional planning has been necessary at other current and formerly used military sites where containers and buried munitions have been inventoried (the "nonstockpile" sites) (Opresko et al., 1998; NRC, 1999, 2003). More recent global events have focused attention on the potential threat of chemical terrorism, especially at transportation hubs (Tucker and Raber, 2008). The deliberate release of nerve agent sarin at lethal concentrations in the Japanese cities of Matsumoto (1994) and Tokyo (1995) by a Japanese domestic terrorist group has illustrated that such attacks can be a reality and require advance emergency preparedness planning (Morita et al., 1995; Okumura et al., 1996, 2007; Sidell, 1996; Cannard, 2006; Yanagisawa et al., 2006; Tu, 2007).

Information provided on agent toxicity, risk assessment, treatment options, and other related topics will be useful to communities and facilities developing and updating emergency preparedness plans for accidental or intentional release of nerve agents; this information can also be used to support environmental decision-making where nonstockpile material and military gear have been found. In response to various public laws and international agreements such as the Chemical Weapons Convention (PL 99-145, 1986; PL 102-484, 1993; CWC, 1997), existing emergency guidance and military policy documents currently reflect the identified criteria and information (OASA, 1999; CSEPP, 2003, 2006a,b; NRT, 2008).

Although there are different applications for the information provided in this evaluation, in general the toxicological focus for emergency preparedness and response applications is that of acute exposures associated with a one-time release. Typically, the scenarios considered include a single-source airborne release from either an intentional terrorist attack, or accident involving an agent container or munition from a military site. It is widely recognized that vapor inhalation is the exposure route of greatest concern for such an event (Sidell, 1997; ATSDR, 2007). In contrast, the toxicological focus of environmental site remediation plans for military installations and formerly used defense sites where buried CW agent residues may occur requires consideration of long-term release and potential incidental ingestion of media such as soil particles or water with relatively low levels of contamination. To reflect these critical applications and information needs, the current evaluation will also primarily focus on

- single-source, one-time nerve agent releases and exposure routes involving agent vapor inhalation or direct ocular vapor exposures.

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- long-term (chronic or subchronic) exposure from residual nerve agent contamination.

It is acknowledged that there exists a rich and valuable body of repeat-exposure studies using serial vapor or serial injection exposures for the nerve agents soman (GD), sarin (GB), and VX (see recent excellent reviews and analyses by Shih et al., 2006; McDonough and Romano, 2008; and experimental studies by Dabisch et al., 2005, 2007a); the interested reader is encouraged to examine these and related resources, because the current evaluation does not highlight experiments that apply serial exposure protocols.

BACKGROUND

Development of Organophosphate Formulations as CW Agents

The G-series nerve agents evaluated are all toxic ester derivatives of phosphonic acid containing either a cyanide (GA) or a fluoride (GD, GE, GF) substituent and are commonly termed "nerve" agents as a consequence of their anticholinesterase properties as well as their effects on both the peripheral nervous system (PNS) and central nervous system (CNS). The "G" series military nomenclature used by NATO member nations has historically been considered to be an abbreviation for "German," with the second letter of the code ("A", "B", and so on) identifying the order in which these compounds were found and analytically identified by Allied forces investigating materials found in captured German military facilities at the close of WWII (Sidell, 1997). Agent VX, a phosphonic acid ester with a sulfur substituent, was industrially synthesized in the United Kingdom in the early 1950s; the code letter "V" is a reported reference to "venom" (Sidell, 1997). Other, less well-characterized V-series compounds include Vx, VE, VM, and VG (trade name Amiton™ when commercially introduced as a miticide in the mid 1950s).

As Cold War deterrents, nerve agents began to be manufactured and weaponized by the United States in the 1950s. When the US CW agent production program was terminated by the Nixon presidential "Statement on Chemical and Biological Defense Policies" of November 1969 (National Security Decision Memorandum 35), the US stockpile of unitary munitions included bulk ("ton") containers, underwing spray tanks, projectiles, rockets, bombs, land mines, and rockets (Carnes, 1989; Sidell, 1997). Nerve agent unitary munitions contained GA, GB, or VX. The US CW agent munition stockpile is obsolete and is presently undergoing destruction and disposal by the US Army Chemical Materials Agency (see www.cma.army.mil) at each of the several unitary stockpile sites, for compliance with the CWC to eliminate the risk of continued storage for these aging CW munitions; current nerve agent stockpile sites are located in Alabama, Arkansas, Indiana, Kentucky, Oregon, and Utah. In December 2008, the US Army Chemical Materials Agency announced that 58% of the US unitary chemical munitions stockpile had been successfully destroyed.

Physical and Chemical Properties of Nerve Agents

The G-agents are all viscous liquids of varying volatility (vapor density relative to air between 4.86 and 6.33) with faint odors ("faintly fruity" or "spicy," "odor of camphor"). Agent VX is an amber-colored liquid with a vapor density of 9.2, and it is considered odorless. Thus, nerve agent vapors possess little to no olfactory warning properties (Table 9.1).

The vapor pressures and acute toxicity of these agents are sufficiently high for the vapors to be rapidly lethal. Within the G-series, GB is considered to present the greatest vapor hazard (order of vapor hazard approximates GB > GD > GF > GA). Agent VX was deliberately formulated to possess a low volatility; VX is approximately 2,000-times less volatile than nerve agent GB (DA, 1990a,b). As a consequence, agent VX is considered a persistent "terrain denial" military compound with the potential to be a contact hazard or generate off-gas toxic vapor concentrations over a period of days after surface application, particularly under cold weather conditions or when bulk-release quantities of liquid agent are involved. Although not readily volatile, VX vapors (if allowed to accumulate) are nevertheless considered more acutely potent than those of agent GB or the other G-series agents (Mioduszewski et al., 1998).

As a consequence of the volatilities exhibited by G-series nerve agents (Table 9.1), the most likely exposure route (and source of primary hazard) is via direct vapor exposure to the eyes and upper respiratory tract tissues and vapor inhalation (with consequent systemic absorption) (Cannard, 2006; Dabisch et al., 2008a); G-agents are considered "nonpersistent" as per definitions used by the US Department of Defense (DOD, 2008). Nerve agent VX is widely considered to present a greater threat from the percutaneous exposure route (when compared with the G-series agents) as well as a vapor inhalation threat at elevated ambient temperatures (e.g., >40°C) (Craig et al., 1977; Sidell, 1997; Benton et al., 2005, 2006a).

Nerve agent V_x exhibits volatility (76.4 mg/m^3 at 25°C) intermediate to that of agents GA and VX, and a vapor density (7.3) intermediate to that of agents GF and VX; V_x is also considered "persistent." There are few data from which to characterize nerve agents VE (*O*-ethyl-*S*-[2-(diethylamino) ethyl]ethylphosphonothioate, CAS No. 21738-25-0) or VM (*O*-ethyl-*S*-[2-(diethylamino) ethyl]methylphosphonothioate, CAS No 21770-86-5).

TABLE 9.1 Physical and Chemical Properties of Organophosphorous Nerve Agents

Parameter	GA	GB	GD	GF	VX	GE	Vx
CAS Registry No.	77-81-6	107-44-8	96-64-0	329-99-7	50782-69-9	1189-87-3	20820-80-8
Chemical name[a]	Ethyl dimethylamido cyanophosphate	Isopropyl methyl-phosphono-fluoridate	Pinacolyl methyl-phosphono-fluoridate	*O*-cyclohexyl methyl-phosphono-fluoridate	*S*-(2-diisopropyl-aminoethyl) *O*-ethyl methyl phosphonothiolate	Isopropyl ethylphosphono-fluoridate	*O*-ethyl *S*-(2-dimethylaminoethyl) methyl-phosphonothiolate
Common name[a,b]	Tabun	Sarin	Soman	Cyclosarin	VX	NA	NA
Chemical formula[a]	$C_5H_{11}N_2O_2P$	$C_4H_{10}FO_2P$	$C_7H_{16}FO_2P$	$C_7H_{14}FO_2P$	$C_{11}H_{26}$ NO_2PS	$C_5H_{12}FO_2P$	$C_7H_{18}NO_2PS$
Molecular weight[a]	162.13	140.10	182.178	180.2	267.38	154.12 (calc)	211.26
Physical state[a,c]	Liquid, vapor	Liquid, vapor	Liquid, vapor	Liquid, vapor	Oily liquid, vapor	Vapor	Liquid
Vapor pressure (mmHg)[a]	0.037 (20°C)	2.10 (20°C)	0.40 (25°C)	0.056 (20°C)	0.0007 (25°C)	NA	6.73×10^{-3} (25°C)
Volatility (mg/m^3 at 25°C)[a,c]	610	22,000	3,900	548 at 20°C; 817 at 25°C	10.5	11.6 mg/L at 25°C (saturated concentration)	76.4
Liquid density (g/mL)[a]	1.073 (25°C)	1.102 (20°C)	1.0222 (25°C)	1.1327 (20°C)	1.006 (20°C)	1.0552 (25°C)	1.06 (25°C)
Vapor density (air = 1)[a]	5.63	4.86	6.33	6.2	9.2	NA	7.3 (calculated)
Melting point (°C)[a–c]	−50	−56	−42	−30	−39 (calculated)	NA	NA
Boiling point (°C)[a–c]	245	158, 150	198	239	298	67–68	256 (extrapolated)
Water solubility[a,c]	98 g/L (25°C); 72 g/L (20°C)	Miscible	21 g/L (20°C)	0.37% (20°C)	30 g per 100 g (25°C)	NA	Slightly
Hydrolysis half-life[d] (20°C and pH 7)	8.5 h	39–41 h; 80 h	80–83 h; 45 h at pH 6.65	42 ha	400–1,000 h	NA	NA
Log K_{ow}[e]	1.18	0.15	1.02	NA	NA	NA	NA
Odor[a–c,f]	Faintly fruity; no odor when pure	Odorless when pure	Fruity, odor of camphor when impure	Perceptible; fruity; no agreement on odor description; odorless when pure	Odorless when pure	NA	Odorless
Odor threshold (mg/m^3)[a–c,f]	Undefined	<1.5	~1.5 to ~7.0	~10.4 to ~14.8	Odorless when pure	Undefined	Undefined
Henry's law constant[e,g] (atm m^3/mol)	$1.52 \times 10 - 7$	$5.34 \times 10 - 7$	$4.56 \times 10 - 6$	NA	$3.5 \times 10 - 9$ (est.)	NA	NA
Conversion factors[h] in air	ppm = (0.15) × mg/m^3 mg/m^3 = (6.6) × ppm	ppm = (0.17) × mg/m^3 mg/m^3 = (5.7) × ppm	ppm = (0.13) × mg/m^3 mg/m^3 = (7.5) × ppm	ppm = (0.14) × mg/m^3 mg/m^3 = (7.4) × ppm	mg/m^3 = (10.936) × ppm ppm = (0.0914) × mg/m^3	NA	NA

Source: Adapted from NRC (2003) with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington, DC.

[a] *Gates and Renshaw (1946); Buckles (1947); DA (1990a,b); Abercrombie (2003); Tevault et al. (2003).*

[b] *DA (1992).*

[c] *DA (1974); Yang (1999).*

[d] *Clark (1989); DA (2005).*

[e] *Britton and Grant (1988).*

[f] *Dutreau et al. (1950); McGrath et al. (1953); DA (2005).*

[g] *Small (1984); Opresko et al. (1998).*

[h] *Calculated from molecular weight.*

MECHANISM OF ACTION

All of the OP nerve agents under consideration are anticholinesterase compounds and induce accumulation of the neurotransmitter acetylcholine (ACh) at neural synapses and neuromuscular junctions (NMJs) by phosphorylating acetylcholinesterase (AChE). Depending on the route of exposure and amount absorbed, the PNS and/or CNS can be affected and muscarinic and/or nicotinic receptors may be stimulated. Interaction with other esterases may also occur, and direct effects to the nervous system have been observed.

Exposure to acutely toxic concentrations of nerve agents can result in excessive bronchial, salivary, ocular, and intestinal secretions, sweating, miosis, bronchospasm, intestinal hypermotility, bradycardia, muscle fasciculations, twitching, weakness, paralysis, loss of consciousness, convulsions, depression of the central respiratory drive, and death (Grob and Harvey, 1953; Grob, 1956; Sidell, 1997; Yanagisawa et al., 2006; Marrs, 2007; Okumura et al., 2015). Minimal effects observed at low vapor concentrations include miosis (contraction of the pupils of the eye, with subsequent decrease in pupil area), tightness of the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989; Dunn et al., 1997). Pupillary contraction, resulting in varying degrees of miosis characterized by measures of pupil diameter, is consequent to local inhibition of ocular AChE activity with pupillary sphincter contraction (Dabisch et al., 2007b, 2008a,b; Dabisch and Taylor, 2010).

Reactivation of inhibited cholinesterase by dephosphorylation is not possible once the nerve agent–cholinesterase complex undergoes "aging," which is thought to be the consequence of the loss of an alkyl or alkoxy group. Agent GD ages very rapidly when bound to red blood cell cholinesterase (RBC–ChE), with a $t_{½}$ (time required for 50% of the enzyme to become resistant to reactivation) of 1.3 min (Harris et al., 1978). The aging half-time for agent GA with RBC–ChE is 46 h (calculated; De Jong and Wolring, 1978), and the $t_{½}$ for agent GB with RBC–ChE is 5 h (Sidell and Groff, 1974). The complex formed between RBC–ChE and agent VX does not age significantly (half-life of approximately 48 h) (Sidell and Groff, 1974; Dunn et al., 1997).

Direct Nervous System Effects

Although nerve agents exert toxic effects on the CNS and PNS indirectly through AChE inhibition (Koelle, 1975, 1981), nerve agents may also affect nerve impulse transmission by additional mechanisms at NMJs (see reviews by Somani and Husain, 2001; Marrs, 2007; Gupta and Milatovic, 2010) and at neurotransmitter receptor sites in the CNS (Myhrer and Aas, 2010; Weissman and Raveh, 2010). Rao et al. (1987) reported that VX caused an increase in ACh release at NMJs in the frog by an interaction with the nicotinic ACh receptor–ion channel complex. Aas et al. (1987) reported alterations in muscarinic receptors in rat bronchi and lung tissue after subacute inhalation exposures to agent GD. In the CNS, nerve agents may act directly on muscarinic, nicotinic, and glutamate receptors in manners unrelated to cholinesterase inhibition (Bakry et al., 1988; Chebabo et al., 1999; Lallement et al., 1991a,b; Rocha et al., 1998, 1999). Chebabo et al. (1999) reported that 0.3–1 nM of agent GB reduced the amplitude of GABA-mediated postsynaptic currents (GABA; neurotransmitter γ-aminobutyric acid), but had no effect on the amplitude of glutamatergic-mediated postsynaptic currents; this selective reduction in action potential–dependent release of GABA might account for GB-induced seizures. Lallement et al. (1991a,b) had suggested that GD-induced overstimulation of glutamatergic receptors contributed to maintenance of seizures.

Although these electrophysiological data indicate that nerve agents may have direct effects on the nervous system unrelated to AChE inhibition, the data do not provide a means of determining a dose conversion to an integrative whole-body endpoint such as lethality or qualitative/quantitative comparisons directly relevant to adverse effects.

It should be further noted that the effects of nerve agents on GABAergic transmission in the CNS may have implications for behavioral effects in laboratory animals and humans, and may also contribute to the induction of convulsions at higher doses (Bakshi et al., 2000). Nevertheless, given the present undefined application of noncholinergic data to whole-body estimations, reliance on the primary assumption of AChE action is consistent with recognized opinion (Bakshi et al., 2000).

Binding with Blood Cholinesterases

The activity of RBC–ChE, as well as that of plasma cholinesterase (plasma–ChE, plasma butyrylcholinesterase or BuChE), has been used to monitor exposure to, and recovery from, anticholinesterase pesticides as well as nerve agents. There is some historical evidence that RBC–ChE can be as sensitive as brain–ChE to the anticholinesterase effects of nerve agents. Grob and Harvey (1958) reported that *in vitro* concentrations producing 50% activity depression of brain–ChE and RBC–ChE were equivalent in the case of GA (1.5×10^{-8} mol/L), and comparable in the case of GB (3.0×10^{-9} versus 3.3×10^{-9} mol/L). The *in vivo* animal studies conducted by Jimmerson et al. (1989) disagree, which is further supported by the fact that blood ChE activity may not always be correlated with exposure or with signs and symptoms of toxicity (Holmstedt, 1959; Sidell, 1992, 1997) (Table 9.2). This was also observed during clinical treatment of cases after the Matsumoto and Tokyo

TABLE 9.2 Human Experimental Data for Single Exposures to GB Vapor

Concentration (mg/m^3)	Exposure Duration	Ct ($mg\,min/m^3$)	Signs and Symptoms	Reference
0.05	20 min	1	Headache, eye pain, rhinorrhea, tightness in chest, cramps, nausea, malaise	Harvey (1952)
0.05	20 min	1	Threshold (<1 mm pupil diameter decrease) to mild (1–2 mm pupil diameter decrease) miosis[a] in test subjects	Johns (1952)
0.06	20 min	1.2	No reported effects	McKee and Woolcott (1949)
0.06	40 min	2.4	Miosis; slight tightness in chest ($n = 4$)	McKee and Woolcott (1949)
0.3	0.5 min	0.15	Rhinorrhea in 16/16; chest tightness in 7/16	Fairley and Mumford (1948)
0.5	30 min	15.0	Miosis, dyspnea, photophobia, 40% inhibition of RBC–ChE, subclinical SFEMG[b] changes	Baker and Sedgwick (1996)
0.6	1 min	0.6	Miosis and slight tightness in chest	McKee and Woolcott (1949)
2	2 min	4	Miosis "moderate"; no other signs of ChE inhibition	Rubin et al. (1957)
NA	10 min to 5 h	3.13	50% pupil area decrement	Callaway and Dirnhuber (1971)
NA	10 min to 5 h	13.85	90% pupil area decrement	Callaway and Dirnhuber (1971)
4.19 (average)	2 min	8.38	Average 47% inhibition of RBC–ChE; no other effects (breathing rate 5.6–8.4 L/min through nose or mouthpiece)	Oberst et al. (1968)
20.7 (average)	2 min	41.4	Average 49% inhibition of RBC–ChE; no other effects (breathing rate 47–65 L/min through nose or mouthpiece)	Oberst et al. (1968)
2.8–4.3	1–2.25 min	4.5–5.0	Miosis of unprotected (unbandaged)[c] eyes of 10 military servicemen; min pupil size of 1.8 mm	Sim (1956)
4.0–4.5	2–2.25 min	8.3–9.8	Miosis of unprotected (unbandaged)[c] eyes of 22 military servicemen; min pupil size of 1.6 mm	Sim (1956)
9.5	1 min, 3 s	10	Miosis of unprotected (unbandaged)[c] eyes of 12 military servicemen; min pupil size of 1.7 mm	Sim (1956)
5.5–7.6	1.75–2.5 min	13.1–15.4	Miosis of unprotected (unbandaged)[c] eyes of 54 military servicemen; min pupil size of 1.5 mm	Sim (1956)
12.8–15.3	1–1.2 min	14.4–15.0	Miosis of unprotected (unbandaged)[c] eyes of 38 military servicemen; min pupil size of 1.5 mm	Sim (1956)

Source: Adapted from NRC (2003) with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington, DC.
[a]*Mild miosis defined by Johns (1952) as "decrease of 1–2 mm" in pupil diameter; reversible within 24 h.*
[b]*Single fiber electromyography (SFEMG).*
[c]*Note that a similar experimental exposure protocol employed by Sim (1956) for subjects with bandaged eyes ("protected") resulted in no clinical miosis in any subject.*

chemical terrorist incidents of GB exposure to the public (Nozaki et al., 1997; Yanagisawa et al., 2006).

It is generally considered that systemic effects in humans after acute nerve agent exposures are likely when RBC–ChE is inhibited by 75–80% (e.g., to 20–25% of normal activity levels) (Sidell, 1992). Nevertheless, it is well-known that local signs and symptoms of the eye and nose in humans and animals (e.g., miosis, rhinorrhea) can occur in the absence of any measurable change from baseline ChE activity in the blood after vapor or aerosol nerve agent exposure (Harvey, 1952; Craig and Woodson, 1959; Sidell, 1992) and are attributable to the local and direct effects of agent on tissues of the eye and upper respiratory tract (Grob, 1956; Dabisch et al., 2008a) (Table 9.2). When systemic exposure (e.g., other than direct ocular or direct nasal) occurs, miosis and rhinorrhea are not usually observed as first noticeable effects (NRC, 2003; Dabisch et al., 2008a).

EPA science policy guidelines regarding use and application of cholinesterase activity inhibition data generally consider blood ChE activity inhibition to be an imperfect measure, and there appears to be no fixed

percentage of blood ChE activity change that can distinguish adverse from nonadverse effects (Storm et al., 2000; USEPA, 2000). A number of investigations have noted the poor association between blood (RBC and plasma) cholinesterase activity and nerve agent intoxication (Rubin and Goldberg, 1957; Sidell, 1992, 1997; Koelle, 1994; Mioduszewski et al., 2002a; Cannard, 2006; Yanagisawa et al., 2006); minimal blood ChE activity has been observed in association with normal tissue function (Sidell, 1992). In a clinical situation, measurement of blood ChE activity has forensic utility and is helpful as a measure of recovery but is not a quantitative measure of absorbed dose (Cannard, 2006).

Binding with Other Enzymes

Nerve agents also interact with detoxification enzymes such as carboxylesterases (CarbE) and A-esterases (e.g., arylesterase and paraoxonase), and the degree of such interaction can alter the magnitude and extent of the toxic cascade after AChE inhibition (Gupta et al., 1991; Pope, 1999; Pope and Liu, 2002; Fonnum and Sterri, 2006) as well as species-specific characteristics. Observed spontaneous reactivation of soman-inhibited plasma CarbE in the rat indicates that "aging" does not occur for the GD–plasma CarbE complex (in contrast to that observed for GD and RBC–ChE) (Dunn et al., 1997), and further suggests that endogenous plasma CarbE may be a principal functional scavenger for agent GD (Maxwell and Brecht, 2001). Recent studies indicate that full characterization of the OP-protective capabilities of CarbEs requires assessment not only of the amount, but also of the affinity exhibited by CarbEs for the inhibitor, as well as the total CarbE activity unlikely to be inhibited (inhibitor resistant esterase activity) (Chanda et al., 2002). The detoxification potential of CarbEs is multifaceted and is an area requiring further experimental characterization (Fonnum and Sterri, 2006, 2015).

TOXICITY

Effects

Nerve agents are toxic anticholinesterase compounds by all routes of exposure and exhibit a steep dose–response. Detailed descriptions of nerve agent toxicity may be found in reviews by Munro et al. (1994), Mioduszewski et al. (1998), Opresko et al. (1998), Sidell (1997), NRC (1999, 2003), Bakshi et al. (2000), Somani and Husain (2001), Marrs (2007), and Weissman and Raveh (2010).

Anticholinesterase effects of nerve agent exposure can be characterized as muscarinic, nicotinic, or CNS. Muscarinic effects occur in the parasympathetic system and, depending on the amount absorbed, can be expressed as conjunctival congestion, miosis, ciliary spasm, nasal discharge, increased bronchial secretion, bronchoconstriction, anorexia, emesis, abdominal cramps, sweating, diarrhea, salivation, bradycardia, and hypotension. Nicotinic effects are those that occur in somatic (skeletal/motor) and sympathetic systems, and can be expressed as muscle fasciculations and paralysis. CNS effects may be manifested as confusion, reflex loss, anxiety, slurred speech, irritability, forgetfulness, depression, impaired judgment, fatigue, insomnia, depression of central respiratory control, and death (Sidell and Groff, 1974; Sidell, 1992, 1997; Opresko et al., 1998; Bakshi et al., 2000). Minimal effects observed at low concentrations in human subjects include miosis, a feeling of "tightness" in the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989) (Table 9.2).

Although RBC–ChE inhibition in the blood is considered an operationally acceptable surrogate for CNS inhibition, plasma ChE is more labile and is a less reliable reflection of enzyme activity change at neuro-effector sites (Young et al., 1999; USEPA, 2000).

In the whole-body agent vapor exposure studies of Mioduszewski et al. (2002a; SD rat single exposures to GB vapor) and Benton et al. (2006a; SD rat single exposures to VX vapor), miosis was usually not correlated with or accompanied by reductions of circulating AChE, BuChE, or CarbE. For the VX vapor exposure study of Benton et al. (2006a), and among those rats exhibiting only one sign (either whole-blood AChE activity inhibition or miosis), miosis developed in the absence of blood AChE activity depression "90% of the time." The findings of Mioduszewski et al. (2002a) for SD rats are consistent with those for human volunteers exposed to GB vapor in the study by Rubin and Goldberg (1957). These results further document the fact that miosis alone, and in the absence of signs such as ChE or CarbE activity inhibition, is a local effect and reflects an exposure much less than that required for generation of systemic clinical effects. Thus, consideration of a local effect such as miosis as a critical endpoint for decision criteria and exposure guideline determination allows a useful margin of protection against the potential for agent exposures sufficiently large to generate systemic effects.

Minimal Potential for Delayed Neuropathy

A continuing area of public concern regarding nerve agent exposure is the possibility of chronic neurological effects, particularly delayed neuropathy, given that neuropathic effects have been observed after high levels of occupational exposure to the lipophilic agricultural pesticides. Exposure to some OP anticholinesterase compounds results in delayed neurotoxic effects (ataxia, distal neuropathy, paralysis), which are collectively described as organophosphate ester–induced delayed

neuropathy (OPIDN). OPIDN is characterized by myelin sheath and axon degeneration and was once thought to be the consequence of inhibition and aging of neuropathy (or neurotoxic) target esterase (NTE) (Abou-Donia, 1993; Ehrich and Jortner, 2002; Gupta and Milatovic, 2012). With greater knowledge and recent data pointing out that NTE-knockout mice may also develop OPIDN (Abou-Donia, 2003; O'Callahan, 2003; Winrow et al., 2003), the NTE theory has been replaced with one involving a noncholinergic, proteolytic mechanism involving cytoskeletal proteins found in neurofilaments (De Wolff et al., 2002). The resulting proteolysis, accompanied by perturbed ionic gradients, cellular edema, and myelin debris, can generate neuropathy.

A number of well-conducted studies using USEPA guidelines for experimental determination of delayed neurotoxicity (USEPA, 1998) have been performed for the G-agents and agent VX (Gordon et al., 1983; Willems et al., 1984; Goldman et al., 1988; Wilson et al., 1988). The USEPA protocol requires toxicological testing with the domestic hen, an OPIDN-sensitive laboratory animal. In general, exposure to the standard threat nerve agents (e.g., GA, GB, GD, GF, VX) is not considered neuropathic in humans (Marrs, 2007) given that agent VX is not neuropathic in standard challenge tests with hens and that G-agent concentrations necessary to induce OPIDN would be supralethal, and human survival would be highly unlikely.

Evaluation of Other Potential Effects

Animal data from vapor, oral, and injection exposure studies for the G-series nerve agents and agent VX indicate that these agents do not induce reproductive or developmental effects in mammals (Van Kampen et al., 1970; Denk, 1975; Schreider et al., 1984, 1988; LaBorde and Bates, 1986; Goldman et al., 1988; Bates et al., 1990; Bucci et al., 1993; LaBorde et al., 1996). Incidental data from the Tokyo subway incident (Ohbu et al., 1997) documenting the birth of healthy children to women who had received exposures to toxic GB concentrations at 9–36 weeks of gestation support this finding.

Neither agent GB nor agent VX was genotoxic in a series of microbial and mammalian assays (Crook et al., 1983; Goldman et al., 1987, 1988), whereas agent GA has been reported to be weakly mutagenic in similar cellular assays (Wilson et al., 1994). Experimental results indicate that agents GB, GA, and VX have no carcinogenic potential (Weimer et al., 1979; Goldman et al., 1988; Bucci et al., 1992a,b).

Inhalation/Ocular Toxicity in Human Subjects

It is noted that the most complete experimental data set for the nerve agents evaluated in all species is that for agent GB. The following analysis reflects that emphasis (Table 9.2). Human study reports evaluated have been previously judged by the US Environmental Protection Agency National Advisory Committee for Acute Exposure Guideline Levels for Hazardous Substances and the National Research Council (NRC) Committee on Toxicology to be consistent with acceptable criteria and procedures regarding informed consent and appropriate clinical supervision (NRC, 2001, 2003).

Agent GB

Fairley and Mumford (1948) exposed 16 male volunteers to 0.3 mg GB/m^3 for 0.5 min. Nine of the test subjects reported that they could detect the agent by smell; seven reported tightness of the chest and 16 reported rhinorrhea.

McKee and Woolcott (1949) evaluated the effects of low concentrations of agent GB on 14 male volunteers. A single exposure to 0.6 mg GB/m^3 for 1 min or 0.06 mg GB/m^3 for 40 min resulted in miosis and slight tightness of the chest; within 24 h, signs and symptoms resolved in subjects exposed for 1 min, whereas more than 48 h was required for resolution in subjects exposed for 40 min.

In a study reported by Harvey (1952), 128 adult male volunteers were exposed in a chamber to GB concentrations ranging from 0.05 to 3.0 mg/m^3 for 2–20 min. The corresponding cumulative exposures ranged from 1.0 to 6.0 mg min/m^3. The most common signs and symptoms resulting from the GB exposures were headaches, eye pain, rhinorrhea, tightness in the chest, cramps, nausea, and concentration difficulties.

When evaluating data from the Harvey (1952) study, Johns (1952) reported on the occurrence of miosis in exposed individuals. Regression analysis of 150 observations, including 55 controls, indicated that the concentration at which a 50% decrease in pupil diameter would be attained was approximately 4.1 mg min/m^3, with 90% confidence limits of approximately 2.7 and 5.7 mg min/m^3. Johns (1952) defined "mild miosis" as a "decrease of 1–2 mm" in pupil diameter, which usually disappeared within 24 h. Although mild miosis as defined was observed in some subjects at the lowest Ct tested (Ct = 1.0 mg min/m^3), other subjects exhibited mean maximal pupil decreases of less than 1 mm, indicating attainment of a response threshold at this level of exposure. Untreated controls exhibited a pupil diameter decrease of 0.33 mm or more; Johns (1952) attributed this difference to observer bias and pointed out that there was still a relative difference between the control group and the exposed groups.

Oberst et al. (1968) conducted inhalation studies in which 125 volunteers were exposed to low concentrations of GB vapor to measure levels of GB retention and changes in RBC–ChE activity. In one series of tests in which resting subjects were exposed to GB for 2 min,

the calculated retained dose was 3.4–3.8 μg/kg and the percent inhibition of RBC–ChE activity was 39–63% (average 49%). In a second series of tests in which exercising men were exposed to GB for 2 min, the calculated retained dose was 3.2–4.0 μg/kg and the percent inhibition of RBC–ChE activity was 29–58% (average 47%). The reported 2 min ChE_{50} dose for all 125 subjects (grouped data) was 3.95 μg GB/kg. From these data, the 2 min EC_{50} for cholinesterase inhibition can be estimated as approximately 21 mg/m^3 for resting men breathing approximately 7 L/min and approximately 4 mg/m^3 for exercising men breathing approximately 50 L/min.

Baker and Sedgwick (1996) exposed eight human volunteers to 0.5 mg GB/m^3 for 30 min in a chamber; test subjects walked at a rate of 96 paces per minute while breathing normally. The exposure resulted in a 60% inhibition of RBC–AChE activity; subjects exhibited miosis, some photophobia and mild dyspnea. Respiratory symptoms resolved within minutes and the ocular effects within 48 h after exposure. There were no clinical neuromuscular signs or symptoms; however, small and nonclinical changes in single fiber electromyography (SFEMG) of the forearm were measured at 3 h and 3 days after exposure; SFEMG changes were not detectible 15–30 months after exposure.

The results of agent GB vapor exposure studies conducted with human volunteers indicate that the threshold for miosis and other minimal toxic effects is in the range of 0.05–0.5 mg/m^3 for 10 min to 30 min exposures (Table 9.2 and summaries).

Rubin et al. (1957) evaluated the effects of agent GB on the visual threshold of three adult volunteers. The test individuals were exposed to 2 mg GB/m^3 for 2 min with the eyes exposed or protected. With the eyes unprotected, the exposure resulted in moderate miosis with no other obvious signs of cholinesterase activity inhibition, but with a significant elevation of the absolute visual threshold in the dark-adapted eye.

Callaway and Dirnhuber (1971) evaluated the "miotogenic potency" of GB vapor in humans (62 miosis responses in 26 human volunteers). Exposure time periods ranged from 10 min to 5 h. Callaway and Dirnhuber reported 50% and 90% decrements in pupil area (Table 9.2). There are acknowledged weaknesses in the protocol and data of Callaway and Dirnhuber (1971), such as limited 1970s-era capabilities for measuring agent vapor concentrations, semi-subjective protocols for measuring miosis in human eyes, and incomplete documentation of miosis incidence.

Based on human and animal data, McNamara and Leitnaker (1971) estimated that the EC_{50} for miosis in humans would be 0.0083 mg/m^3 for 8 h exposure duration or 0.0028 mg/m^3 for 24 h exposure duration. McNamara and Leitnaker (1971) did not expect miosis to occur at 0.001 mg/m^3 for 8 h or 0.0003 mg/m^3 for 24 h.

Agents VX and Vx

No experimental data are available for direct characterization of acute VX vapor toxicity in humans after inhalation exposure. Based on lethality data for several animal species, Bide and Risk (2000, 2004) estimated the 10 min LCt_{50} value for a VX aerosol to be 7 mg min/m^3 for a 70 kg man breathing 15 L/min for 10 min.

One of the few experimental attempts to evaluate human exposure to VX vapor for durations longer than a few minutes is the historically important study of Bramwell et al. (1963), in which eight individuals were exposed to VX vapor concentrations ranging from 0.23 mg/m^3 to 5 mg VX/m^3 for durations ranging from 2.25 s to 24 min (Cts = 0.7–25.6 mg min/m^3). The Bramwell et al. (1963) study is not considered credible because of its seriously flawed exposure protocol; both C and t were varied (resulting in no replicate cumulative exposures), and the organic solvent benzene was used to help disperse the agent in the exposure (carrier solvent may have altered agent absorption) (Reutter et al., 2000).

Koon et al. (1959) evaluated the minimum odor detection limits of VX in 16 volunteers. Each subject sniffed the agent both during the morning and afternoon on 2 successive days (presumably only one sniff at each time point). The estimated total doses for the four exposures ranged from 0.01 to 0.13 μg/kg. No significant changes in RBC or plasma ChE activity were observed in the test subjects. Three subjects reported headaches the evening of the last test, and three other subjects reported slight chest tightness, dryness of the mouth, and nasal irritation for 30 min after the test.

Recent multiservice (Army, Marine Corps, Navy, and Air Force) guidance on agent-specific exposure limits estimates the VX ECt_{50} for mild toxicity in humans (miosis, rhinorrhea) to be 0.10 mg VX-min/m^3 for 2–360 min exposures (DA, 2005). The inhalation/ocular ECt_{50} for severe effects in humans (i.e., muscular weakness, tremors, breathing difficulty, convulsions, paralysis) was estimated to be 10 mg min/m^3 for 2–360 min exposures for a respiratory minute volume of 15 L/min (DA, 2005).

Agent V_x is considered toxic via inhalation exposure or direct contact with the eye and/or skin (DA, 2005) but has been poorly studied. Because of lack of data suitable for analysis, DA (2005) has determined that no toxicity estimates for Vx can be developed at this time.

Inhalation/Ocular Toxicity in Laboratory Species

G-Series Agents

Lethal Levels

There are considerable data regarding the acute lethality of G-series agents for short-term exposures (Table 9.3) (see also NRC, 2003 for a detailed review).

TABLE 9.3 Acute Inhalation Lethality (LC_{50}; LCt_{50}) for Nerve Agent Vapor in Laboratory Animals

Agent	Species	LC_{50} (mg/m^3)	LCt_{50} (mg min/m^3)	Duration (h)	Reference
GB	Rat (f)	18.1	–	0.16[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	22.6	–	0.16[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	8.51	–	0.50[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	8.84	–	0.50[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	6.39	–	1[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	7.55	–	1[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	4.46	–	1.5[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	4.81	–	1.5[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	3.03	–	4[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	4.09	–	4[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	2.63	–	6[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	2.89	–	6[a]	Mioduszewski et al. (2001, 2002a)
GB	Mouse (m)	21.5	–	0.33	Bide and Risk (2004)
GB	Mouse (m)	9.0	–	1	Bide and Risk (2004)
GB	Mouse (m)	5.0	–	3	Bide and Risk (2004)
GB	Mouse (m)	3.4	–	6	Bide and Risk (2004)
GB	Mouse (m)	3.1	–	12	Bide and Risk (2004)
GB	Guinea pig (m)	3.99	–	1	Whalley et al. (2007)
GE[b]	Rat	–	260 to < 350	0.16	Gates and Renshaw (1946)
GE[b]	Mouse	–	245	0.08	Gates and Renshaw (1946)
GE[b]	Mouse	–	330–1,000	0.16	Gates and Renshaw (1946)
GE[b]	Mouse	–	570	0.5	Gates and Renshaw (1946)
GE[b]	Guinea pig	–	>210–1,000	0.16	Gates and Renshaw (1946)
GE[b]	Rabbit	–	230–1,000	0.16	Gates and Renshaw (1946)
GE[b]	Cat	–	170	0.16	Gates and Renshaw (1946)
GE[b]	Dog	–	230	0.16	Gates and Renshaw (1946)
GE[b]	Monkey	–	210	0.16	Gates and Renshaw (1946)
GF	Rat (f)	25.2	–	0.16[a]	Anthony et al. (2003, 2004)
GF	Rat (m)	36.9	–	0.16[a]	Anthony et al. (2003, 2004)
GF	Rat (f)	5.49	–	1[a]	Anthony et al. (2003, 2004)
GF	Rat (m)	6.60	–	1[a]	Anthony et al. (2003, 2004)
GF	Rat (f)	2.2	–	6[a]	Anthony et al. (2003, 2004)
GF	Rat (m)	2.48	–	6[a]	Anthony et al. (2003, 2004)
GF	Minipig (f)	8.69	–	0.16	Hulet et al. (2006b)
GF	Minipig (m)	7.25	–	0.16	Hulet et al. (2006b)
GF	Minipig (f)	2.12	–	1	Hulet et al. (2006b)
GF	Minipig (m)	1.76	–	1	Hulet et al. (2006b)
GF	Minipig (f)	0.97	–	3	Hulet et al. (2006b)
GF	Minipig (m)	1.01	–	3	Hulet et al. (2006b)
VX	Rat (f)	5.44	–	0.16	Benton et al. (2006b, 2007)

(*Continued*)

TABLE 9.3 (Continued)

Agent	Species	LC_{50} (mg/m^3)	LCt_{50} (mg min/m^3)	Duration (h)	Reference
VX	Rat (m)	4.85	–	0.16	Benton et al. (2006b, 2007)
VX	Rat (f)	0.74	–	1	Benton et al. (2006b, 2007)
VX	Rat (m)	0.65	–	1	Benton et al. (2006b, 2007)
VX	Rat (f)	0.16	–	4	Benton et al. (2006b, 2007)
VX	Rat (m)	0.16	–	4	Benton et al. (2006b, 2007)

m, male; f, female.
[a]*Lethality assessed over 14 days.*
[b]*LCt_{50} values summarized from numerous obscure sources by Gates and Renshaw (1946).*

In studies conducted by Mioduszewski et al. (2001, 2002a), acute lethality of agent GB to male and female SD rats was evaluated for time periods of 10, 30, 60, 90, 240, and 360 min in a whole-body dynamic chamber. GB concentrations ranged from approximately 2–56 mg/m^3, and lethality was assessed at 24 h and at 14 days after exposure. Female rats were reported to be significantly ($P < 0.01$) more sensitive than males to GB vapor toxicity over the range of exposure concentrations and durations studied.

In studies conducted by Bide and Risk (2004), male CD-1 strain mice were exposed to whole-body GB for time periods ranging from 20 to 720 min. LC_{50} values for 3–12 h were progressively higher (toxicity lower) than that predicted by either Haber's rule or the Ten Berge relationship (Ten Berge et al., 1986). In studies conducted by Anthony et al. (2004), male and female SD rats were exposed to whole-body agent GF for 10, 60, or 240 min, and lethality was assessed 24 h and 14 days after exposure (Table 9.3). Females were more sensitive than males.

Hulet et al. (2006b) exposed male and female Göttingen minipigs to whole-body lethal concentrations of agent GF vapor for 10, 60, or 180 min (Table 9.3). No significant gender differences were observed in the GF lethality values.

In the latter years of WWII, agent GE underwent acute inhalation toxicity characterization at a number of research facilities managed by the Office of Scientific Research and Development (National Defense Research Committee). These results, for which the research protocols and exposure concentrations are not available for comparison, were summarized by Gates and Renshaw (1946) and are provided in Table 9.3 as LCt_{50} values.

Sublethal Levels

A consistent endpoint for sublethal effects determination is miosis. This information is summarized in Table 9.4.

Van Helden et al. (2003, 2004a,b) exposed male and female marmosets (Callithrix jacchus, Harlan, UK) (whole-body) to mean GB vapor concentrations of 0.27–0.91 μg/m^3 and male Dunkin–Hartley guinea pigs to 0.02–0.43 μg/m^3 for 5 h. The lowest cumulative exposure at which the internal dose became measurable (based on fluoride-regenerated GB from blood BuChE) was 0.04 ± 0.01 mg min/m^3 in marmosets and 0.010 ± 0.002 mg min/m^3 in guinea pigs.

Miosis, EEG effects, and visual-evoked response (VER) were examined after 5 h exposures at concentrations ranging from 7.5 to 150 μg GB/m^3. Significant miosis (as measured by the ratio of pupil diameter to iris diameter; $P < 0.05$) was attained for marmosets and guinea pigs (Table 9.4) (Van Helden et al., 2003; 2004a). Significant ($P < 0.05$) threshold change in EEG parameters for marmosets occurred at 0.2 mg min/m^3, whereas significant threshold VER changes occurred at 25 mg min/m^3 (Van Helden et al., 2004b).

Mioduszewski et al. (2002a,b) exposed young adult (8- to 10-week-old) male and female Sprague–Dawley (SD) rats to whole-body GB vapor concentrations of 0.01–0.48 mg/m^3 for three exposure periods of 10, 60, and 240 min in a dynamic airflow inhalation chamber. Rat pupil diameters were assessed and blood samples were also collected for RBC–AChE, BuChE, and plasma carboxylesterase (CarbE) activity determinations. Animals were also observed for development of clinical signs during a 7-day postexposure period; EC_{50} values for miosis were reported (Table 9.4; miosis EC_{50} points defined as the statistical concentration required for postexposure pupil diameters of 50% or less of the pre-exposure pupil diameter in 50% of the exposed population) (Mioduszewski et al., 2002a,b). Gender differences (females more susceptible) were observed. Whole-body exposure to GB vapor did not result in significant activity inhibition for any blood enzyme monitored (RBC–AChE, plasma–BuChE, or CarbE) for any GB vapor concentration and duration tested. Mioduszewski et al. (2002a,b) concluded that clinical signs associated with whole-body GB vapor exposure were limited to miosis.

Kassa et al. (2001) exposed male albino Wistar rats for 60 min in an inhalation chamber once, or repeatedly to GB concentrations of 0.8, 1.25, or 2.5 mg/m^3. Animals exposed to the lowest concentration (level 1) were asymptomatic based on clinical and laboratory

TABLE 9.4 Experimental Ocular Toxicity (Miosis) Values for G-agents and VX Vapor Exposures

Species	Toxicity Value						Reference
	GB (mg/m^3)	GB ($mg\,min/m^3$)	GD ($mg\,min/m^3$)	GF (mg/m^3)	GF ($mg\,min/m^3$)	VX (mg/m^3)	
Human (10 min–5 h, ECt_{90})	–	13.85	–	–	–	–	Callaway and Dirnhuber (1971)
Human (20 min, ECt_{50})	–	4	–	–	–	–	Johns (1952)
Human (10 min–5 h, ECt_{50})	–	2.33	–	–	–	–	Callaway and Dirnhuber (1971)
Human (20 min, no effect)	–	1.2	–	–	–	–	McKee and Woolcott (1949)
Marmoset (5 h, threshold)	–	2.5	–	–		–	Van Helden et al. (2004a)
Rabbit (10 min–5 h, ECt_{50})	–	1.32	0.59	–	0.75[a]	–	Callaway and Dirnhuber (1971)
Rabbit (10 min–5 h, ECt_{90})	–	2.71	2.19	–	1.79[a]	–	Callaway and Dirnhuber (1971)
Guinea pig (5 h, threshold)	–	1.8	–	–	–	–	Van Helden et al. (2004a)
Rat (m) (10 min, EC_{50})	0.087	–	–	0.184	–	0.01	Benton et al. (2005, 2006a)
Rat (m) (60 min, EC_{50})	0.030	–	–	0.042	–	0.004	Benton et al. (2005, 2006a)
Rat (m) (240 min, EC_{50})	0.024	–	–	0.029	–	0.002	Benton et al. (2005, 2006a)
Rat (f) (10 min, EC_{50})	0.068	–	–	0.080	–	0.007	Benton et al. (2005, 2006a)
Rat (f) (60 min, EC_{50})	0.020	–	–	0.024	–	0.002	Benton et al. (2005, 2006a)
Rat (f) (240 min, EC_{50})	0.012	–	–	0.017	–	0.001	Benton et al. (2005, 2006a)
Göttingen minipig (m) (10 min, EC_{50})	0.244	–	–	0.161	–	–	Hulet et al. (2006a)
Göttingen minipig (m) (60 min, EC_{50})	0.043	–	–	0.047	–	–	Hulet et al. (2006a)
Göttingen minipig (m) (180 min, EC_{50})	0.032	–	–	0.022	–	–	Hulet et al. (2006a)
Göttingen minipig (f) (10 min, EC_{50})	0.214	–	–	0.190	–	–	Hulet et al. (2006a)
Göttingen minipig (f) (60 min, EC_{50})	0.044	–	–	0.058	–	–	Hulet et al. (2006a)
Göttingen minipig (f) (180 min, EC_{50})	0.022	–	–	0.037	–	–	Hulet et al. (2006a)
African green monkey (f) (10 min, EC_{50})	0.469	–	–	–	–	–	Genovese et al. (2008)

m, male; f, female.
[a]*Data for T2715 (2-methylcyclohexyl methyphosphonfluoridate) analog for agent GF.*

measurements. Animals exposed to the second concentration (level 2) were asymptomatic based on clinical signs but experienced significant inhibition of RBC–AChE activity (by 30%). The highest test concentration (level 3) was reported to be a nonconvulsive symptomatic exposure. Three months after exposure, control and exposed animals were evaluated for GB-induced effects using biochemical, hematological, neurophysiological, behavioral, and immunotoxicological methods. None of the exposed animals showed any clinical signs of intoxication; their body weight did not differ significantly from control values, and there were no changes in hematological or biochemical parameters, including blood and brain cholinesterase activity. The only significant effect ($P < 0.05$) observed in rats exposed once to 1.25 mg GB/m^3 (level 2) was an increase in stereotypical behavior. In a continuation of these studies, Kassa et al. (2004) reported that at 3 months after exposure, the level 3 animals showed significant increases in two biochemical markers of stress, plasma corticosterone, and liver tyrosine aminotransferase activities. The latter was also significantly increased in level 2 test animals. In spatial discrimination tests, animals tested at all three GB concentrations showed significant increases in reaction time up to 2 h after exposures. In the level 3 animals, the effects lasted for 3 weeks.

Walday et al. (1991) exposed male Wistar rats to 0.05 or 0.2 mg GD/m^3 for a single 40 h period. No clinical

signs of toxicity were seen during the exposures. AChE, BuChE, and CarbE activities were significantly inhibited in airway and lung tissue at both doses. Brain BuChE and CarbE activity exhibited significant effects at either dose; brain AChE activity did not significantly change from baseline at 0.05 mg GD/m^3, but did so at 0.2 mg GD/m^3.

Genovese et al. (2004) evaluated cognitive and general performance effects of GB on adult male SD rats. The test animals received a single whole-body exposure for 60 min once at 1.7–4.0 mg GB/m^3. Cognitive and behavioral performance testing began 48 h after inhalation exposure and was conducted during 55 sessions occurring over approximately 11 weeks after exposures. Single exposures did not significantly affect performance and no delayed performance onset was observed.

Genovese et al. (2008) characterized the miosis EC_{50} for sarin in a nonhuman primate (African green monkey; *Chlorocebus aethiops*) after 10 min exposures (Table 9.4). Evaluation of potential behavioral change by performance on a serial probe recognition test indicated no change from baseline for all subjects. No other clinical sign was observed.

Genovese et al. (2006) also evaluated cognitive and general performance effects of GF on adult male SD rats using the same protocol as that for GB. The test animals were exposed for 60 min to 0, 1.6, 3.7, or 5.2 mg GF/m^3. The highest test concentration resulted in a significant decrease in the response rate in the behavioral task for the first two postexposure sessions; however, the deficit was not persistent and recovery occurred rapidly. None of the exposures to GF caused a significant effect on completion time in the maze (cognitive) task.

In tests conducted by Allon et al. (2005), freely moving male albino SD rats were exposed to whole-body 34.2 ± 0.8 μg GB/L for 10 min, after which electrocardiograms of exposed and control animals were monitored every 2 weeks for 6 months. One and 6 months after exposure, rats were challenged with epinephrine under anesthesia and the threshold for cardiac arrhythmia was determined. Surviving treated rats displayed agitation, aggression, and weight loss compared with nonexposed rats, and approximately 20% experienced sporadic convulsions. GB-challenged rats with severe signs demonstrated QT segment prolongation during the first 2 to 3 weeks after exposure. Epinephrine-induced arrhythmias appeared at a significantly lower blood pressure in the treated group during the first month after exposure and lasted for up to 6 months.

Callaway and Dirnhuber (1971) evaluated the miotogenic potency of GB vapor in rabbits exposed to GB under goggles (43 miosis responses in ten albino rabbits). Exposure time periods ranged from 10 min to 5 h (Table 9.4).

Bartosova-Sevelova and Bajgar (2005) exposed rats to agent GB vapors for 4 h at four different concentrations (0.30, 0.43, 0.58, and 0.82 mg/m^3) in a whole-body exposure chamber. Convulsions and hypersalivation were observed in one animal exposed to 0.82 mg/m^3. There was a significant decrease in blood AChE activity in all but the low-dose test groups and the controls. AChE activity in the brain was significantly decreased only in animals exposed to 0.58 mg/m^3, and only in the pontomedullar area. No significant alterations in AChE activity were seen in the frontal cortex or in the basal ganglia. AChE activity in the pontomedulla was lowest at the greatest dose (0.82 mg/m^3), but the data were too variable for statistical significance.

Sekowski et al. (2004) evaluated gene and protein level changes in the brain of male and female SD rats exposed to low-level doses (0.004–0.033 mg/m^3) of aerosolized agent GB and GF via whole-body inhalation for 4 h. Preliminary results indicate that exposure to nerve agents results in differential expression of a number of neuronal genes, including a group that affects cellular processes critical to neurological injury and regeneration, and gender-associated differences in the level and type of gene expression response were significant.

Whalley et al. (2004) exposed adult male and female SD rats to a series of whole-body agent GF vapor concentrations for 10, 60, or 240 min. Miosis (defined as a 50% reduction in pupil area compared with baseline) measured approximately 30 min after exposure indicates that females were significantly more sensitive than males ($P < 0.05$) (Table 9.4).

In studies conducted by Hulet et al. (2006a,c, 2007), male and female Göttingen minipigs were exposed to whole-body agent GB or GF for 10, 60, or 180 min (Table 9.4). Male minipigs were significantly ($P = 0.022$) more sensitive to the effects of GF exposure than females.

Conn et al. (2002) exposed male F344 rats to 0, 0.2, or 0.4 mg GB/m^3 for 1 h/day for 1 or more days. Animals were maintained at either 25°C or 32°C to evaluate the effects of heat stress. Body temperature and activity were monitored by telemetry continuously during exposure and for 1 month after the exposures. Although RBC–ChE activity was reduced in the exposed animals (quantitative data not provided), the test protocol did not significantly alter temperature regulation or locomotive activity of the rats.

Agent VX

Lethal Levels

Benton et al. (2006b, 2007) experimentally determined the LCt_{50} and LC_{50} in male and female adult SD rats exposed to whole-body VX vapor for 10, 60, and 240 min in a dynamic exposure chamber (Table 9.3); study protocol was similar to that for agent GB in the studies conducted by Mioduszewski et al. (2001, 2002a). Experiments testing the role of decontamination

less than 24 h after exposure provided clear evidence for percutaneous toxicity induced by whole-body vapor exposure to the persistent nerve agent VX. For severe and lethal VX vapor exposure effects, females were not more susceptible than males for the exposure durations examined.

Bide and Risk (2000) exposed outbred male CD1 (SD) BR rats, outbred male CD1 (ICR) BR mice, and outbred male (HA) BR guinea pigs to NaCl aerosols containing entrained VX in a nose-only inhalation system for an exposure time of 12 min. Observed LCt_{50} values are summarized in Table 9.3.

Bide and Risk (2000, 2004) also cite several previous studies in which LCt_{50} values for mice, guinea pigs, rabbits, hamsters, and dogs were reported (Table 9.3).

For exposure to VX vapors, Koon et al. (1960) reported 10 min LCt_{50} values for mice with exposure to either whole-body or head only, as well as for goats. Carroll et al. (1957) also reported female mouse LCt_{50} values for nose-only and whole-body protocols. However, Carroll et al. (1957) reported that the concentration of VX in the exposure chamber was not measured directly but was estimated from the mortality level, which was correlated with the LD_{50} for i.v. injection.

Sublethal Level

Benton et al. (2005, 2006a, 2007) have characterized miosis as well as severe effects (severe tremors and/or prostration, convulsions and/or gasping) in male and female SD rats exposed to whole-body VX vapor (0.00037–0.016 mg VX/m^3) under study protocols similar to those for agent GB in the studies conducted by Mioduszewski et al. (2001, 2002a). Miosis EC_{50} endpoints were derived for VX vapor exposure durations of 10, 60, and 240 min (Table 9.4). At the highest VX concentrations tested for each exposure duration, significant (>99.9% confidence) differences between control and experimental whole-blood AChE activity were observed; no other signs (e.g., tremors, salivation) were observed and delayed pupil effects were minimal. For the miosis endpoint, female rats are considered more susceptible than males to VX vapor exposure.

For severe effects (tremors, prostration, etc.), the ECt_{50} values (mg min/m^3) reported by Benton et al. (2007) were as follows: 10 min, 40.9 (female) and 35.2 (male); 60 min, 30.0 (female) and 31.2 (male); and 240 min, 31.5 (female) and 29.9 (male). EC concentrations were not reported.

After single 60 min VX vapor exposures in the range of 0.016–0.45 mg VX/m^3, Genovese et al. (2007) examined blood AChE activity, dose estimation by regeneration assay, transient miosis, and behavior parameters in adult male SD rats. Behavioral evaluation included a radial maze task and a variable-interval schedule-of-reinforcement task. At all concentrations tested, transient miosis and AChE activity inhibition were observed and some subjects exhibited transient ataxia and slight tremor. After 3-month postexposure evaluations of behavior, the authors concluded that performance deficits were minor and transient at these concentrations. Further, no delayed effects were observed.

RISK ASSESSMENT

Application of standard risk assessment methods by numerous authorities and agencies to the toxicological data summarized has generated exposure guidelines that provide objective and health-based foundations for responsible and efficient response after nerve agent release, as well as a basis for site recovery and decontamination decision criteria. The health-based nerve agent exposure guidance summarized here has been derived in an open and transparent manner and judged scientifically valid and protective (Opresko et al., 1998, 2001; NRC, 1999, 2001, 2003; Krewski et al., 2004, Watson et al., 2006a,b; see also www.epa.gov/oppt/aegl/). Although initially developed to facilitate disposal of the US stockpile of CW munitions and to support remediation or closure at sites where CWs were historically processed, nerve agent exposure guidance became a subject of interest for homeland defense applications after the events of September 2001.

For reasons described previously, the air exposure pathway has been a primary focus of risk assessment activity for nerve agents (NRC, 2003; Cannard, 2006; ATSDR, 2007; Dabisch et al., 2008a; others). In situations where long-term agent release is a concern, and where agent residuals may be found, potential exposure to relatively low levels of ingested agents is a priority.

In all cases, it is important to appropriately safeguard public health without defaulting to overly conservative actions (e.g., to "nondetect") that would divert limited resources without significant benefit. The following sections summarize toxicological support and developmental rationale for the two primary criteria of interest to community decision-makers managing response to an intentional, or accidental release of nerve agent(s) to the environment.

Acute Exposure Guideline Levels

Credible short-term nerve agent exposure limits, designed to aid state and local government agencies in developing emergency response plans in the event of accidental or deliberate atmospheric release, have been derived. These short-term values have also proved useful in deployed force health protection, and in establishing health-based CWA performance goals for detection system development (USACHPPM, 2004, 2008).

TABLE 9.5 Summary of AEGL Values for G-series Nerve Agents and VX (mg/m^3)

Agent	Classification	10 min	30 min	1 h	4 h	8 h
GA	AEGL-1	0.0069	0.0040	0.0028	0.0014	0.0010
	AEGL-2	0.087	0.050	0.035	0.017	0.013
	AEGL-3	0.76	0.38	0.26	0.14	0.10
GB	AEGL-1	0.0069	0.0040	0.0028	0.0014	0.0010
	AEGL-2	0.087	0.050	0.035	0.017	0.013
	AEGL-3	0.38	0.19	0.13	0.070	0.051
GD	AEGL-1	0.0035	0.0020	0.0014	0.00070	0.00050
	AEGL-2	0.044	0.025	0.018	0.0085	0.0065
	AEGL-3	0.38	0.19	0.13	0.070	0.051
GF	AEGL-1	0.0035	0.0020	0.0014	0.00070	0.00050
	AEGL-2	0.044	0.025	0.018	0.0085	0.0065
	AEGL-3	0.38	0.19	0.13	0.070	0.051
VX	AEGL-1	0.00057	0.00033	0.00017	0.00010	0.000071
	AEGL-2	0.0072	0.0042	0.0029	0.0015	0.0010
	AEGL-3	0.029	0.015	0.010	0.0052	0.0038

Source: Adapted from NRC (2003) with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington, DC.

Acute exposure guideline levels (AEGLs; expressed in units of mg/m^3 or ppm) are vapor exposure guideline values for numerous hazardous compounds (primarily toxic industrial compounds) that have been published by the National Academy Press (e.g., NRC, 2003, 2007). For each hazardous compound, guideline levels are developed for vapor exposure durations of 10 and 30 min, 1, 4, and 8 h, as well as for three gradations of toxic effect severity. AEGL-1 concentrations are the mildest effect category, whereas AEGL-3 concentrations represent the most severe effect category (NRC, 2001). The point above the AEGL-3 concentration at which level 3 effects would initiate for any given human exposure duration is not identified in the AEGL assessment protocol.

Typically, the AEGL concentration established for any given effect level is often less than the known experimental concentration at which such toxicological effects occur. This protective nature of the AEGL process and values was demonstrated for each of the nerve agents, where observed human thresholds for reversible effects occur at air concentrations greater than AEGL-1 levels (Watson et al., 2006a,b).

Selection protocols for critical effects and studies, AEGL derivation, time scaling, use and selection of uncertainty and modifying factors, and a description of the lengthy and deliberative review process used are all described in NRC (2001) as well as in recent articles by Krewski et al. (2004) and Bruckner et al. (2004). Development of AEGL values includes consideration of uncertainty factors as well as the need for any modifying factors. Because exposure–response data are usually not available for each AEGL-specific exposure duration (NRC, 2001), temporal extrapolation is used in the development of values for some AEGL-specific time periods. The concentration–exposure time relationship for many systemically acting vapors and gases may be described by $Cn \times t = k$, where the exponent n ranges from 0.8 to 3.5 (Ten Berge et al., 1986; NRC, 2001). Some investigators refer to the Ten Berge extrapolation as the "toxic load model" and n as the "toxic load exponent" (Sommerville et al., 2006; Dabisch et al., 2008a). The excellent data collected by investigators at Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), characterizing nerve agent vapor exposure miosis and lethality endpoints for multiple agents and species, has allowed agent-specific determination of n (summarized in Dabisch et al., 2008a). In the case of swine exposed to G-agents and rats exposed to VX, the experimentally determined n values for these endpoints are 1.6 or less, which are less than the n of 2 assumed during AEGL development for these same compounds in 2001–2003 (NRC, 2003), and based on then-available data for SD rats exposed to agent GB (Mioduszewski et al., 2002a,b). It thus appears that G-agent and VX dose response for the miosis and lethality endpoints are less steep than previously indicated and that the published nerve agent AEGL values (Table 9.5) are more protective than originally considered (NRC, 2003; Watson et al., 2006a,b).

For comparison, it is useful to consider other common guideline sources applicable to short-term nerve agent release events. The US Department of Energy, in their development of 1 h Protective Action Concentrations/Temporary Emergency Exposure Levels (PAC/TEELS), has chosen to replicate the published nerve agent-specific 1 h AEGL-1, AEGL-2, and AEGL-3 values as tier 1, tier 2, and tier 3 PAC/TEEL values, respectively (see http://hss.energy.gov/HealthSafety/WSHP/chem_safety/teel.html).

Application of AEGL Values

The AEGL process does not include specific implementing or application guidance, and specific approaches for using the values are left to the discretion of risk managers and appropriate authorities (NRC, 2001). Nevertheless, AEGL application is already broad and continues to expand.

The utility of AEGL values for CW agent emergency preparedness planning was recognized by the Chemical Stockpile Emergency Preparedness Program (CSEPP), when FEMA and Army representatives adopted final nerve agent AEGL concentrations to replace previous agent toxicity criteria for emergency response decision-making (CSEPP, 2003). As of February 2003, standing CSEPP policy guidance for each of the communities hosting agent demilitarization facilities in the US, recommends application of AEGL-2 concentrations as the protective action level for evacuation or shelter-in-place, and application of AEGL-1 concentrations as notification levels (CSEPP, 2003). Since publication of final AEGL levels by NRC (2003) and enactment of the CSEPP Policy Paper (CSEPP, 2003), multiple stockpile states and counties have incorporated the Policy Paper recommendations into their individual community emergency response plans, and used them in making regulatory decisions permitting agent munition disposal operations (CSEPP, 2006a,b).

In February 2008, the US National Response Team (NRT) posted Quick Reference Guides for the G-series nerve agents and VX for public access on its website (www.nrt.org) (NRT, 2008). These Quick Reference Guides are useful summaries of agent characteristics and advisories and are provided as national guidance. While acknowledging that site-specific clean-up decision criteria will be the result of multi-agency agreements and site-specific factors, the NRT considers that attainment of agent-specific air concentrations less than 8 h of AEGL-1 is an acceptable criterion for verification of site decontamination.

In general, agent concentrations less than AEGL 2 are considered to be in a range that poses relatively negligible health consequences for acute exposures.

Other AEGL applications performed or recommended include use as hazard assessment plume modeling criteria for the US Nonstockpile Chemical Material Program and Homeland Defense scenarios, as testing criteria for personal protective equipment intended for use by first responders in a single weapon-of-mass-destruction scenario, as detection performance goals for advanced equipment acquisition and development, and as a tool for assessing potential exposures during military missions such as peacekeeping (USACHPPM, 2008).

Estimated Oral Reference Doses

Development of nerve agent–specific reference dose estimates is critical to remediation and restoration at existing and closing military sites, which is a priority activity for DOD (Opresko et al., 1998, 2001).

A reference dose (RfD; mg/kg/day) was originally designed for estimating noncancer health risks at CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act of 1980) Superfund sites (USEPA, 1989). As such, it is an essential component of the site risk assessment used to assess potential long-term exposures to contaminated media such as soil, where RfDs address the pathway of incidental ingestion of soil particles (USEPA, 1989; Dourson, 1994; Cicmanec et al., 1996; Abernathy et al., 2004).

Methods used to derive oral RfDs for nerve agents follow standard USEPA protocols (USEPA, 1989; Dourson, 1994), use appropriate toxicological data and uncertainty factors, and have undergone review for consistency by the National Research Council (Opresko et al., 1998, 2001; NRC, 1999; Bakshi et al., 2000). Because EPA has not officially verified the derived values for nerve agents, they are identified as estimated RfDs (RfDe) (Table 9.6).

These criteria are selected by the Office of the Army Surgeon General as the most appropriate oral toxicity reference values for use in environmental risk assessments and represent the Army's position (Opresko et al., 2001). The RfDe values have been input to USEPA risk models along with accepted chronic vapor exposure limits (as cited in Watson and Dolislager, 2007) to generate agent-specific Health Based Environmental Screening Levels (HBESLs). These HBESLs are endorsed by military policy as criteria to assess potentially contaminated soils (Table 9.6) (USACHPPM, 1999; OASA, 1999; Watson and Dolislager, 2007).

TREATMENT

Critical Role of Decontamination

Before discussion of antidotes and treatment regimens, it is noted that affected individuals should be removed from the site of agent exposure as quickly as possible, and undergo rapid decontamination to remove

TABLE 9.6 Estimated Reference Doses (RfDe), RfD Uncertainty Factors, and Health-Based Environmental Screening Levels for Nerve Agents

Nerve Agent	RfDe (mg/kg/day)	Composite Uncertainty	Residential Soil Preliminary Remediation Goal (mg/kg)	Industrial Soil Preliminary Remediation Goal (mg/kg)
VX	6E-7	100	0.042 (est.)	1.1 (est.)
GA	4E-5	3,000	2.8 (est.)	68.0 (est.)
GB	2E-5	3,000	1.3 (est.)	32.0 (est.)
GD	4E-6	3,000	0.22 (est.)	5.2 (est.)

Source: From Opresko et al. (1998, 2001), USACHPPM (1999), OASA (1999), Watson and Dolislager (2007).

potential for continued personal exposure and to prevent secondary exposure to responders, health care workers, medical transport vehicles, and treatment facilities (Sidell, 1997; Cannard, 2006; ATSDR, 2007; Okumura et al., 2007; Pulley and Jones, 2008). Decontamination of CW agents is discussed more fully by Gordon (2015) in Chapter 76 of this volume.

Signs and Symptoms Guiding Medical Management

Depending on concentration and duration of exposure, cases of nerve agent intoxication can exhibit a dose-dependent "constellation" of clinical signs and symptoms representing a variety of parasympathetic effects, functional change at NMJs, and altered CNS function (Cannard, 2006). As a consequence, critical care personnel and others responsible for developing and administering treatment protocols should take into account the totality of the case presentation. A good example is drawn from observations made by medical personnel treating subway passengers presenting at Tokyo area hospitals and clinics on day 1 of the sarin release incident. In decreasing order of frequency, the following clinical signs and symptoms were noted: miosis (observed in most patients), headache, dyspnea, nausea, vomiting, muscular weakness, cough, rhinorrhea, chest oppression, muscular fasciculations, and psychological disturbances such as anxiety (Lillibridge, 1995). Similar distributions were observed among subway passengers treated at St. Luke's International Hospital (Okumura et al., 1996, 2007) as well as residents affected in the Matsumoto incident of 1994 (Yanagisawa et al., 2006), and are consistent with classic descriptions of nerve agent intoxication (Sidell, 1997; Leikin et al., 2002; Cannard, 2006). Miosis was found to be a more responsive exposure index than RBC–ChE activity inhibition (Nozaki et al., 1997) or serum cholinesterase activity (Yanagisawa et al., 2006) in cases of sarin vapor exposure during the Tokyo and Matsumoto incidents, respectively (Okumura et al., 2015).

Yanagisawa et al. (2006) classified individuals exhibiting a pupil diameter larger than 3.0 mm as without miosis and not affected by nerve agent vapor exposure given that simultaneous serum ChE activity measurements taken during treatment of the Matsumoto cases displayed no inhibition (e.g., largely ≥100% of normal serum ChE activity with two cases exhibiting activity <90% of normal). This, and additional miosis data from Matsumoto, can be used in determining appropriate treatment for individuals without known or confirmed nerve agent vapor exposure (Cannard, 2006; Yanagisawa et al., 2006); for these individuals, examination, observation without treatment, and discharge are appropriate actions and were successfully implemented during medical responses to the Tokyo subway incident (Lillibridge, 1995; Okumura et al., 2015).

Nerve Agent Antidotes

The choice of appropriate treatment for nerve agent intoxication depends on the agent as well as extent and route(s) of exposure. Very mild exposure to nerve agent vapor may necessitate only decontamination and observation; severe exposure to vapor or liquid requires immediate decontamination, antidote administration, artificial respiration, monitoring, and supportive therapy over hours to multiple days (Sidell, 1997; ATSDR, 2007; Vale et al., 2007; Pulley and Jones, 2008). Convenient triage classifications have been developed by ATSDR (2007) in collaboration with the US Army Medical Research Institute of Chemical Defense.

In cases of massive inhalation exposure, immediate care is vital to prevent death from respiratory failure and because the agent–AChE complex becomes resistant to reactivation by oxime-type antidotes. "Aging" is compound-specific; aging half-times range from minutes (agent GD) to days (agent VX) (Sidell and Groff, 1974; Sidell, 1997). Standard antidotes clinically available in the United States are atropine (anticholinergic) and pralidoxime (Protopam or 2-PAM-Cl). In addition, CNS active drugs such as diazepam (Valium) are strongly recommended if convulsions occur; anticonvulsant treatment is critical for protection against lethality and brain pathology (Shih et al., 2003).

Individuals exposed to vapor and exhibiting miosis only or miosis and rhinorrhea only do not usually require antidote treatment and will resolve without medical intervention (Cannard, 2006; ATSDR, 2007), but they should be observed. If rhinorrhea is problematic in these vapor-only cases, then ATSDR (2007) advises intramuscular atropine (0.05 mg/kg pediatric; 2.0 mg adult) to relieve signs, followed by patient discharge. If eye pain/headache or nausea is problematic in vapor-only

TABLE 9.7 Recommended Antidote Protocol for Emergency Nerve Agent Exposure Therapy[a]

	Antidotes		
Patient Age	**Mild/Moderate Signs and Symptoms[b]**	**Severe Signs and Symptoms[c]**	**Other Treatment**
Infant (0–2 years)	Atropine: 0.05 mg/kg i.m. or 0.02 mg/kg i.v. 2-PAM-Cl: 15 mg/kg i.v. slowly	Atropine: 0.1 mg/kg i.m. or 0.02 mg/kg i.v. 2-PAM-Cl: 15 mg/kg i.v. slowly	Assisted ventilation as needed Repeat atropine (2 mg i.m. or 1 mg i.m. for infants) at 5–10 min intervals until secretions have diminished and breathing is comfortable or airway resistance has returned to near normal Phentolamine for 2-PAM induced hypertension (5 mg i.v. for adults; 1 mg i.v. for children) Diazepam for convulsions (0.2 to 0.5 mg i.v. for infants up to 5 years; 1 mg i.v. for children >5 years; 5 mg i.v. for adults)
Child (2–10 years)	Atropine: 1.0 mg i.m. 2-PAM-Cl: 15 mg/kg i.v. slowly	Atropine: 2.0 mg i.m. 2-PAM-Cl: 15 mg/kg i.v. slowly	
Adolescent (>10 years)	Atropine: 2.0 mg i.m. 2-PAM-Cl: 15 mg/kg i.v. slowly	Atropine: 4.0 mg i.m. 2-PAM-Cl: 15 mg/kg i.v. slowly	
Adult	Atropine: 2.0–4.0 mg i.m. 2-PAM-Cl: 15 mg/kg (1 g) i.v. slowly	Atropine: 6.0 mg i.m. 2-PAM-Cl: 15 mg/kg i.v. slowly	
Elderly, frail	Atropine: 1.0 mg i.m. 2-PAM-Cl: 5–10 mg/kg i.v. slowly	Atropine: 2.0 mg i.m. 2-PAM-Cl: 5–10 mg/kg i.v. slowly	

[a]*Contents reproduced from ATSDR (2007) (public domain).*
[b]*Mild/moderate signs and symptoms include localized sweating, muscle fasciculations, nausea, vomiting, weakness, dyspnea.*
[c]*Severe signs and symptoms include unconsciousness, convulsions, apnea, flaccid paralysis.*

cases, then ATSDR (2007) further advises administration of topical atropine or homatotropine to the eye for relief, eye protection from bright light, and discharge.

If liquid/droplet exposure is known or suspected in an individual exhibiting miosis only or miosis and rhinorrhea only, it is recommended that the individual receive no antidote treatment but be closely observed for at least 18 h, because toxic effects of liquid percutaneous exposure may not manifest for several hours (Sidell, 1997; Cannard, 2006). Toxic effects from vapor-only exposure usually occur quickly (within minutes) (Sidell, 1997). Current medical management guidelines and recommended medication protocols are summarized in ATSDR (2007), Vale et al. (2007), and Pulley and Jones (2008). The ATSDR antidote treatment protocol for civilian emergency management is summarized in Table 9.7. For additional information about antidotal treatment, readers are referred to Chapters 67, 68, and 71 of this volume.

Ongoing Antidote Development

Given that termination of seizure activity protects against development of neuropathological lesions (especially neuronal necrosis) in brain tissues of experimental animals (Martin et al., 1985; Shih et al., 2003; Marrs and Sellström, 2007), focus on anticonvulsant therapy is critical. Reduced potential for permanent brain damage in human cases by preventing or limiting the duration of status epilepticus is a primary goal. In challenge tests against multiple LD_{50} doses of agents GA, GB, GD, GF, VX, and VR in guinea pigs, the anticonvulsants midazolam and trihexyphenidyl were more effective than diazepam for seizure control, and midazolam was the most rapidly effective (Shih et al., 2003). The diazepam pro-drug avizafone is also effective (Lallement et al., 2000, 2004) and is available via auto-injector administration (as is diazepam). For more rapid seizure control during acute treatment phases, Marrs and Sellström (2007) recommend midazolam administered intramuscularly because of its more rapid intramuscular absorption.

Although pralidoxime is an effective and well-tolerated reactivator, it is not very potent. The search has thus continued for oximes that would combine high reactivator effectiveness against nerve agents with low toxicity and good chemical stability; several promising drugs (the oxime HI6; trimedoxime, or TMB-4; and obidoxime, or LüH-6) have emerged but are not equally effective against all agents and forms of exposure (Dawson, 1994; Marrs et al., 2006; Antonijevic and Stojiljkovic, 2007; Eyer and Worek, 2007; Wetherell et al., 2007; Kuca et al., 2009; Kassa and Kuca, 2010). Marrs et al. (2006) point out that, in the absence of pyridostigmine pretreatment, there are at present "no clinically important differences" between the standard oxime pralidoxime and the alternative oximes HI-6 and obidoxime in the treatment of nerve agent intoxication.

As a combinatorial drug with atropine, galantamine has been effective and safe in counteracting lethal GD and GB doses in the guinea pig (Albuquerque et al., 2006; Pereira et al., 2008); galantamine also protects against neurodegeneration at doses of LD50 or more in the guinea pig, and it shows promise as a pretreatment before GD or GB exposures.

Lethality was prevented by treatment with nasal atropine (atropine methyl nitrate), and postexposure treatment with atropine methyl bromide instillation, in combination with pulmonary therapeutic surfactants or liquevents in guinea pigs exposed to approximate LC_{50} concentrations of VX aerosol (Nambiar et al., 2007). This concept shows promise for operational application in emergency response.

Pretreatment When Exposure Is Likely

In certain military deployment settings when threat of nerve agent exposure exists, pretreatment with an anticholinesterase carbamate compound has been fielded to protect personnel. The pretreatment carbamate of current choice, pyridostigmine bromide (PB), reversibly sequesters (and thereby protects) a fraction of AChE from bonding with circulating nerve agents; the carbamate moiety spontaneously hydrolyzes from the AChE molecule within hours and allows AChE to again become available for normal physiological function. Such a pretreatment concept and drug enhances the effectiveness of atropine and oximes in treating lethal doses of GA, GB, and VX (Gall, 1981; Inns and Leadbeater, 1983). US combat units already supplied with atropine and pralidoxime have been equipped with 30 mg PB tablets for oral administration every 8 h; the current maximum pretreatment period is 21 days (Sidell, 1997; Scott, 2007).

A related carbamate, physostigmine, has been shown to protect animals against not only nerve agent lethality but also incapacitation (Leadbeater et al., 1985). When co-administered with hyoscine, physostigmine effectively reduced incapacitation and prevented death in guinea pigs exposed to agent GD (Wetherell, 1994), and prevents lethality and reduced/prevented incapacitation in guinea pigs exposed to GA, GB, GD, GF, and VX (Wetherell et al., 2002). Transdermal patch administration of physostigmine and hyoscine, or physostigmine alone, in the guinea pig has protected against GD intoxication (Meshulam et al., 1995).

Other developmental pretreatment options include NMDA receptor antagonist memantine in combination with atropine sulfate (Gupta and Dettbarn, 1992; McLean et al., 1992), pre-exposure loading with an excess of circulating ChE or BuChE (Bajgar et al., 2007; Lenz et al., 2008; Podoly et al., 2008; Saxena et al., 2008; Van der Schans et al., 2008; Masson, 2015), or CarbEs (Maxwell et al., 1987) to bind nerve agent before the agent can reach tissue AChE sites.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A good deal of nerve agent toxicity research performed and published since the mid 1990s has served to bring a more widespread awareness of the knowledge that detectable (either by means of devices or manifestation of signs) low-level nerve agent exposures under a variety of scenarios are both sublethal and also potentially without significant long-term adverse health impact. Although such points are also inherent in the development of LD_{50}s and related values, much of the original focus of nerve agent toxicological work was, not surprisingly, on lethal or severe toxicological endpoints. To the extent that it adds detail and rigor to the estimation of exposure levels for nonlethal effects as well as insightful information on specifics of the mechanisms of nerve agent action, the more recent research has been highly useful in validating past conclusions on the subject. These recent data also further direct attention toward responsible consideration of the consequences for transient presence of residual, low-level agent concentrations in a number of scenarios. Having a transparent, robust, and strongly data-based framework within which to evaluate likely consequences of potential low-level nerve agent exposures, will greatly aid in planning and evaluating response operations, and reducing the magnitude of disruption to affected communities and facilities.

It is hoped that additional species and agents will be evaluated under comparable experimental protocols for the endpoints of miosis and lethality, as well as for more intermediate toxicological endpoints.

Standard treatment and pretreatment guidance is also summarized, as well as consideration of novel antidote development and promising approaches. Additional clinical trials will assist in developing protocols and practices for expanding available treatment options. Fielding of improved seizure management drugs and protocols should be expedited. The detoxification potential of CarbEs is multi-faceted and is an area that would benefit from further characterization.

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CHAPTER

10

Russian VX

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INTRODUCTION AND BACKGROUND

One of the most abundant and most toxic agents in the chemical arsenals of the United States and Russia is VX and Russian VX (RVX), respectively, whose development in the middle of the twentieth century indicated the peak of warfare chemistry. V-gases are low-volatile liquids with high boiling points, so they are much more persistent than the higher-volatility organophosphorus nerve agents, such as sarin or soman. V-series compounds are more toxic that other organophosphates (OPs). For example, in comparison with sarin (GB), VX is estimated to be approximately twice as toxic by inhalation, 10 times as toxic by oral administration, and approximately 170 times as toxic by skin exposure (Munro et al., 1994). V-series nerve agents are quite effective when exposed through skin contact (especially as tiny drops) and commonly cause death. Poisoning occurs regardless of exposure method (specifically inhalation, ingestion of vaporous and liquid agents through intact or injured skin or eye mucosa, and contact with contaminated surfaces).

The name *VX* relates to a group of *O,S*-diesters of methylphosphonic acid $ROPO(CH_3)S(CH_2)_2N(R1)_2$. *O*-isobutyl *S*-2-(diethylamino)ethyl methylphosphonothioate (R = *i*Bu, R1 = Et), produced since 1972 exclusively in the former Soviet Union, was generally referred to as *RVX* (CAS #159939-87-4). The synonyms for this substance are as follows:

- VR
- VA
- Phosphonothioic acid methyl-, *S*-[2-(diethylamino) ethyl] *O*-(2-methylpropyl) ester
- *O*-isobutyl *S*-2-(diethylamino)ethyl methylthiophosphonate
- *O*-isobutyl *S*-(*N,N*-diethylaminoethyl) methylphosphonothioate
- Russian V-gas

The brutto formula of RVX is $C_{11}H_{26}SNPO_2$ (MW 276.37). The structural formula of RVX is presented in Figure 10.1.

RVX is a colorless transparent liquid resembling glycerol in mobility, with a boiling point of 300.0°C, melting point of 35.0°C, ρ^{20} of 1.0083 g/cm^3, and volatility of C_{max}^{20} 0.0105 mg/dm^3. The agent is poorly soluble in water (less than 5% at 20°C) and highly soluble in organic solvents. Technical products can be colored from yellow to dark brown and smell like fried sunflower seeds.

Research into the environmental behavior of highly toxic chemicals is an important branch of analytical toxicology. We take the word *behavior* to mean the persistence, mechanisms of possible transformation, composition of such transformation products, and toxicity of these substances. Among known toxicants, V-series compounds tend to undergo perhaps the most varied transformations due to their unique structure. Possessing both high reactivity and polyfunctionality, these compounds incorporated into multicomponent matrices are capable of concurrently reacting with several components. Therewith, the reactions may involve different active centers within the same molecule. The routes and results of such reactions cannot be predicted in advance. The situation is even more complicated by the fact that active components of a natural or technogenic matrix are not always known. In view of this, research aimed at identifying transformation products of

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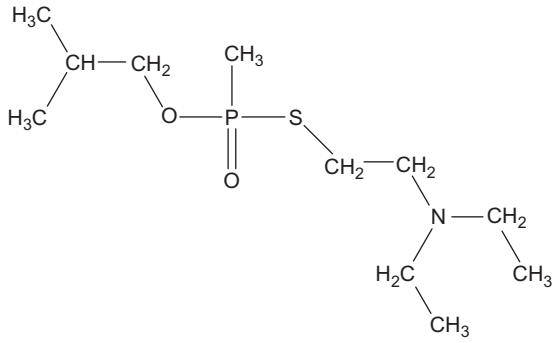


FIGURE 10.1 Structural formula of Russian VX.

VX in various media are of particular importance. The degradation of such compounds is suggested to be initiated by electron addition to phosphorus *via* reaction with anionic nucleophiles (Yang, 1999), such as hydroxide ion, water, alcohols, amines, or unsaturated organic compounds. The problem of determination of RVX in complex matrices and identification of RVX transformation products is still more complicated by the scarcity of available reference information.

Cholinesterases (ChEs) are well-known targets for OPs, and RVX is no exception. Much less information is available about other enzymes that could be primary targets upon exposure to low doses of OP, and on biochemical markers of possible delayed effects of OP intoxication when the level of ChE activities is the same as the control. However, this problem is very important for various reasons, among which is fulfillment of chemical warfare agents (CWAs) nonproliferation conventional programs, and the inherent possibility of accidental exposure of personnel to RVX, as well as the chronic effects of subsymptomatic concentrations of RVX that could arise from the stockpiles of chemical agents. Among other reasons, the terrorist threat is a well-recognized reason for concern.

MONITORING OF RVX

A group of measures for assessing levels of toxic chemicals in the environment is called *ambient monitoring*. The possible objects of ambient monitoring are air, water, soil, food, wastes, building materials, and other media through which toxicity factors can be delivered to the human organism. Biomonitoring is based on sampling and analysis of body fluids and tissues and is an indicator of internal dose that provides a measurement of exposure to a toxic chemical. It is quite understandable that toxicological studies are mainly focused on biomonitoring rather than on the levels of toxic compounds in environmental media. At the same time, ambient monitoring is the only tool for revealing sources of toxic pollutants affecting an organism.

Ambient Monitoring and Environmental Persistence of RVX

The composition of VX degradation products and admixtures has been studied in detail by hybrid chromatography–mass spectrometry (MS) methods. The objects of admixture studies were the contents of containers in which VX had been stored for a long time. Dozens of admixtures and stabilizers could be identified. There has been much work on the identification of admixtures in VX and its degradation products. A systematic review of VX transformation products is presented by Munro et al. (1999), but there are no such systematic data for RVX. The degradation of RVX in various media always produces complex mixtures of products that commonly contain dozens of both volatile and nonvolatile organic compounds. The prevalent volatile products are phosphorus-free alkyl diethylaminoethyl monosulfides and polysulfides. The electron impact (EI) mass spectra of these compounds are quite similar to each other and most commonly contain a single strong peak at *m/z* 86, formed by the $[(Et)_2NCH_2]^+$ ion. Among phosphorus-containing products, methylphosphonic acid (MPA) and its monoisobutyl and diisobutyl esters (iBuMPA and iBu_2MPA) are almost always detected in certain quantities. These compounds are also present as admixtures in technical RVX samples in varied amounts (from tenths of a percent to several percent). The P–S bond cleavage in RVX forms diethylaminoethanethiol and iBuMPA. The latter slowly hydrolyzes to form MPA. Conditions favoring P–O bond cleavage to form the highly toxic *S*-2-(diethylamino)ethyl methylphosphonothioic acid are unknown.

The most hazardous known VX hydrolysis products are persistent bis[2-(diisopropylamino)ethyl] disulfide, and highly toxic and persistent *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate. The decomposition of RVX gives rise to structural analogs of the above products: bis[2-(diethylamino)ethyl] disulfide and *S*-[2-(diethylamino)ethyl] methylphosphonothioate. The latter is commonly called *monothiol*. Systematic data on the behavior of these products in the environment, as well as on their acute and chronic toxicity to humans and mammals and ecotoxicity, are lacking. Components of the complex mixture of RXV decomposition products present as low-informative EI mass spectra; therefore, these compounds are hardly possible to identify solely on the basis of mass spectral data. Chemical-ionization (CI) mass spectra are much more characteristic, but CI mass spectra—in view of their irreproducibility and lack of databases—are of limited use for identification. Like VX, RVX does not possess a strong electron-acceptor center favoring decomposition but still is not a complete analog of VX in this respect. In dilute aqueous solutions, RVX proved to be much more persistent: a half-life of 12.4 days as

opposed to 4.8 days for VX (Crenshaw et al., 2001). The mechanism of the neutralization of VX and RVX with an equimolar amount of water was first described by Yang et al. (1996). It was found that autocatalytic hydrolysis is possible exclusively in V-series nerve agents since it should involve the protonated amino group.

We performed experimental research on the stability assessment of RVX and identification of its transformation products under the action of equimolar or excess amounts of water. The method for analysis was gas chromatography–electron impact mass spectrometry (GC-EIMS). In a dilute aqueous solution (10 mg/mL) in the presence of 5% phosphoric acid, the concentration of RVX after exposure for 20 days at room temperature without stirring was 2.7 mg/mL, or 27% of the initial amount. Since among RVX hydrolysis products both volatile and nonvolatile compounds could be expected, we chose three schemes for sample preparation: (i) evaporation to dryness followed by silylation; (ii) organic solvent extraction; and (iii) silylation of the extract obtained by procedure (ii).

Table 10.1 lists the principal products of RVX hydrolysis with excess water in an acid medium, with specified analytical fractions with the highest contents of each compound. It should be noted that even after 100-day exposure, we could detect 1% of the initial amount of RVX in the solution, which implied a fairly uniform hydrolytic degradation.

Experiments on RVX hydrolysis with an equimolar amount of water were performed as follows. A mixture of 74 μL of RVX and 5.6 μL of water was exposed at room temperature for 3.5 months without stirring. An ash-gray thick uniform material formed after hydrolysis and completely dissolved in 5 mL of acetonitrile. The solution was diluted 100 times with acetonitrile and analyzed by GC-EIMS (sample D). An aliquot of this sample was mixed with an equal volume of bis-trimethylsilyltrifluoroacetamide (BSTFA), and the mixture was heated at 70°C for 30 min and then analyzed by GC-EIMS (sample E). The autocatalytic hydrolysis of RVX was almost complete by the end of the experiment, since the RVX content in the sample was no more than 0.01%. Qualitatively, the reaction mixture in the latter case was much poorer than in the hydrolysis with excess water. Among the volatile hydrolysis products, the following were detected by GC-EIMS in sample D: 2-(diethylamino)ethanethiol (4%), diisobutyl methylphosphonate (2%), bis[2-(diethylamino)ethyl] sulfide (2%), RVX (<0.01%), and bis[2-(diethylamino)ethyl] disulfide (80%). Of nonvolatile products in sample E, MPA (3%) and isobutyl ester of MPA (isobutyl MPA) (96%) were identified as trimethylsilyl ester. Isobutyl MPA, which catalyzes RVX degradation, was detected as the major components of the reaction mixture. As the second most abundant component we expected 2-(diethylamino)ethanethiol, according to Yang et al. (1996), but we found instead that it almost completely converted into bis[2-(diethylamino)ethyl] disulfide. This result seems feasible since once the autocatalytic degradation of RVX was complete, nothing would prevent thiol from being converted into disulfide. In a dilute aqueous solution of RVX, this conversion occurs less rapidly, and even after 100 days, the solution contains much less 2-(diethylamino) ethanethiol than bis(2-diethylaminoethyl) disulfide.

The Chemical Weapons Convention requires signatory states to completely destroy not only their

TABLE 10.1 Products of RVX Hydrolysis with Excess Water in an Acid Medium

RT (min)	Compound	Formula	Fraction	Content in the Reaction Mixture[a] (%[b])
6.709	*N,N*-Diethylformamide	$HCONEt_2$	**B**[c]	1
7.480	2-(Diethylamino)ethanethiol	$Et_2NCH_2CH_2SH$	**B**, C	10
7.917	*N,N*-Diethylacetamide	$MeCONEt_2$	**B**	<1
10.275	MPA (as bis(trimethylsilyl) derivative)	$MePO[OSi(Me)_3]_2$	**A**, C	5
11.442	Isobutyl hydrogen methylphosphonate (as TMS derivative)	$CH_3PO(OiBu)OSi(Me)_3$	**A**, C	22
12.217	*O*-Isobutyl *S*-hydrogen methylphosphonothioate (as S-TMS derivative)	$CH_3PO(iBu)SSi(Me)_3$	A, **C**	11
12.674	Diisobutyl methylphosphonate	$MePO(iBuO)_2$	**B**, C	3
16.733	Bis(2-diethylaminoethyl) sulfide	$(Et_2NCH_2CH_2)_2S$	**B**, C	10
18.50	RVX	$MePO(OiBu)SCH_2CH_2NEt_2$	**A**, B, C	26
18.898	Bis(2-diethylaminoethyl) disulfide	$(Et_2NCH_2CH_2)_2S_2$	**B**, C	1

[a]*Hydrolysis time 20 days.*
[b]*Dozens of minor and insignificant components altogether amounted near 10% and not shown here.*
[c]*Bold-faced is the fraction in which quantitative analysis for the component was performed.*

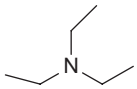
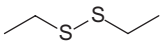
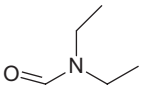
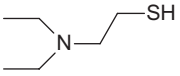
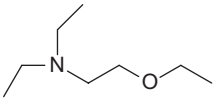
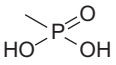
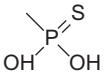
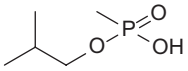
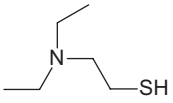
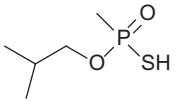
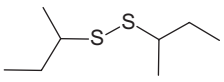
stockpiled chemical weapons (CWs), but also the corresponding industrial facilities, including buildings and other constructions. Toxicity and hazard assessment of demolition wastes of former VX production facilities is quite a challenging problem in view of the high absorption capacity of building materials for VX-series agents. Whether V-series agents are possible to detect in one or another material is difficult to predict because of the paucity of data on their persistence in various media. An ion-trap secondary ionization MS study showed that VX undergoes complete degradation when in contact with concrete surfaces (Groenewold et al., 2002). The process follows first- or pseudo-first-order kinetics, and the half-life of VX is about 3 h. The affinities and persistence of VX in certain materials were studied by Love et al. (2004). The test materials included powdered activated charcoal, as well as natural iron and aluminum oxyhydroxide minerals: goethite (yellow ochre) and montmorillonite (clay), respectively. It was shown that VX has a high affinity for charcoal, a moderate affinity for montmorillonite, and a very low affinity for goethite. The adsorption on goethite was increased in the presence of dissolved organic matter. VX degraded rapidly on a dry goethite than in the presence of water. We performed experiments with RVX applied on the soil, concrete, bricks, and a polymeric material (polyisobutylene used in hydroinsulation) at concentration ranges of 10^{-6}–10^{-5} mg/kg, which corresponded to the tentative maximum allowable concentrations accepted in the Russian Federation. Conditions for the effective extraction of RVX could not be found for any of the materials tested. The extremely low recoveries of RVX from materials of various natures can be explained by both irreversible sorption and degradation. The problem of simulating the behavior of V-series nerve agents, including RVX, in various materials is complicated by the fact that such factors as the presence of water, organic compounds, or both strongly affect both RVX sorption and degradation kinetics. In any case, in alkaline materials (concrete, cement, plaster, or lime), RVX undergoes fast degradation, especially in the presence of water, whereas in organic hydrophobic media (polymers, lubricants), it can persist for a long time.

During the operation of an RVX production or destruction facility, its inner surfaces might have been treated with various reagents, and therefore it is quite difficult to predict the transformation routes and degradation products of the toxic agent. These products may include previously unknown toxic compounds. We faced problems in assessing the toxicity and hazard of samples (specifically wall and ceiling fragments) from a former RVX production facility (Radilov et al., 2007). This research was required for developing technical regulations for demolition of the facility. Table 10.2 lists compounds isolated from the sample building materials, taken in working areas of the former RVX production facility, which might have contacted RVX. Aqueous and organic extracts of the samples were prepared according to the Organization for the Prohibition of Chemical Weapons recommended operational procedures (Rautio, 1994). Analysis was performed via GC-MS in the EI and positive chemical ionization (reactant gas methane) modes. According to the procedures, aliquots of all extracts were subjected to BSTFA silylation and diazomethane methylation.

Along with RVX transformation products, the samples contained more than 100 organic compounds that are matrix components of building materials, solvents, plasticizers, reaction products of degassing agents with matrix components, among other materials. In brick and concrete samples, we detected in high concentrations (up to 10 g/kg) so-called fyrols (components of foam fire-extinguishing compositions). In terms of chemical composition, fyrols are mixtures of tris(dibromoalkyl) phosphates with phosphoric acid. Among the RVX transformation products in Table 10.2, components with RI 1,685, 1,888, and 2,241 were relatively abundant (up to 5 mg/kg) and were assigned two almost equally probable structures (which appear in bold in the table). Such products could not be expected in advance; as a result, they were not detected by target analysis. However, their detection can be considered as evidence that the samples in hand were contaminated with RVX in the past.

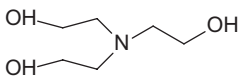
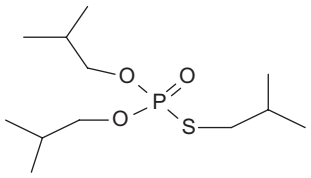
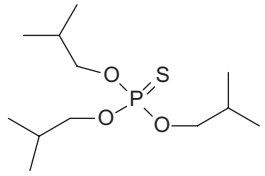
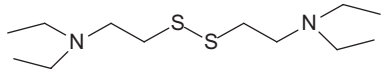
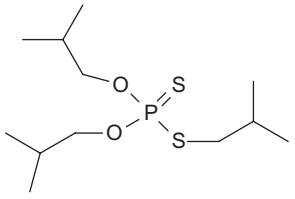
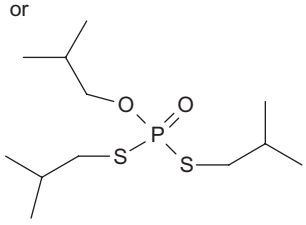
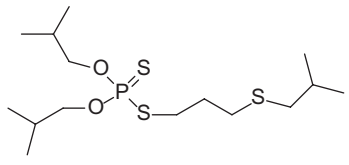
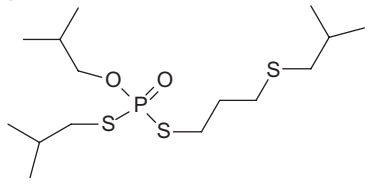
Environmental monitoring of RVX chemical weapon storage and destruction facilities (CWDFs) is carried out by sanitary regulation units, and development of highly sensitive and productive biochemical analytical express methods for such monitoring is of great significance for governmental bodies. An alkali-peroxide formulation is applied at CWDF as a basic degassing solution for decontamination of RVX. In our first study on determination of warfare nerve agents with microplate biochemical methods, we showed that hydrogen peroxide at concentrations in the micromolar range substantially distorted results obtained with the Ellman method. For the Hestrin method, the IC_{50} for hydrogen peroxide was shown to be about 2.5 orders of magnitude higher than that found via the Ellman method, so the former method was considered more specific for quantitative determination of OPs in the samples containing hydrogen peroxide (Prokofieva et al., 2010). In our next study, to quantitatively determine RVX in aqueous solutions for the purposes of environmental monitoring at places of storage or destruction of CWs, we developed new microplate versions of the Ellman and the Hestrin methods in order to avoid interferences from admixtures that could otherwise give rise to false results (Prokofieva et al., 2012). The linear range of the Hestrin method (74.8–1,120 pM) was 3.1-fold wider than that of the Ellman method (37.4–374 pM). Limits of detection

TABLE 10.2 RVX-Related Organic Compounds Identified in Samples of Building Materials (Radilov et al., 2007)

RT (min)	Compound	Structural Formula, Molecular Weight (MW)	RI
4.19	Triethylamine	 MW 101	
7.35	Diethyl disulfide	 MW 122	929
7.64	*N,N*-Diethylformamide	 MW 101	946
7.76	Ethylamine (as TMS derivative)	 MW 45	952
8.44	2-(Diethylamino)ethanethiol	 MW 133	996
8.72	(2-Ethoxyethyl)diethylamine	 MW 145	1,014
10.79	MPA (as bis-TMS derivative)	 MW 96	1,147
11.75	Methylphosphonothioic acid (as TMS derivative)	 MW 112 (256)	1,210
11.92	Isobutyl hydrogen methylphosphonate (as TMS and M derivatives)	 MW 152	1,223
12.46	2-(Diethylamino)ethanethiol (as TMS derivative)	 MW 133	1,261
12.58	*O*-Isobutyl *S*-hydrogen methylphosphonothioate (as TMS derivative)	 MW 165 (240)	1,269
12.76	Diisobutyl disulfide	 MW 178	1,281

(*Continued*)

TABLE 10.2 (Continued)

RT (min)	Compound	Structural Formula, Molecular Weight (MW)	RI
17.19	Tris(2-hydroxyethyl)amine (as TMS derivative)	 MW 149	1,635
17.79	*O,O,S*-Triisobutyl phosphorothioate or *O,O,O*-triisobutyl phosphorothioate	 or  MW 282	1,685
19.26	Bis(2-diethylaminoethyl) disulfide	 MW 264	1,822 1,828
19.94	*O,O,S*-Triisobutyl phosphorodithioate or *O,S,S*-triisobutyl phosphorodithioate	 or  MW 298	1,888
23.20	*O,O*-Diisobutyl *S*-[(3-isobutylsulfanyl)propyl] phosphorodithioate or *O,S*-Diisobutyl *S*-[(3-isobutylsulfanyl)propyl] phosphorodithioate	 or  MW 372	2,241

and quantification of RVX for both methods were below the maximal allowable concentration of RVX in water-soluble washouts. One of the early products of RVX hydrolysis, *N*,*N*-diethylaminoethanethiol, caused false negative results via the Ellman method at >10 μM; individual blanks were necessary to eliminate these effects. The Hestrin method showed greater specificity (about 3 orders of magnitude) for analysis of samples containing mercaptans. Thus, the principal merit of the Hestrin-based approach is that potentially dangerous false negative results can be avoided. A new biochemical procedure of RVX determination at technological surfaces were certified and introduced to the Federal Register of measurement procedures.

TABLE 10.3 Characteristics of the Procedures for Determination of *O*-isobutyl MPA as a Marker of Exposure to RVX, in Urine and Blood Plasma

Method	Detection Limit (ng/mL)	Matrix	Total Analysis Time per Sample (h)
GC-MS	10	Plasma	10
SPME-GC-MS	5	Urine	1.5
GC-MS-MS	1	Plasma	10
HPLC-MS	1.0	Plasma	1.5
HPLC-MS-MS	0.8	Plasma	1.5

Biomonitoring and Toxicokinetics of RVX

GC-MS and HPLC-MS Analyses of RVX Metabolites

Identification and quantitative assessment of toxic chemicals and their metabolites in biomedical samples can be performed by the following methods: (i) establishment of the fact and factor of exposure of humans and animals to chemical accidents; (ii) clinical diagnosis of poisoning; (iii) forensic expertise; and (iv) biomonitoring of people that deal with highly toxic chemicals. RVX, like other highly toxic readily metabolizing chemicals, is difficult to detect in body fluids and tissues even for very short periods after exposure. Successful identification and quantitative assessment of the toxicity factor in biomedical samples is possible under the following conditions (Savelieva et al., 2003):

- Right choice of biological matrix (the object for analysis)
- Right choice of a biomarker pertinent to the level and nature of exposure
- Measurable biochemical or physiological effect
- Availability of a reliable and sufficiently selective and sensitive analytical procedure
- Availability of reference compounds and criteria for correct data interpretation.

Analytical procedures applied to diagnosis and retrospective verification of exposure to OP include (Worek et al., 2005): (i) biochemical determination of ChE activity; (ii) identification of unbound OP; (iii) identification of decomposition products; (iv) fluoride-induced reactivation of inhibited ChE, followed by analysis of the inhibitor; and (v) analysis of phosphyl-protein-adducts after tryptic digestion of the protein. The last procedure is regarded to be the most specific and sensitive, but it has the drawback of being strongly dependent on the analysis of butyrylcholinesterase (BChE), the most abundant plasma serine esterase with a half-life of about 16 days.

It is well known that hydrolysis is the major metabolism pathway of G-series agents (Beck and Hadad, 2008). The enzymatic hydrolysis of these agents primarily involves phosphorylyl phosphatases and produces *O*-alkyl methylphosphonic acids (*O*-alkyl MPA). V-series agents (VX, RVX) are not typical substrates for phosphorylyl phosphatases, which probably explains the higher persistence of these agents in the organism. Free *O*-alkyl MPAs formed by hydrolysis of OP agents can be detected in body fluids and tissues and are used as markers of exposure to these agents. The final hydrolysis product is MPA, but its fraction is smaller compared to *O*-alkyl MPA. The low-molecular RVX hydrolysis products are actively excreted within the first few days after intoxication and can serve as suitable targets for retrospective analysis for no longer than 2–3 weeks. However, 2–3 days after exposure, the body levels of *O*-alkyl MPA and MPA become much more demanding in terms of detection sensitivity. We have developed a series of procedures for identification and quantitative assessment of *O*-isobutyl MPA as a marker of exposure to RVX in urine and blood plasma. Characteristics of the procedures are presented in Table 10.3.

The GC-MS procedure involves sample deproteinization, purification, evaporation to dryness, *tert*-butyldimethylsilylation of redissolved dry residue, and GC-EIMS analysis in the Selective Ion Monitoring (SIM) mode. The solid-phase microextraction (SPME)-GC-MS procedure involves extraction of *O*-alkyl MPA from urine on the microfiber, their *tert*-butyldimethylsilylation directly on the microfiber, and thermodesoption of the resulting derivatives in a GC injector. SPME of *O*-alkyl MPA on microfiber is an effective approach for urine since it allows one to avoid the stage of sample desalination associated with the inevitable losses of target compounds. However, SPME is much less effective with plasma because of its high protein content. The high-performance liquid chromatography with mass spectrometry (HPLC-MS) procedure involves sample centrifuging, solid-phase extraction on Diapak C16M

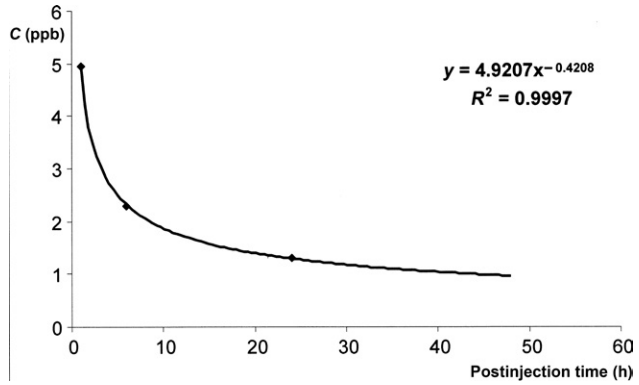


FIGURE 10.2 Concentration of *O*-isobutyl MPA in blood plasma versus time after intramuscular injection of RVX in rats at a dose of 0.8 LD_{50}

cartridges, elution with an acidic (pH 3.0–4.0) methanol, evaporation and redissolution of the eluate, and HPLC-MS analysis in the chemical ionization mode at atmospheric pressure with registration of negative ions (Rodin et al., 2007). The latter procedure has been used in toxicokinetic experiments for measuring the concentration of *O*-alkyl MPA in rat plasma 1, 6, 24, and 48 h after intramuscular (i.m.) injection of OP agents at a dose of 0.8 LD_{50} (0.0144 mg/kg). The toxicokinetic curve for *O*-isobutyl MPA is presented in Figure 10.2.

The HPLC-MS (quadrupole analyzer) and HPLC-MS-MS (medium-class ion-trap analyzer) procedures for the determination of OP metabolites in blood plasma were found to be comparable in terms of sensitivity, accuracy, and performance, but HPLC-MS is preferred in terms of the availability and cost of equipment and maintenance.

MS/MS Analysis of Human Albumin

Albumin has been demonstrated to be an OP hydrolase (Sogorb et al., 1998). Bovine albumin binds diisopropylfluorophosphate (DFP) in equimolar proportions (Murachi, 1963), and binding of DFP to Tyr was shown simultaneously (Sanger, 1963). MS identified the active site to be Tyr410 for bovine albumin and Tyr411 for human albumin (Schopfer et al., 2005; Li et al., 2007). A characteristic feature of this Tyr is a low pK_a (near 8), in contrast to pK_a values of almost 10 for other tyrosines (Means and Wu, 1979), which is believed to be due to a pocket with nearby basic residues of Arg410 and Lys414 in human albumin (Sugio et al., 1999; Li et al., 2007). Various warfare agents were shown to readily bind to Tyr411, though VX needs more time and a higher concentration for this chemical binding, which is explained by a lower potential of the $SCH_2CH_2N(iPr)_2$ substituent in VX to leave in a noncatalyzed reaction, as compared with F^- or CN^- (Williams et al., 2007).

In order to determine the specificity of RVX binding with albumin, we first obtained a mass spectrum of human albumin after *in vitro* incubation of blood serum with RVX, and the shift in the spectrum of about 400 Da suggested at least two sites of RVX binding to albumin (Radilov et al., 2009). To locate the binding sites, we incubated RVX with commercially available human albumin (Sigma), and then digested it with trypsin to obtain MS/MS peptide spectra. Two sites of binding RVX to human albumin were revealed, one of them being the well-known Tyr411 and the second site being an unexpected Tyr150. Taking into consideration that 144–160 amino acids in human albumin are RRHPYFYAPELFFAKR (http://myhits.isb-sib.ch/cgi-bin/motif_scan), we suggest that Tyr150 could be activated by positively charged RRH and KR, which could serve to lower its pK_a.

Up to recently, studies of V-agents degradation in *in vivo* or even *in vitro* experiments (under conditions maximally approximated to those *in vivo*) were quite problematic because of methodological and technical difficulties: there were no analytical equipment with sufficient sensitivity for assessment and quantitative analysis of metabolic transformations of these OPs in complex protein mixtures. There is only one study (Tsuchihashi et al., 1998) on identification of VX metabolites in blood serum of a human victim, where metabolic pathways of VX degradation were designated. The analysis of blood serum revealed ethyl methylphosphonic acid (EMPA), as well as methyl thioether of 2-(diisopropylamino) ethanethiol, in contrast to thiol alone, which was not revealed because of its high reactivity. There is evidence to suggest that thiols in human organism are rapidly methylated via thiol *S*-methyltransferase (Weisinger and Jakoby, 1980), leading Tsuchihashi et al. (1998) to conclude that VX is hydrolyzed to produce EMPA and thiol, which was then methylated by the enzyme. However, the pH of human blood and intercellular and intracellular fluids are within a narrow span of 7.2–7.4. Therefore, one can suppose that hydrolysis of V-agents could proceed not only by breaking the P–S bond, but also by breaking the P–O bond. Indeed, the formation of EA-2192 was shown first, both in *in vivo* and *in vitro* experiments, by Reiter et al. (2011).

In the human or animal body, nerve agents are rapidly bound with their targets, so analysis of blood samples for presence of the intact agents is possible soon after poisoning with rather high doses. A more helpful approach can be the analysis of biological samples for presence of alkyl methylphosphonic acids (AMPAs), which are the major products of nerve agent hydrolysis (Noort et al., 2002; Riches et al., 2005). Besides AMPA, the long-living biochemical markers are considered to be covalent adducts of nerve agents with blood proteins (Riches et al., 2005). In this case, the half-life of the marker depends not only on the quantity of a nerve

agent entering the organism, but also on metabolic stability of the adduct and the lifetime of the protein (Noort et al., 2002).

All the adducts of nerve agents with proteins that can be revealed presently are derivatives of a corresponding AMPA and serine and/or tyrosine amino acids (Bao et al., 2012; Read et al., 2010). In the case of G-agents, these adducts are the only ones to be formed. In contrast, V-agents are broken up so that two parts with similar molecular weights are produced, such as AMPA and the corresponding thiol. Therefore, in the case of intoxication with RVX, one should seek not only adducts of isoBMPA with serine and tyrosine residues, but also adducts of *N*,*N*-diethyl-2-aminoethanethiol (hereinafter referred to as *thiol*) with cysteine residues. From the point of view of its diagnostic value, the adduct of thiol with cysteine would be less specific than adducts of isoBMPA and tyrosine residue of albumin or serine residue of ChEs, but its sensitivity should be much higher than these adducts due to high reactivity of the thiol.

A molecule of albumin has several disulfide bonds and also one reduced cysteine residue, Cys34. It is well known that most thiol groups present in blood plasma belong to Cys34 of albumin (Ogasawara et al., 2007; Oettl and Stauber, 2007). That is why in order to obtain *in vitro* preparation of covalent adducts of proteins with RVX, we have mixed RVX solutions not only with undiluted blood serum and ethylenediaminetetraacetic acid (EDTA)–plasma, obtained from two outbreed male rats and nine healthy human volunteers, but also with human and rat serum albumins (HSA and RSA, respectively, in amounts of 50 mg/mL). Samples of human plasma/serum were pooled, and albumin solutions were prepared in 10 mM phosphate buffer saline (PBS) and pH 7.2, containing 137 mM NaCl and 2.7 mM KCl. The final concentrations of RVX in the samples were 37 nM, 0.37 μM, 3.7 μM, and 37 μM. The samples were incubated at 37°C for 24 h and then hydrolyzed by pronase according to Read et al. (2010). Solutions of RVX served as control samples, with PBS as the blank sample. HPLC-MS/MS analysis was carried out on a Thermo Scientific LTQ Orbitrap Velos mass spectrometer coupled with an Accela 1250 LC system and an autosampler. Data were acquired and processed using Xcalibur software. Chromatographic separation was carried out on a Hypersil Gold C18 column (150 mm × 4.6 mm i.d., particle size 1.8 μm): mobile phase, 0.05% trifluoroacetic acid (TFA) in water (A) and 0.05% TFA in acetonitrile (B); flow rate 400 μL/min; gradient elution conditions, 55% B (0–1.5 min), 5–90% B (1.5–10.0 min), 90% B (10–30 min), 5% B (30.01–35.0 min); injection volume 10 μL. The MS conditions were as follows: heated electrospray ionization (HESI-II) probe, positive ionization mode, spray voltage 3 kV, ion transfer tube (capillary) temperature 400°C, and sheath gas and auxiliary gas flow rates of 50 and 17 arbitrary units, respectively. Transitions were monitored at the collision energy 18 eV.

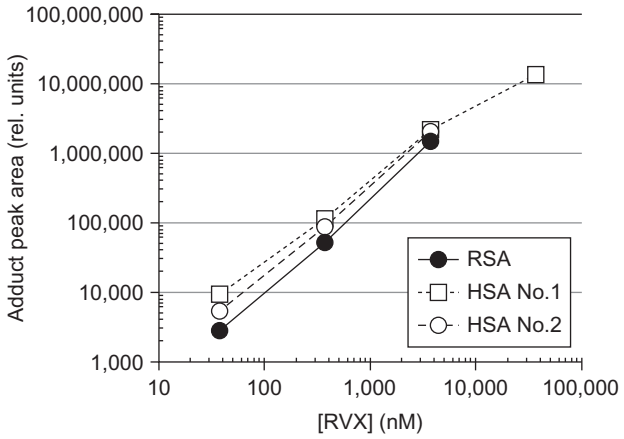


FIGURE 10.3 Relationship between thiol–cysteine adduct formation and amount of RVX introduced into albumin solution. Logarithmic coordinates.

Analysis for detection and quantification of the adduct was preceded by *in situ* synthesis of an RVX adduct with cysteine. To this end, we incubated a mixture of RVX with L-cysteine in PBS at 37°C for 24 h in molar ratios of about 10:1 and then analyzed the resulting mixture by HPLC-MS/MS. Along with RVX and L-cysteine spikes, a new strong peak at 4.4 min was detected in the reconstructed mass chromatograms of the reaction mixtures at the exact mass corresponding to a protonated molecular ion of the thiol–cysteine adduct: *m/z* 253.10161 ($[(C_2H_5)_2NCH_2CH_2S\text{-}SCH_2CH(NH_2)COOH^+H]^+$; theoretical mass 253.10389). The structure was confirmed by the primary transition *m/z* 253.10161→*m/z* 132.08307 ($((C_2H_5)_2NCH_2CH_2S^+$; theoretical mass 132.08469) and secondary transition *m/z* 132.08307→*m/z* 86.09697 ($[(C_2H_5)_2NCH_2]^+$; theoretical mass 86.096974) (not shown in this chapter).

Then we detected the thiol–cysteine adduct in the samples obtained after joint incubation of HSA and RSA (50 mg/mL) and RVX for 24 h, followed by pronase hydrolysis. The data reflecting the ability of these albumins to bind RVX are given in Figure 10.3. As seen, both RSA and HSA cysteine residues showed a similar ability to form adducts with RVX. Taking HSA as an example, we analyzed two different samples (HSA №1, HSA №2) at two different times and obtained good reproducibility of results. Figure 10.4 shows data on thiol adduct formation with cysteine residues of blood plasma or serum proteins, obtained after their reaction with different quantities of RVX. We observed a linear correlation between the initial concentration of RVX and the amount of the thiol–cysteine adduct. In contrast to albumin solutions, a greater spread of data was obtained for different concentrations of RVX. Moreover, there was

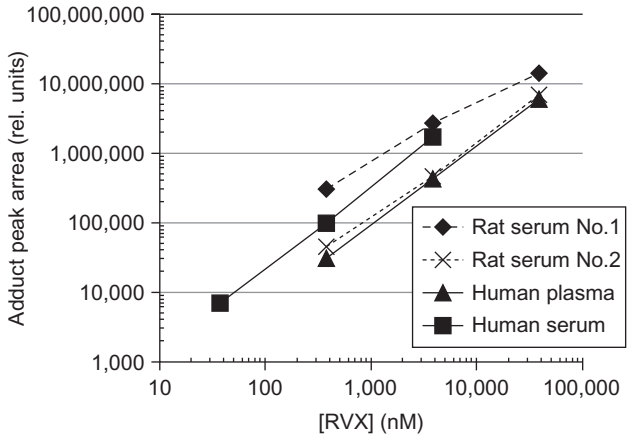


FIGURE 10.4 Relationship between the adduct formed and the amount of RVX introduced into serum/plasma. Logarithmic coordinates.

a considerable variability in the abilities of sera obtained from different animals (rats 1 and 2 in Figure 10.4) to form thiol adducts. The peak areas of the thiol–cysteine adduct differ from each other by an order of magnitude relative units, which could be attributed to different redox status of the animals' serum samples.

Thus, we have revealed a new thiol–cysteine adduct, produced by interaction of plasma/serum proteins with RVX *in vitro*. We have also shown that cysteine of serum albumin plays the major role in this formation. However, there is a need for further increase of sensitivity for the sake of solid-phase microextraction, which makes it possible to concentrate the sample before HPLC-MS/MS analysis. Presently, the sensitivity of the method is not high enough to reveal the adduct in rat serum samples two days after the sublethal poisoning with RVX.

MECHANISMS OF ACTION AND PRINCIPLES OF THERAPY

Three types of damages induced by OPs have been identified: acute poisoning, the so-called intermediate syndrome (IS), and OP-induced delayed polyneuropathy (OPIDP) (Ray, 1998). The IS symptoms and signs usually occur after apparent recovery from the acute cholinergic syndrome, but before OPIDP develops (Karalliedde et al., 2006). OPIDP also occurs almost exclusively in patients with preceding acute cholinergic toxicity related to severe acute exposure to an OP compound; neuropathy target esterase (NTE) is considered to be the principal molecular target for OPIDP (Lotti and Moretto, 2005). However, there is no data to indicate that VX has any potential at high or low doses for the induction of OPIDP in its classic manifestation in humans or other species, either with acute or long-term exposure. The reason could be that the ability of VX to inhibit NTE is near 1,000-fold less than that of GB (Vranken et al., 1982; Gordon et al., 1983). Single i.m. injections of VX at 5 LD_{50} in atropine-protected chickens produced neither inhibition of NTE nor histological or behavioral evidence of OPIDP (Wilson et al., 1988a). There was no ability of VX at subchronic exposure (0.04 mg/kg for 90–100 days) to induce OPIDN in antidote-protected chickens (Wilson et al., 1988b). According to other data, NTE activity in brain areas and soleus muscles of rats was significantly depressed after they were subacutely exposed to VX in the absence of supporting therapy for 14 days at doses at the LD_{50} level and higher, and surviving animals exhibited muscle myopathy in the soleus muscles (Lenz et al., 1996). Blood AChE activity was depressed to zero throughout the experiment, so there is no contradiction on the lack of clear OPIDP signs under severe intoxication with VX.

Thus, molecular mechanisms of delayed and chronic effects of VX are still poorly understood, and scientific data on these effects under RVX intoxication is much scarcer. It seems that chronic intoxication should be a separate type of damage with OPs. The problem requires searching for new criteria to determine intoxication. One should differentiate at least two aspects of this problem: molecular and functional. The former concerns revealing new molecular targets of OP action. For instance, plasma and liver carboxyesterases (CarbEs) may be more sensitive targets compared to AChE in cases of chronic action of OP low concentrations (Ray and Richards, 2001). Although CarbE are responsible for the metabolism of a variety of xenobiotics, this fact has been ignored over a long period of time. Another esterase is involved in testosterone biosynthesis metabolizing cholesterin esters in the testis (Jewell and Miller, 1998); one more (surfactant convertase) is also involved in cholesterin metabolism in the lung (Krishnasamy et al., 1997); and a number of brain CarbE and other hydrolases are also sensitive to OPs (Chemnitius and Zech, 1983; Richards et al., 2000; Nomura et al., 2005). Various OPs are potent inhibitors of fatty acid amide hydrolase and monoacylglycerol lipase (Quistad et al., 2001, 2006), the principal enzymes of the endocannabinoid system.

The second (functional) aspect of this problem concerns the development of pathologic symptoms, the molecular causes of which are not due to AChE inhibition. One of the first cases of OP non-ChE effects (when the association between the OP molecular target and the functional disturbance has been proved) involved inhibition of kinurenin formamidase of the hen egg yolk sac membrane responsible for teratogenic effects (Seifert and Casida, 1978). Another example of the OP teratogenic effect is abnormal development of the conjunctive tissue of Xenopus embryos due to inhibition of lysyloxidase and incomplete post-translational modification of collagen (Snawder and Chambers, 1993).

Acute Intoxication with RVX

A contributing factor to the high toxicity of VX may be its preferential reaction with AChE. Moreover, unlike the G-agents, VX depresses AChE activity significantly more than BChE in humans (Sidell and Groff, 1974); the result is that more VX is available to react specifically with the target enzyme, AChE. At the same time, indirect primary (connected with AChE inhibition) and secondary (not connected with AChE inhibition) effects of RVX have also been described. Development of after-intoxication immunodeficient or immunotoxic states relate to the first group of effects (Germanchuk and Zabrodskii, 2005). In experiments with rats that were administered RVX at a dose of 0.75 LD_{50}, a suppression of immune reactions was found: Th-1 cells significantly lost their functions and T-dependent immune reactions were depressed. In addition, an optimal balance of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in lymphocytes necessary for their proliferation and differentiation was disturbed (Zabrodskii et al., 2007). On the other hand, after a severe intoxication of laboratory rats with RVX (2 LD_{50} with therapeutic treatment), there were no aftereffects concerning the capability of the central nervous system (CNS) to produce conditioned reflex reactions, neither in the early post-intoxication period (2 weeks) nor at remote terms (1–6 months) (Novikova et al., 2007). Four different effects of RVX were ascribed to the second group (Prozorovskii and Chepur, 2001): (i) sensitization and desensitization of cholinoceptors to acetylcholine that is characteristic of M-cholinoceptors; (ii) influence on acetylcholine release by nerve terminals: an inhibition after stimulation of M-cholinoceptors and facilitation after stimulation of N-cholinoceptors; (iii) direct interaction with cholinoceptors, mainly with nicotinic ones, leading either to their activation or inactivation; (iv) interaction with ionic channels, mainly with that of N-cholinoceptors.

Hypoxic syndrome is one of the principal clinical manifestations under acute intoxication with RVX. It is triggered by disturbance of ventilation, which in its turn is caused by bronchospasm, bronchorrhea, convulsions, and central deregulation of respiration. A series of pathological mechanisms leads to reduction of circulating blood volume, decrease of blood vessel tone, and deregulation of vessel wall permeability. Abnormalities of blood rheology, caused by the loss of body fluids (salivation, bronchorrhea, etc.), and aggregation of blood cells significantly increase the dynamic viscosity of blood and aggravate disturbances of microcirculation. Disturbances of oxygen transport and its delivery to tissues induce secondary metabolic disorders and involvement in a pathological process of related biological systems that facilitates oxygen utilization by the tissues (Shestova and Sizova, 2005). An imbalance of electrolytes has also been described: in blood plasma under acute intoxication, there was decrease of sodium and potassium, the latter being more expressed than the former (Rybalko et al., 2005). Indirect secondary effects of OP agents, including RVX, can be induced by excessive amounts of ACh in blood, with its action on cells having no cholinergic innervations. This can lead to deformation of red blood cells and endothelial cells, activation of basophiles and degranulation of mast cells (Prozorovskii and Chepur, 2001). In this content, one should keep in mind that endothelial cells have got all the attributes of autonomic cholinergic regulation. There have been not only M-cholinoceptors, but also N-cholinoceptors revealed (Hsu et al., 2005), as well as activity of AChE (Carvalho et al., 2005; Santos et al., 2007), the system of synthesis of acetylcholine (cholinacetyl transferase) and vesicular system of ACh transport out of the cells (Kirkpatrick et al., 2003).

Delayed Effects: Chronic and Subchronic Intoxication with RVX

In the context of realization of the conventional programs for CW destruction, chronic effects of subsymptomatic concentrations of RVX are of particular interest, since risk of exposure of personnel of the CW destruction facilities to chemical agents cannot be completely ruled out. In addition, members of the rescue service engaged in the decontamination process are regarded to be a group at high risk of exposure. The difficulty of diagnosis of delayed effects and chronic intoxication with RVX relates to polymorphism of the clinical manifestations (Savateev et al., 2001). Long-term monitoring of personnel staff engaged in production of RVX revealed slowly progressing signs of chronic intoxication (Gur'eva et al., 1997). The clinical signs of this may consist of functional and organic disorders of the CNS together with vegetative dysregulations, such as peripheral angiodystonic syndrome, vegetosensory polyneuropathy or complex motor-sensory-vegetative pathology of axonopathic or myelinopathic type. Neuromuscular effects, visual and gastrointestinal disorders, immunodeficiency, and metabolic disorders have also been described (Gur'eva et al., 1997; Yanno et al., 2000). Cessation of contact with RVX does not lead to involution of the clinical picture of chronic intoxication. Along with nervous, gastrointestinal, and motor disorders, there can be visual and cardiovascular diseases. Moreover, the chronic occupational pathology of the former workers of the facilities may develop in 3–6 years, even though they were exposed to no more than 10 times the maximum allowable concentrations of RVX and had no acute intoxications in their anamneses (Filippov et al., 2005).

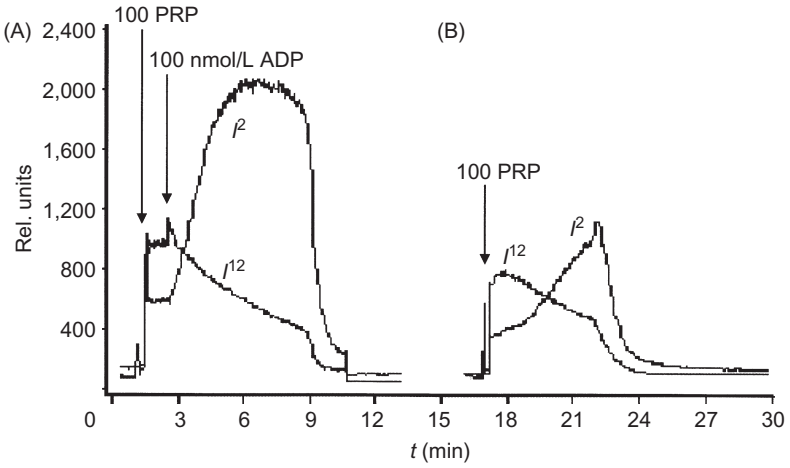


FIGURE 10.5 (A) ADP-induced activation and aggregation of blood platelets of control rats. (B) Spontaneous activation and aggregation of rat blood platelets immediately after three months' RVX exposure at a dose of 10^{-4} mg kg^{-1}.
Inscriptions: *100 PRP* means 100 µL of Platelet-Rich Plasma; I^{12} and I^2 indicate low angles of 12 and 2 degrees, respectively, at which the light scattering was recorded. *Source: Reprinted from Mindukshev et al. pp. 247–257. Copyright (2005), with permission from IOS Press.*

Experimental results were reported on exposing male and female rats to VX (0.00025, 0.001, or 0.004 mg/kg, s.c.) daily for 30, 60, and 90 days (Goldman et al., 1988). RBC-AChE activity was significantly depressed in rats at all VX doses for 30, 60, and 90 days. BChE was significantly depressed in rats given 0.001 mg/kg VX for 30 days and in both genders of the high-dose group for all exposure periods. No dose-related changes were reported in clinical chemistry and histopathology. The authors concluded that VX exposure sufficient to significantly depress RBC-AChE activity produced no subchronic toxic effects.

Taking into consideration these clinical manifestations of delayed and chronic effects of RVX, an experimental search was undertaken to seek new possible mechanisms of the pathogenesis and novel functional signs of intoxication (Goncharov et al., 2003; Mindukshev et al., 2005). To model chronic intoxication in experiments with animals, RVX was dissolved daily in drinking water to concentrations of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} g/100 mL. A group of 5 rats consumed 20 mL of RVX aqueous solution daily. So, during the 3-month test, animals of the first group consumed daily RVX with drinking water in a dose of 10^{-5} mg per 1 kg body weight (I), animals of the second group consumed a dose of 10^{-4} mg (II), and those of the third group consumed 10^{-3} mg (III). Measurement of AChE activity in red blood cells (RBC-AChE) was performed by Ellman's method, and the functional activity of platelets was investigated by a novel method of low-angle light scattering that allows all stages of the platelet transformation to be assessed (Mindukshev et al., 2005). Investigation of monosynaptic miotatic reflex and conduction rate through the peripheral nerve fiber was conducted with *N. tibialis.*

Comparative analysis of biochemical and physiological parameters studied is indicative of the complete absence of significant changes of RBC-AChE activity in rats of all three groups relative to the control, after exposure to the RVX doses given above (not shown in this chapter). On the contrary, platelets of test animals exposed to RVX differed from the control by their pronounced instability, an indication of which was development of their spontaneous activation and aggregation (Figure 10.5).

After 3 months of intoxication, two kinetic parameters of aggregation (namely, normalized maximal rate U_{max} and effective concentration EC_{50}) were significantly increased in groups II and III. Two months after cessation of the chronic intoxication with RVX (i.e., the rehabilitation period), significant differences of both kinetic parameters were found only for group III. Groups I and II showed significant increases of EC_{50}, but only a tendency ($P<0.1$) to increase U_{max}. The final estimation of kinetic parameters of aggregation, made 6 months after the rehabilitation, showed significant decrease of U_{max} in group III and increase of EC_{50} in all three intoxicated groups (Figure 10.6). Significant increase of U_{max} suggests the sensitization of platelets with the primary activation of signaling ways *via* protein kinases, the action of which tends to increase the expression of GPIIb/IIIa receptors (Shattil et al., 1998). The increase of the EC_{50} parameter with further elevation of U_{max} points to a partial desensitization of $P2X_1$ and $P2Y_1$ receptors, as does the growing activity of the abovementioned kinases.

Stimulation of the peripheral nerve trunk of intact animals leads to generation of muscle action potentials of three types. According to the duration of latent periods, they fall into the following order: (i) M-response

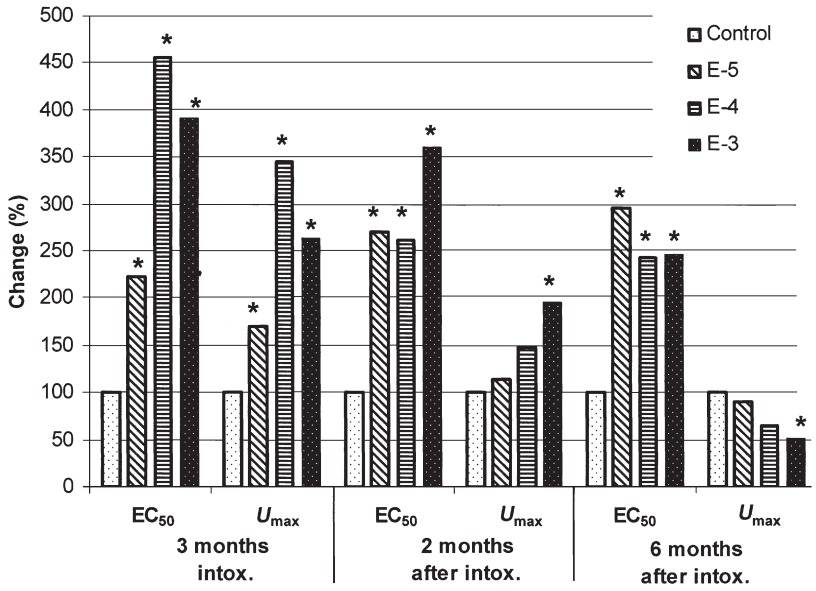


FIGURE 10.6 Kinetic parameters of rat platelet aggregation immediately after three months of RVX exposure, and at 2 and 6 months after cessation of the intoxication (*$P < 0.05$ relative to control). E-5, E-4, and E-3 relate to doses of RVX (1×10^{-5}, 1×10^{-4}, and 1×10^{-3} mg/kg) consumed by rats daily with drinking water.
Inscriptions: U_{max} means normalized maximum rate of platelet aggregation. *Source: Reprinted from Mindukshev et al. pp. 247–257. Copyright (2005), with permission from IOS Press.*

TABLE 10.4 Electrophysiological Parameters of the Peripheral Nervous System of Rats After RVX 3 Months' Chronic Exposure

	Control	I	II	III
t_M	1.36 ± 0.29	1.50 ± 0.19	1.72 ± 0.33[a]	1.07 ± 0.14[b]
$t_{peak\ M1}$	2.33 ± 0.54	2.79 ± 0.38	3.06 ± 0.42[a]	1.74 ± 0.44[b]
T_M	5.02 ± 1.87	6.73 ± 2.26	7.29 ± 1.70[a]	3.62 ± 0.96[b]
t_H	4.00 ± 0.41	3.51 ± 0.20	3.78 ± 0.20	4.02 ± 0.58
$t_{peak\ H1}$	4.60 ± 0.74	4.12 ± 0.33	3.95 ± 0.56[b]	4.35 ± 0.70

Symbols: t_M, M-response latent period; $t_{peak\ M1}$, latent period of the first maximum component of M-response; T_M, M-response duration; t_H, H-response latent period; $t_{peak\ H1}$, latent period of first maximum component of H-response.
[a]*Differences are significant with $P < 0.01$.*
[b]*Differences are significant with $P < 0.05$.*

(the result of the direct stimulation of α-motorneuron axons), (ii) H-response (the monosynaptic response), and (iii) polysynaptic responses with the variable latent period from 8–12 up to about 40 ms. In test animals of the III group, the changes of temporal parameters refer mainly to the latent period and duration of M-response (Table 10.4). Polysynaptic responses occur at all intensities of excitation and have a more pronounced character than in intact rats. A marked level and more distinct differentiation of the peaks of the complex action potential were noted.

The results obtained after examining the rats in group II differ from the control group more significantly. Along with normal action potentials (of the "spike" type), there were slow waves of depolarization of up to 30 ms duration. Another significant difference is the absence of the "subthreshold border"; that is, graduation of the increase of the amplitude of the action potential when the irritation stimulus enhances (Figure 10.7). Such an event can usually take place in newborn animals and is caused by a slight differentiation of motor neurons (Bursian, 1983). The latent period of M-response, the rate of rising action potentials, and their duration increased significantly. The results of testing the rats of group I did not differ from the control. At the same time, some animals had the entire spectrum of pathologic reactions observed in rats of groups II and III: fasciculations, the presence of slow (local, depolarized) potentials, and paradoxical discharges. The opposite character of the changes in velocity of nerve impulse conduction within

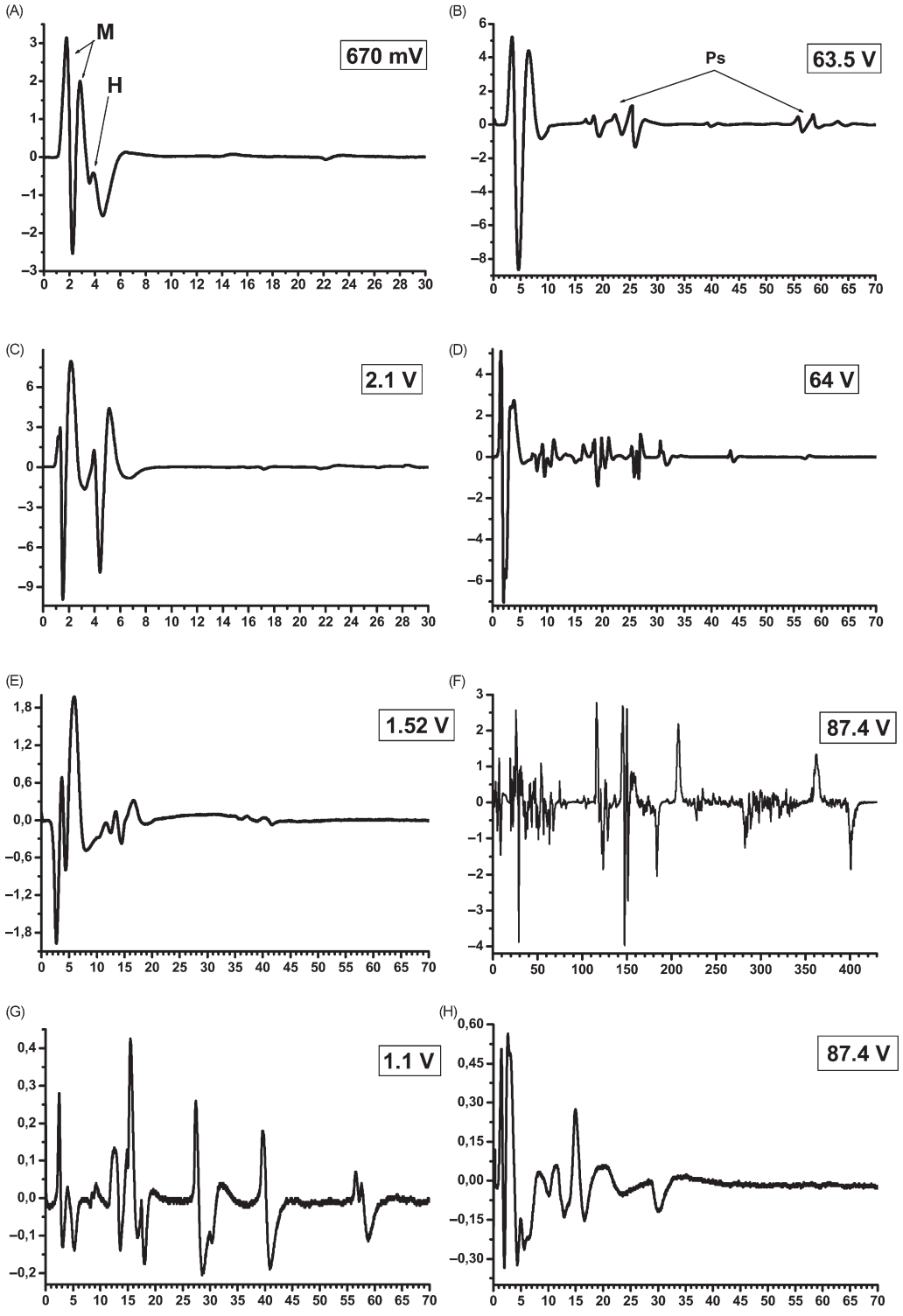


FIGURE 10.7 Action potentials of the rat gastrocnemius muscle under stimulation of *N. tibialis*: Effect of direct stimulation of the muscle (M-response, M), mono- (H-response, H), and polysynaptic (Ps) responses of control rats (A and B), rats of group III (C and D), II (E and F), and I (G and H). Ciphers within frames indicate value of the stimulus. The *x*-axis shows time of registration in ms; the *y*-axis shows amplitude of action potential in mV.

groups II and III could be caused by different intensities of demyelinization of nerve fibers with different diameters.

Earlier in this chapter, contradictory functional disorders in response to exposure to OPs were described, for which no appropriate interpretations were suggested from the point of view of molecular sources. For example, daily administration of DFP to rats for 20 days at a dose of 17% of LD_{50} caused an increase of velocity of nerve impulse conduction (Anderson and Dunham, 1985), although dimethoate or dichorphos treatment at subsymptomatic doses for at least 8 weeks caused reduction of peripheral nerve conductivity (Desi and Nagimajtenyi, 1999).

Delayed Effects: Embryo- and Gonadotoxicity, Mutagenesis, and Carcinogenesis

To study the embryotoxicity of RVX, it was administered to pregnant female rats perorally at a dose of 1/100 LD_{50} (Kiryukhin et al., 2007). RVX had a toxic effect in the females judged by a decrease in their RBC-AChE activity; at the same time, AChE activity in tissues of fetoplacental complex complied with age-specific control levels. These results may be regarded as evidence for impossibility (or at least low probability) of transplacental transfer of RVX from maternal organism to fetus. However, manifestation of intoxication symptoms by pregnant rats indicates that RVX can induce an embryotoxic effect, which is apparent from disorders of prenatal and postnatal ontogenesis (Tochilkina and Kiryukhin, 2007). It should be mentioned that embryotoxicity of VX to rat fetuses was also shown after single LD_{50} doses to the mother (Guittin, 1988), and after repeated doses of 0.005 mg/kg (near 1/5 LD_{50}) at varying times during fetal development (Guittin et al., 1987). On the other hand, no teratogenic potential of VX was found in sheep, rats, and rabbits (Van Kampen et al., 1970; Goldman et al., 1988).

Gonadotoxicity of RVX was investigated in chronic experiments for different methods of introduction. Dermal exposure of male rats to RVX demonstrated that general toxic effects were the governing factor of gonadotoxicity. There were no gonadotoxic effects at the level of threshold dose, which was estimated to be 4×10^{-6} mg/kg (Maslennikov and Kiryukhin, 2003). Other research has not revealed changes in the weight parameters of left and right testicles and epididymis, nor their total and specific weight parameters (Shabasheva et al., 2007). It has been concluded that RVX poses no danger concerning development of specific disorders of male reproductive function, and this is in agreement with available data on VX: neither acute nor chronic VX exposure had deleterious effects on reproductive potential (Van Kampen et al., 1970).

Studies of mutagenicity of RVX in the Ames test revealed no point mutations in the indicative bacteria. However, studies conducted by a micronuclear test have demonstrated that a single intragastric introduction of RVX at a dose of 1/10 LD_{50} stimulated in rats an enhancement of frequency of polychromatophilic erythrocytes with extranuclear inclusions. In cases when the cytotoxic effects were absent, these alterations could be interpreted as being of a mutagenic nature (Maslennikov and Ermilova, 2005). At the same time, the threshold dose of general toxic effect of RVX had neither mutagenic nor embryotoxic or gonadotoxic effects. Therefore, the delayed effects of RVX can appear at doses exceeding those that induce general toxic effects, indicating the nonspecific character of its action.

In studies involving bacteria or yeast, VX was tested both with and without metabolic enzyme activation to determine if VX metabolites might be mutagenic; the results were negative (Crook et al., 1983; Goldman et al., 1988). In addition, negative results were obtained for VX in the *Drosophila* mutagenicity assay (Crook et al., 1983), and VX was considered to be a nonmutagen in a test with mouse lymphoma cells (Goldman et al., 1988).

In the available literature, we could not find experimental data on the carcinogenicity of RVX. Nevertheless, epidemiologic data on tumor and pretumor diseases of the people that were engaged in production of RVX did not reveal an increase of oncological morbidity compared to a control group of human subjects (Fedorchenko et al., 2003). In agreement with this, McNamara et al. (1973) reported that there was no association of increased cancer in personnel working daily with VX.

Principles of Therapy

Acute toxicity of OPs in general and RVX in particular has been much more extensively investigated than chronic toxicity, so it is not surprising that an effective therapy has been developed for acute intoxications only. The most effective antidote complex for treating acute intoxications with RVX consists of an antagonist of M-cholinoceptors, a reversible inhibitor of ChE, and a reactivator of ChE; in addition, anticonvulsants can be used in cases where convulsions occur. In experiments with guinea pigs, they were pretreated with pyridostigmine (0.026 mg/kg, i.m.), and then immediately after RVX intoxication ($2 \times LD_{50}$), animals were given pralidoxime chloride (25 mg/kg, i.m.) and atropine sulfate (2, 8, or 16 mg/kg, i.m.); diazepam (5 mg/kg, i.m.) was administered to animals that displayed seizures and convulsions (Chang et al., 2002). It was also shown that in cases of acute intoxication with RVX, higher doses of atropine should be administered compared to those for VX. Of several oximes (pralidoxime, obidoxime, and

TABLE 10.5 Parameters of RVX Toxicity for Animals and Humans

Parameter	Rout of Exposure	Dose
LCt_{50} (human)	Inhalation	0.04 mg/min/dm^3
LC_{50} (mouse)	Inhalation	1.8–4.5 × 10^{-5} mg/kg
LCt_{50} (mouse)	Inhalation	0.011 mg/min/L
LD_{50} (rabbit)	Percutaneous	0.014 mg/kg
LD_{50} (cat)	Percutaneous	0.01 mg/kg
LD_{50} (dog)	Percutaneous	0.0157 mg/kg
LD_{50} (mice)	Percutaneous	0.016 mg/kg
LD_{50} (human)	Percutaneous	0.1–0.01 mg/kg

TABLE 10.6 RVX Safety Standards in the Russian Federation (Uiba et al., 2007)

MAC for working air (mg/m^3)	5 × 10^{-6}
MAC for reservoir water (mg/dm^3)	2 × 10^{-6}
TSEL for ambient air (mg/m^3)	5 × 10^{-8}
MPL for equipment surface (mg/dm^2)	2 × 10^{-6}
MPL for human skin (mg/dm^2)	3 × 10^{-8}
MAC for soil (mg/kg)	5 × 10^{-5}
EEL for ambient air (mg/m^3)	
After 1 h	1.6 × 10^{-5}
After 4 h	4.1 × 10^{-6}
After 8 h	2.0 × 10^{-6}
After 24 h	6.6 × 10^{-7}

Abbreviations: MAC, maximal allowable concentration; TSEL, tentative safety exposure level; MPL, maximal permissible level of pollution; EEL, emergency exposure limit.

HI-6), the most effective was HI-6, as it was in the case of VX, sarin, cyclosarin, and soman poisonings (Kassa et al., 2006).

Looking for therapies to treat chronic, low-dose intoxication, one should keep in mind that the VX-AChE complex has been found to undergo a significant degree of spontaneous reactivation in humans (at a rate of about 1%/h over the first 70 h after intravenous/i.v. administration of VX); another feature of VX toxicity is the lack of aging or stabilization of the VX-AChE complex and the relative ease of reactivation of VX-poisoned enzymes by oxime antidotes in humans (Sidell and Groff, 1974). Because there are no major differences in the reactivation process of both VX and RVX-inhibited ChE (Kuca et al., 2006), natural inactivation with plasma and liver paraoxonase (PON1) of RVX, VX, and other warfare agents could play a bigger role in prophylactic therapy of acute and chronic intoxications and their delayed effects (Costa et al., 2005). PON1 is a catalytic bioscavenger, in contrast to the stoichiometric bioscavenger BChE. A constituent of red wine, known as resveratrol, has been shown to be a natural and rather effective up-regulator of PON1, and it has good potential for protecting living cells against CWAs (Curtin et al., 2008).

TOXICOMETRY AND HYGIENIC REGULATIONS

In countries dealing with destruction of CWAs, control limits for exposure via surface contact of drinking water are needed, as are detection methods for their low levels in water, soil, or foodstuffs. Some of the toxicity parameters of RVX for humans and animals are available in the *Textbook of instructive and technical documentation on the problem of chemical weapon destruction* (Anon, 2001) and are given in Table 10.5.

In the event of contact of human skin with the fabric of a protective suit, toxicometric parameters of RVX have been experimentally estimated with laboratory rats (Zhukov et al., 2007): LD_{50}=0.55 ± 0.09 mg/kg, or 6.9 × 10^{-3} mg/cm^2; Lim ac (integr.) = 0.056 ± 0.001 mg/kg, or 7.0 × 10^{-4} mg/cm^2; Lim ac (sp) = 0.0051 ± 0.001 mg/kg, or 6.4 × 10^{-5} mg/cm^2; Lim ch = 4.75 × 10^{-5} mg/kg, or 5.9 × 10^{-7} mg/cm^2. The maximum concentration limit of RVX for the fabric of protective suits has been estimated to be 3.1 × 10^{-8} mg/cm^2, taking into consideration the reserve coefficient, an average body weight, and total area of the skin coverlet.

For the purposes of sanitary regulations, it has been estimated that RVX within the range of concentrations 0.01–1.0 mg/L has no negative influence on the natural purification of aquatic reservoirs, on growth and decay of saprophytic and pathogenic microflora, and on nitrification processes. The noneffective dose of RVX has been estimated to be 1 × 10^{-7} mg/kg, the threshold dose 1 × 10^{-6} mg/kg, and the effective dose 1 × 10^{-5} mg/kg (Maslennikov and Ermilova, 2006). Studies on RVX effects on the soil microflora have revealed that actinomyces and micromycetes proved to be the most vulnerable, whereas *Nitrobacteria* was the least vulnerable species (Gorbunova and Maximova, 2003). The hygienic regulations for RVX are presented in Table 10.6.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The ambient monitoring and biomonitoring of RVX and its destruction products within the areas of CW storage and destruction facilities is an important task for the State Sanitary Inspection Units of the Russian Federation. In this regard, development of chemical analytical and

bioanalytical methods and procedures should be among the main purposes of applied sciences. At the same time, estimation of probability of exposure to RVX and other CWAs calls for the solving of fundamental problems of molecular and functional diagnostics of the pathogenesis, as well as the development of effective therapy or prophylaxis.

In this chapter, we presented the principal products of RVX hydrolysis with excess water in an acid medium. It has been shown that after 3 months of exposure, nearly 1% of the initial amount of RVX in the solution could be detected. According to data on RVX hydrolysis with an equimolar amount of water, the autocatalytic hydrolysis of RVX was almost completed after 3 months since the RVX content in the sample was no more than 0.01%. Moreover, the reaction mixture was much poorer than in the case of hydrolysis with excess water. Isobutyl MPA, which catalyzes RVX degradation, was detected as the major component of the reaction mixture. Yang et al. (1996) found 2-(diethylamino)ethanethiol to be the second major component of the mixture, but in our experiment, it was almost completely converted into bis[2-(diethylamino)ethyl] disulfide.

We have not found conditions for effective extraction of RVX in experiments with its application on soil, concrete, bricks, and polyisobutylene at concentrations 10^{-6}–10^{-5} mg/kg. The extremely low recoveries of RVX from materials of various natures can be explained by both irreversible sorption and degradation. A summarized list of compounds isolated from samples of building materials was presented, which were taken from some areas of a former RVX production facility and might have contacted RVX. RVX degradation products were found, along with organic components of building materials, solvents, plasticizers, reaction products of degassing agents with matrix components, and other materials. High concentrations of fyrols were detected in some brick and concrete samples.

Procedures for identification and quantitative assessment of *O*-isobutyl MPA (a marker of exposure to RVX) in urine and blood plasma have been developed, and then toxicokinetic experiments for measuring *O*-alkyl MPA in rat plasma after intramuscular injection of RVX at a dose of 0.8 LD_{50}. In addition, we have obtained a mass spectrum of human albumin after *in vitro* incubation of blood serum with RVX. Incubation of commercially available human albumin with RVX with subsequent trypsinolysis and MS/MS analysis has revealed two sites of binding RVX to human albumin, Tyr411 and Tyr150. These data could contribute to development of sensitive and specific diagnostic methods.

Having analyzed our own experimental data and available scientific literature, we came to the conclusion that the toxicological features of RVX were not properly studied. On the one hand, pathogenesis of acute intoxication has been described, and toxicometric parameters and hygienic regulations have been developed. On the other hand, mechanisms of nonspecific effects and chronic intoxication, pathogenesis of delayed manifestations need further clarification. That is why there is a lack of effective prophylactic and therapeutic means of treating the delayed effects of RVX (and many other OPs) that cannot be equated or reduced to development of OPIDP. The search for non-ChE targets is still far from its final stage and not all the functional consequences of an OP exposure have been revealed. Various OPs can covalently bind to tyrosine residues of tubulin, near the GTP binding sites or within loops that interact laterally with protofilaments (Grigoryan et al., 2008), and this could partly explain an impairment of fast axonal transport and neuropsychological effects of chronic, low-dose exposure to OPs (Stephens et al., 1995; Terry et al., 2007). Previously, assumptions have been made about a direct influence of OPs on the humoral components of hemostasis and the complement system in terms of abundance of serine proteases in these systems (O'Neill, 1981). An interrelation of hemostasis and neuromuscular conduction is of undoubted interest considering the low "percentage" of endoneural capillaries in the structure of nerve fibers, and dependence of the trophism of any tissue upon the integrity of the endothelial monolayer and upon adequate functioning of hemostatic mechanisms. An idea about dependence of the trophism of nerve fibers upon the vascular bed comes from data on their ratio: the volume of capillaries amounts to just 2–4% of the total volume of nerve fiber (Odman et al., 1987). Moreover, peripheral nerves have no lymphatic vessels, which could provide an effective outflow of the capillary infiltrate and prevent development of edema (Low, 1984; Olsson, 1984).

Our data support the existence of a key role of nonsynaptic mechanisms in developing effects under chronic exposure to RVX. The morphofunctional changes at the level of the microcirculatory bed that influence the functional state of platelets may prove to be significant factors in the etiology of delayed effects of chronic intoxication. Since the life span of blood platelets is about 10 days, the changes observed are likely to reflect multiple microangiopathies, when clinically pronounced manifestations are prevented by compensatory mechanisms, such as receptor desensitization, endothelial and plasmatic proteases, and molecular effectors providing the feedback regulation.

It is well known that OPs cause the inhibition of several major enzymes, and this fact can be easily proved by simple methods of laboratory and clinical biochemistry. On the other hand, diagnostics of a chronic, low-dose action of OPs, or their delayed effects after subacute intoxication is a big problem because of lack of inhibition or recovery of the enzymes' activities. Determination of a peptide spectrum of blood plasma or serum could

serve as an alternative and more sensitive method for diagnosing the intoxications.

An adequate interpretation of the experimental data would lead to a proper understanding of the therapeutic approaches, which could prevent or even reverse the development of delayed effects of RVX and other warfare or pesticide OPs. Resveratrol, being an effective up-regulator of PON1, is now extensively studied in different laboratories as a possible remedy (Curtin et al., 2008). In view of our understanding of the pathogenesis of delayed effects, it is important to mention another property of resveratrol as a protector of endothelial progenitor cells, which contribute to renovation of injured blood vessels (Gu et al., 2006).

In conclusion, we outline some present and future areas of study on the analytical chemistry, biochemistry, and toxicology of RVX:

- Improvement of the methodology of chemical and biochemical monitoring in environmental objects and human organisms.
- Development of prognostic modeling for probable risk assessment in case of elevation of the allowable levels of RVX in various media.
- Studies on the quantitative relations dose-effect, time-effect, and dose-time-effect for ChE and non-ChE effects, for both acute and chronic exposure to RVX.
- Studies on novel molecular and functional effects under acute and chronic exposure to RVX.
- Studies on mechanisms of development of delayed effects after intoxication with RVX.
- Development of novel effective means for prophylaxis and treatment of delayed effects of intoxications with RVX.

Fulfillment of these studies surely would contribute to fundamental and applied knowledge well beyond the toxicology of RVX.

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CHAPTER

11

Riot Control Agents

Corey J. Hilmas

INTRODUCTION

Nonlethal agents are a broad class of compounds intended to produce transient incapacitation of an individual or individuals. Both incapacitating agents and riot control agents (RCA) are separate classes of nonlethal agents. Although the two classes share the characteristic to incapacitate, a distinction must be drawn between these two types of agents. RCAs differ from incapacitating agents in several respects. RCAs possess relatively short onset and limited duration of action. RCAs induce short-term toxic effects that subside within minutes after termination of the exposure. Additionally, modern RCAs have a very high safety ratio compared with incapacitating agents and first-generation RCAs. Many incapacitating agents were developed during the Cold War that produced either limited lethality and/or prolonged morbidity. Consequently, incapacitating agents have been banned by international treaties recognized by the United States, including the Chemical Weapons Convention (CWC). Specifically, the CWC has placed a ban on the development, production, and possession of any chemical weapon intended to cause death or "temporary incapacitation." The United States considers these broad incapacitating agents as chemical warfare agents (CWAs). However, the United States does not recognize RCAs as CWAs; therefore, US policy considers them to be legal for use by civilian police or the military. The CWC does prohibit their use in times of war. Thus, the United States has opted not to utilize RCAs in Iraq during the early twenty-first century against organized and armed insurgents.

Although the field of nonlethal agents is diverse and interesting, we limit our discussion to only those agents considered to be RCAs. The goal of RCAs is to temporarily incapacitate through irritating the skin and mucosal membranes of the eyes, airways, and digestive tract. As a result of their short-term toxicity, they are effective agents used by military and law enforcement personnel to disperse crowds, clear buildings, and quell riots. Whereas RCAs are often thought of as "tear gas" or pulmonary irritants, they encompass more than this terminology would suggest. They are neither gases nor exclusively pulmonary irritants. Historically, RCAs were categorized as lacrimators, sternutators, and vomiting agents based on their predominant toxicity in the eyes, lungs, or digestive tract. This nomenclature is outdated because modern RCAs affect a wide variety of organ systems. This fact will be clearly evident in the subsequent discussion concerning their mechanism of action and toxicity. Today, RCAs comprise a diverse array of chemical compounds with similar toxic effects because of their introduction on the battlefield in the early part of the past century. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Food and Drug Administration.

HISTORY

The Chinese were perhaps the first to use pulmonary irritants with their stink bombs (Smart, 1996). The smoke emanating from them was a primitive sternutator designed to harass the enemy. RCAs were used during the Peloponnesian War in the fifth century BC when the Spartans used smoke from burning coal, sulfur, and pitch to temporarily incapacitate and confuse occupants of Athenian strongholds (Thoman, 2002). During antiquity, the Romans used irritant clouds to drive out their Spanish adversaries from hidden dwellings (Robinson, 1971). Almost all of these examples involved the use of incapacitating agents as an offensive tactical weapon as opposed to controlling crowds for defensive purposes.

World War I (WWI) marked the birth of RCAs as well as the modern age of CWAs (Figure 11.1). Both German

Handbook of Toxicology of Chemical Warfare Agents.
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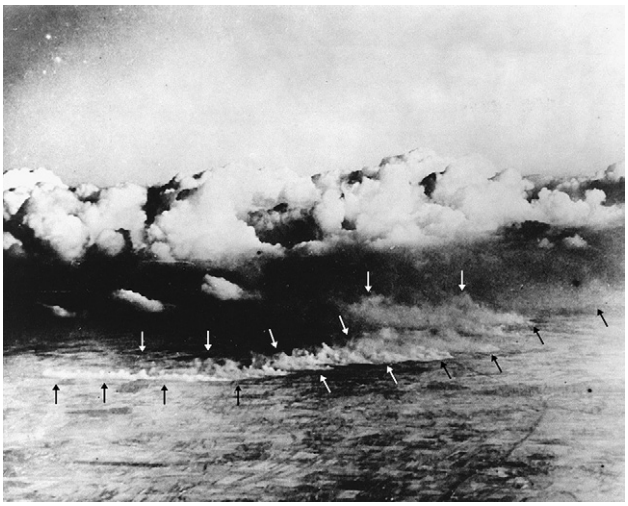


FIGURE 11.1 The birth of CWAs in WWI. The photograph depicts the initial chlorine gas attack by Germany at Ypres, Belgium, on April 22, 1915. The German Army released chlorine gas from cylinders to form a poisonous cloud (indicated by black and white arrows) directed toward the French lines by the prevailing winds.

and French forces used a wide variety of irritating agents, such as acrolein (papite), chloropicrin (PS), and diphenylaminearsine (DM; Adamsite); however, bromoacetone (BA) was the most widely used lacrimator agent at that time. At the end of WWI, the US military investigated the use of chloroacetophenone (CN) as a chemical irritant. First developed by Graebe in 1869 and formulated as Chemical Mace®, CN was the most widely used RCA until World War II (Olajos and Stopford, 2004).

Two chemists, Carson and Stoughton (1928), synthesized 2-chlorobenzylidene malononitrile (CS); however, it was not adopted by the military as an official RCA until 1959. As a more chemically stable compound and having a greater potency with less toxicity than CN, it gradually replaced CN as the preferred RCA. CS was widely used during the Vietnam War to flush the Viet Cong out of the labyrinth of underground tunnels and bunkers throughout Southeast Asia (Figure 11.2). In the years following the Vietnam War, other militaries adopted CS. Saddam Hussein's forces used it against Iran during the Iran–Iraq War of the 1980s. Today, CS is commonly used by law enforcement agencies and militaries for riot control training, respirator training in boot camps, temporary incapacitation of an assailant, and civil disturbances. A famous case of RCA use by the US Federal government involved CS dissemination on the Branch Davidian cult members in 1993. Because of its high flammability rating, CS was believed to be a large contributor to the inferno that burned down the Waco, TX, compound and its inhabitants. Even before fire broke out and destroyed the compound, it is believed that CS concentrations ranged from five-times to 60-times the amount required to deter individuals (Bryce, 2000).

FIGURE 11.2 Top: US Army Engineers unpack and test a Mitey-Mite blower in the jungles of Vietnam. The Mighty-Mite aerosolized and disperse smoke, CS powder, or other RCA as a means of tunnel denial. Bottom: American soldiers ("tunnel rats") wearing M28 protective masks just before entry into underground tunnels previously saturated with CS. *Source: Photograph (top): Courtesy of the US Army Engineer School, Fort Belvoir, VA.*

During the 1980s and 1990s, the use of CS gas was rapidly on the decline and slowly being replaced by oleoresin capsicum (OC) spray. OC, an extracted resin from *Capsicum* pepper plants, was first developed in the 1970s as an alternative to CN and CS agents. Commercially available OC sprays used by the public are approximately 1% capsaicin, whereas formulations used by law enforcement agencies can contain up to 15% capsaicin. Most recently, a synthetic form of capsaicin called nonivamide, marketed as Captor, gained popularity as a defensive aerosol in the early 1990s (Olajos and Stopford, 2004).

Under the CWC of 1997, RCAs were banned from use as a method of warfare because in high concentrations RCAs are toxic chemicals with the potential to incapacitate individuals for prolonged periods, produce long-term sequelae, and cause death. The CWC allows RCAs to be used in domestic riot control as well as for enforcement of domestic law and "extraterritorial law enforcement

activities undertaken by military forces" (Rosenberg, 2003). These boundaries and definitions, although vague, were clarified in 2003 by President George W. Bush (Wade and Schmitt, 2003). Bush authorized the use of tear gas against Iraqi troops for defensive purposes as allowed in Executive Order 11850 of 1975. Many experts believed this would violate the CWC (which was not signed by Iraq) and give Saddam Hussein the power to use chemical agents against the United States under the authority of the Geneva Protocol (Wade and Schmitt, 2003). In the end, RCAs were never used during that conflict.

BACKGROUND

The Agents and Their Physicochemical Properties

Unlike the majority of chemical agents that are liquid at room temperature, modern RCAs are crystalline solids with low vapor pressure (see Table 11.1). RCAs are typically administered as fine particles, aerosol sprays, or in solutions; therefore, they are not true gases. The inhalation toxicity of RCAs, as well as CWAs, is often indicated by the expression *Ct*. This term is defined as the product of concentration (*C*) in mg/m^3 multiplied by exposure time (*t*) in minutes ($mg\,min/m^3$). LCt50 and ICt50 are conventional terms used to describe airborne dosages that are lethal (L) or incapacitating (I) to 50% of the exposed population. The intolerable concentration (mg/m^3), ICt50, and minimal lethal concentration (mg/m^3) are provided in Table 11.1 for the most common RCAs. The ocular irritancy threshold (minimal irritant or minimal effective dose), estimated human LCt50, and safety ratio are provided in Table 11.2 for these same RCAs. The modern RCAs are characterized by a high LCt50, low effective Ct50, low ICt50, low minimal irritating concentration, and large safety index ratio (LCt50/irritancy threshold). As a rule of thumb, clinical signs and symptoms from RCA exposure generally subside within 30 min but may persist depending on dose and duration of exposure (Blain, 2003). Orthochloroacetophenone (CN) and chlorobenzylidene malononitrile (CS) are the classic representative agents of this class of compounds. The toxicity of CN and CS is discussed in depth because of the vast volume of literature available for these compounds.

Chloroacetophenone (CN)

CN is a crystalline solid with a strong, pungent odor (Figure 11.3). It is dispersed as a smoke, powder, or liquid formulation from grenades or other devices. It is perhaps better known under the trade name Chemical

TABLE 11.1 Physical Characteristics and Toxicity Data for the Common RCAs

		Physical Characteristics			Toxicity Data			
Agent	**Discovered in**	**Solubility**	**Vapor Pressure (mmHg at 20°C)**	**Vapor Density**	**Onset**	**Intolerable Concentration (mg/m^3)**	**ICt50 ($mg\,min/m^3$)**	**Minimal Lethal Concentration[a] (mg/m^3)**
CS	1928 (Carson and Stoughton)	Insoluble in water Soluble in organic solvents	0.00034	6.5	Immediate	5	3–10	2,500
CN	1871 (Graebe)	Poorly soluble in water	0.0054	5.3	Immediate	35	20–40	850–2,250
DM	1915 (Wieland)[b] and 1918 (R Adams)[c]	Insoluble in water Poorly soluble in organic solvents except acetone	2×10^{-13}	9.6	Delayed with long recovery period	5	22–150	1,100–4,400
CR	1962 (Higginbottom and Suschitzkey)	Sparingly soluble in water Stable in organic solvents	0.00059	6.7	Immediate	1	1	10,000
Bromobenzyl cyanide (CA)	1881 (Riener)[d]	Insoluble in water Soluble in organic solutions	0.12	4.0	Immediate	0.8	30	1,100

Source: References: Maynard (1999); Sidell (1997); Smith and Stopford (1999); Olajos and Salem (2001).
[a]*Estimate for minimal lethal concentration (10 min exposure).*
[b]*Wiegand (1915); Wieland and Rheinheim (1921).*
[c]*Sartori (1939).*
[d]*Prentiss (1937).*

TABLE 11.2 Health Risk Considerations for the Common RCAs

Agent	Irritancy Threshold[a] (mg/m^3)	Estimated Human LCt50[b] ($mg\,min/m^3$)	Safety Ratio[c]	Adverse Effects
CN	0.3[a]	8,500–22,500	28,000	Danger of permanent eye injury, vesiculation, bronchopneumonia, reactive airways, documented fatality cases
CS	0.004[a]	25,000–150,000	60,000	Same as CN, but fatality cases not authenticated, enhanced persistence compared to CN and CS
CR	0.002[a]	100,000	100,000	No significant respiratory toxicity
OC	0.0003[d]	not available	>60,000	Eye, skin, respiratory toxicity, significant morbidity in neonate, fatality involving case of in-custody use
DM	~1[a]	11,000–44,000	11,000	No longer used
CA	0.15[a]	11,000	11,000	Predominantly a lacrimatory agent, no longer used

[a]*Ocular irritancy thresholds unless indicated otherwise.*
[b]*Values obtained from references: Sidell (1997); Maynard (1999); Smith and Stopford (1999); Olajos and Salem (2001).*
[c]*Values derived from estimate of the human LCt_{50} (lower bound)/irritancy threshold (minimal effective dose). Therefore, ranges are not provided for the safety ratios.*
[d]*Threshold for respiratory complaints by capsaicinoids: Stopford and Sidell (2006).*

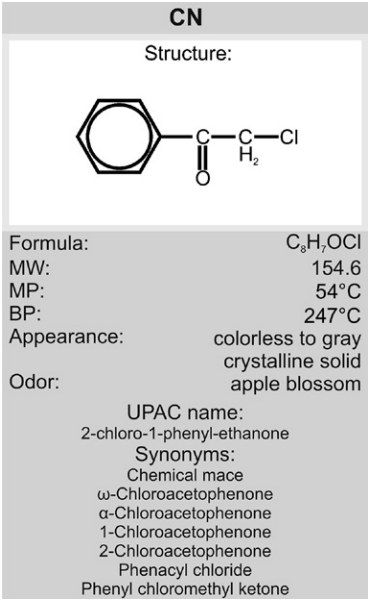


FIGURE 11.3 Chemical structure and physicochemical properties of chloroacetophenone (CN).

FIGURE 11.4 US soldier in protective clothing disseminating CN aerosol using the M33A1 disperser.

Mace® and was once used widely for self-protection. It was also the standard tear gas used by the military (Figure 11.4) and police personnel. It has been replaced in favor of the less toxic CS for riot control and capsaicin pepper spray for self-defense.

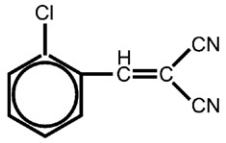
CN exhibits the greatest toxicity among RCAs in use today. Consequently, it has been replaced by compounds with higher safety ratios. CN is 3-fold to 10-fold more toxic than CS in rats, rabbits, guinea pigs, and mice (Ballantyne and Swanston, 1978). Pathological findings in the lungs tend to be more severe and CN causes far greater edema. CN typically causes an acute, patchy, inflammatory cell infiltration of the trachea, bronchi, and bronchioles, in addition to early bronchopneumonia. CN not only demonstrates greater irritation to the skin than CS but also is a more potent skin sensitizer (Chung and Giles, 1972). Patients frequently exposed to CN are at high risk for development of allergic dermatitis (Penneys, 1971).

Ortho-Chlorobenzylidene Malononitrile (CS)

The term CS was adopted after the two chemists, Carson and Stoughton, who synthesized the compound. CS is a white, crystalline powder with a pepper-like odor and low vapor pressure (Figure 11.5). It is rapidly hydrolyzed after contact with water but minimally soluble in ethyl alcohol. CS is the most widely used RCA today, although many countries are switching to even less toxic compounds. CS is used by the US Armed Forces for gas discipline training exercises to help new recruits learn the importance of donning their protective

CS

Structure:



Formula:	$C_{10}H_5N_2Cl$
MW:	188.6
MP:	93°C
BP:	310–315°C
Appearance:	white, crystalline solid
Odor:	pungent, pepper-like

UPAC name:
2-[(2-chlorophenyl) methylidene] propanedinitrile

Synonyms:
2-(2-Chlorobenzylidene) malononitrile
(ortho-Chlorobenzal)malononitrile
2-(Chloro-phenyl)methylenemalononitrile
(2-Chlorophenyl)methylenepropanedinitrile
β,β-Dicyano-ortho-chlorostyrene

FIGURE 11.5 Chemical structure and physicochemical properties of ortho-chlorobenzylidene malononitrile (CS).

FIGURE 11.6 Aerial spraying of a Chemical Warfare School class with CS tear gas during a training event.

masks quickly (Figure 11.6). It was also used by the United States during the Vietnam War for tunnel denial and crowd control (Figure 11.7) and by police forces for dispersing violent protests and incapacitating assailants.

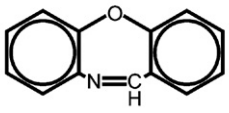
Dibenz(b,f)-1:4-Oxazepine (CR)

Dibenz(*b,f*)-1:4-oxazepine (CR) (Figure 11.8) is a potent sensory irritant with less toxicity than CS or CN (Ballantyne, 1977b). CR causes an immediate and effective irritation of the eyes, nose, and skin without persistent effects in these target organs. The irritation associated with CR is more transient compared with other RCAs. It is 5-times to 10-times greater in potency than CS; therefore, a smaller concentration is needed to cause irritation (low minimal irritant concentration or

FIGURE 11.7 US Army soldiers using CS tear gas in South Vietnam.

CR

Structure:



Formula:	C_8H_7OCl
MW:	195.2
MP:	72°C
BP:	335°C
Appearance:	pale yellow, crystalline solid
Odor:	pungent, pepper-like

Synonym:
Dibenzoxazepine

FIGURE 11.8 Chemical structure and physicochemical properties of dibenz(*b,f*)-1:4-oxazepine (CR).

dose) and incapacitation (low ICt50) (Tables 11.1 and 11.2). CR has a favorable safety ratio; it is safer than other RCAs based on its higher LCt50 (Table 11.1) and greater LCt50/irritancy threshold (safety ratio). In humans, the effects caused by CR are identical to CS. The LCt50 for humans is estimated at >100,000 mg min/m^3. Despite its reduced toxicity in humans, CR is not entirely without risk. CR is fairly stable, resists weathering, and persists in the environment (Sidell, 1997); therefore, enhanced toxicity may occur with prolonged exposure.

Diphenylaminechlorarsine (DM)

Diphenylaminechlorarsine (DM) (Figure 11.9) and Adamsite are pro-emetic agents used in WWI. DM has greater toxicity than other RCAs and has been abandoned in favor of compounds with less toxicity and greater safety ratios. Although toxicity is typically delayed with DM exposure, toxic signs and symptoms can occur within minutes after exposure. Systemic toxicity may also be more pronounced and prolonged.

Symptoms often subside hours after exposure. Because DM is an antiquated RCA, this compound is irrelevant today and will not be discussed further.

Oleoresin Capsicum

OC is an oily resin derivative from capsicums and composed of several related compounds. Capsicums are solanaceous (nightshade species) plants from the genus *Capsicum*. More than 20 species fall within the genus. Capsaicinoids are considered the active ingredients of OC. These active compounds are endocrine products of glands found in the plant placenta and are a mixture of two unsaturated and three saturated homologs (Figure 11.10). Capsaicinoids are isolated through a volatile solvent extraction of the dried, ripened fruit of chili peppers. The capsaicinoids are distilled, dried, and compounded together. The final oleoresin contains several branched-chain alkyl vanillylamides, in addition to capsaicin, the major component in OC. The predominant capsaicinoid components of OC are capsaicin (70%), dihydrocapsaicin (20%), norhydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%) (Salem et al., 2006) (Figure 11.10).

Capsaicinoids cause dermatitis as well as nasal, ocular, pulmonary, and gastrointestinal effects in humans. OC gained popularity in the 1990s as a defensive weapon for civilians and law enforcement agencies because they produce immediate, temporary immobilization and incapacitation when sprayed directly into the face or eyes. It is important to note that hand-held pepper spray formulations can contain OC by themselves or a mixture of OC and CS.

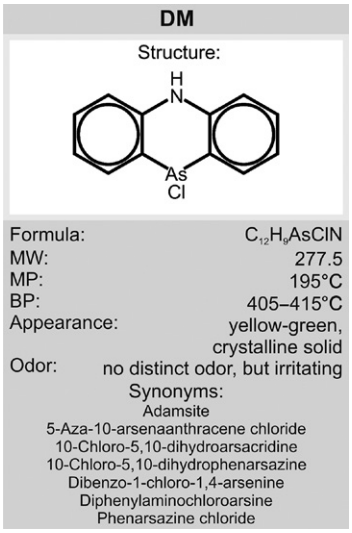


FIGURE 11.9 Chemical structure and physicochemical properties of diphenylaminechlorarsine (DM).

Pelargonic Acid Vanillylamide

Other capsaicinoids are available. Pelargonic acid vanillylamide (PAVA or nonivamide), shown in Figure 11.10, is a "synthetic" form of capsaicin. Nonivamide was first synthesized by Nelson (1919). Nonivamide was originally found to be a minor component in *Capsicum annum* peppers (Constant and Cordell, 1996); however,

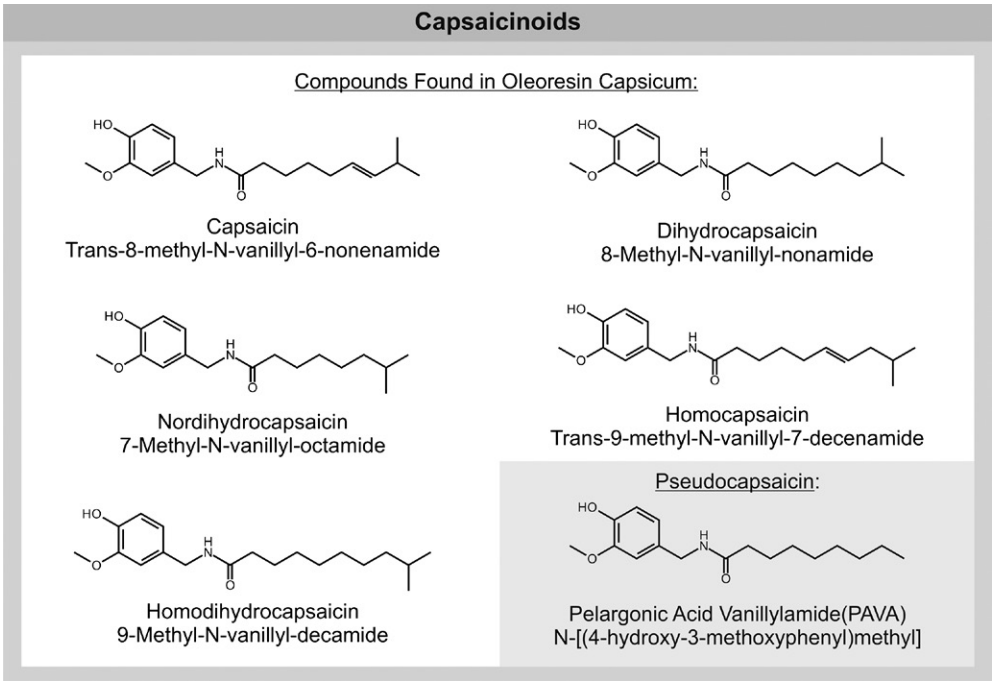


FIGURE 11.10 Chemical structures of the most common capsaicinoids found in OC.

the majority of PAVA is derived from synthesis rather than by extraction from natural plant sources. As a result, the composition and concentration of PAVA can remain consistent (Haber et al., 2007).

For PAVA to work, it must be directed at the subject's eyes. The pain in the eyes is reported to be higher than that caused by CS tear gas (Smith et al., 2004; Association of Chief Police Officers of England, Wales and Northern Ireland, 2006). The effects are immediate but will subside 15–20 min after exposure to fresh air. PAVA does display disadvantages. Although PAVA has a high rate of effectiveness, it has proven to be ineffective against those under the influence of alcohol (Association of Chief Police Officers of England, Wales and Northern Ireland, 2006). Additionally, the Smith et al. (2004) study mentions a number of cases in which PAVA was used without effect. The effect of PAVA was also reported to be almost instantaneous, with the undesirable effect that recovery was also immediate. PAVA is commercially available in two forms, captor I and captor II. Captor I contains 0.3% PAVA with a solvent of equal parts ethanol and water. Captor II contains 0.3% PAVA with propylene glycol, water, and ethanol (Committee on Toxicity of Chemicals in Food, Consumer Products, and the Environment, 2007).

MECHANISM OF ACTION

CS, CN, and CR

The mechanisms of action through which RCAs act are not completely understood. One explanation for the toxic effects of RCAs is the production of hydrochloric acid through reduction of chloride ions on mucosal membranes (Worthington and Nee, 1999). This may help explain the marked, focal irritation and burns on skin resulting from exposure to CS (Anderson et al., 1996). In addition, CS and CN are SN_2 alkylating agents (Cucinell et al., 1971; Ballantyne and Swanston, 1978); in contrast, the vesicant mustard is an SN_1 alkylating agent. The SN_2 moniker describes direct reaction of the agent with nucleophilic compounds in a bimolecular fashion. In particular, they react with intracellular thiol or SH-containing enzymes, thereby inactivating them (Ballantyne, 1977a). Mackworth (1948) first showed that CN and other first-generation lacrimators used during WWI (bromoacetophenone, ethyl iodoacetate, chloropicrin, bromobenzyl cyanide) strongly inhibited thiol-containing succinic dehydrogenase and pyruvic oxidase, major players of crucial metabolic pathways. Some suggest that lactic dehydrogenase is completely insensitive to lacrimators (Mackworth, 1948), but only lacrimators from the iodoacetate family were ever studied by this group. Another group reported that lactic dehydrogenase is, in fact, strongly inhibited by CS (Cucinell et al., 1971). Chloropicrin also interferes with oxygen transport to the tissues by reacting with SH groups on hemoglobin.

In addition, CS reacts with the disulfhydryl form of lipoic acid, a coenzyme in the pyruvate decarboxylase system (Olajos and Salem, 2001). Alteration in dihydrolipoic acid biochemistry can lead to decreased acetyl CoA levels, resulting in cellular injury. Therefore, tissue injury seems to be related to inactivation of these metabolic enzyme systems. The damage is transient because the enzymes can be rapidly reactivated if exposure is terminated (Beswick, 1983).

Based on these studies, it has been suggested that alkylation of nucleophilic sites, including SH-containing enzymes, is the underlying biochemical lesion responsible for lacrimator-induced toxicity. However, pain from RCA exposure can occur without tissue injury. It has been suggested that the irritant and painful effect of CS may be bradykinin-mediated (Cucinell et al., 1971; Olajos and Salem, 2001). CS causes bradykinin release *in vivo* in humans (Cucinell et al., 1971) and *in vitro* (Blain, 2003). Elimination of bradykininogen *in vivo* abolishes the systemic response to CS (USAMRICD, 2000; Salem et al., 2006).

The metabolism of CS to cyanide (see "Toxicokinetics") was once thought to be responsible for agent-induced lethality in animals (Jones and Israel, 1970; Cucinell et al., 1971). Despite reports of alleged fatality cases, mortality in humans after CS administration has not been authenticated (Ballantyne, 1977a; Olajos and Salem, 2001). CS has been demonstrated to cause death in dogs (Cucinell et al., 1971). CS is hydrolyzed to malononitrile and 2-chlorobenzaldehyde (Brewster et al., 1987). Further metabolism of malononitrile yields two potential cyanides, which could interact with sulfur thiols to yield thiocyanate. Cyanide typically causes death immediately, but animals administered CS by inhalation far more than the lethal C*t* do not die immediately; death occurs 12–24 h after exposure. In fact, death seems to be attributable to airway and lung damage (Ballantyne and Swanston, 1978). Studies to ascertain cyanide production after CS exposure in humans showed negligible levels of plasma thiocyanate (Leadbeater, 1973). Another study revealed low levels of cyanide production in mice administered carbon 14–labeled CS (Brewster et al., 1987). In short, cyanide is not liberated in sufficient quantity from CS metabolism to become toxic enough to cause death.

While cyanide inhibition of cytochrome *c* oxidase may not account for the full spectrum of toxicity in CS exposure, cyanide toxicity may include an array of biochemical interactions. These include lipid peroxidation (Johnson et al., 1987), cyanide release of endogenous opioids to cause respiratory paralysis, disruption of neuronal calcium homeostasis (Johnson et al., 1986), and phospholipid hydrolysis (Sakaida and Farber, 1990). The mechanism of action for CN follows very closely that of CS because they are both alkylating agents. The

effect of both agents on SH-dependent enzyme systems has been studied (Cucinell et al., 1971). Less is known regarding the mechanism of action for CR intoxication.

Capsaicinoids

Capsaicinoids interact with a population of neuropeptide-containing afferent neurons and activate a "vanilloid" receptor (Szallasi and Blumberg, 1990). There seems to be a requirement by the receptor for a vanilloid ring and an acyl chain moiety for activity (Szallasi and Blumberg, 1999). Vanilloid receptors are part of a superfamily of transient receptor potential cation channels. Binding of a vanilloid-containing ligand to the receptor causes channel opening, influx of Ca^{2+} and Na^{+}, depolarization of the neuron, and release of neuropeptides (Martling, 1987). In addition to transitory excitation of primary afferents, activation of these receptors leads to a prolonged refractory period, indicative of an apparent nonconducting and desensitized state of the receptor. In this refractory period, primary afferents become unresponsive to further application of capsaicinoids. Furthermore, it has been suggested that influx of Ca^{2+} and Na^{+} may lead to rapid cellular damage and eventual cell death (Jancso et al., 1984), possibly by Ca^{2+}-dependent protease activity. Administration of capsaicin in neonatal rats causes destruction of the dorsal root ganglion neurons (Jancso et al., 1977).

The biological actions of capsaicin are primarily attributable to release of the neuropeptide substance P, calcitonin gene–related peptide (CGRP), and neurokinin A from sensory neurons. These transmitters from primary sensory neurons communicate with other cell types. They produce alterations in the airway mucosa and neurogenic inflammation of the respiratory epithelium, airway blood vessels, glands, and smooth muscle. Alterations in multiple effector organs lead to bronchoconstriction, increased vascular permeability, edema of the tracheobronchial mucosa, elevated mucosal secretion, and neutrophil chemotaxis (Tominack and Spyker, 1987). Capsaicin-induced effects of bronchoconstriction, vasodilation, and plasma protein extravasation are mediated by substance P. In addition, substance P can cause bronchoconstriction through stimulation of c-fibers in pulmonary and bronchial circulation.

TOXICOKINETICS

The uptake, distribution, and metabolism of CS, CR, and capsaicins (but not CN) have been well characterized.

Uptake, Distribution, and Metabolism of CS

CS is rapidly absorbed and distributed throughout the body after inhalation exposure. Pharmacokinetic studies show that CS is removed from circulation quickly with first-order kinetics after inhalation exposure. CS half-life is just less than 30 s (Olajos, 2004). Short half-lives in the circulatory system are also demonstrated for the major CS metabolites (2-chlorobenzyl malononitrile and 2-chlorobenzaldehyde) (Leadbeater, 1973). Currently, it is thought that significant amounts of CS, near the tolerable concentration of approximately $10\,mg/m^3$, would not be absorbed after CS inhalation. The absorption of CS from the digestive tract in cases of exposure by ingestion is unknown at this time. Systemic toxicity has been noted after ingestion of CS pellets (Solomon et al., 2003).

In mammalian species, CS rapidly hydrolyzes to form 2-chlorobenzaldehyde and malononitrile (Leadbeater, 1973). The malononitrile intermediate is further metabolized from two cyanide moieties, which are converted to thiocyanate (Cucinell et al., 1971). The aldehyde intermediate undergoes oxidation to 2-chlorobenzoic acid or reduction to 2-chlorobenzyl alcohol. These metabolites are conjugated and excreted in the urine.

Uptake, Distribution, and Metabolism of CR

Absorption of CR after aerosol inhalation is rapid, with a plasma half-life of 5 min; this is consistent with half-life estimates after intravenous administration (Upshall, 1977) and gastrointestinal uptake (French et al., 1983). Corneal tissue has been demonstrated to take-up CR and to metabolize it to the lactam derivative (Balfour, 1978).

A number of studies have investigated the bioconversion, fate, and elimination of CR in various animal species (Balfour, 1978; French et al., 1983). Human metabolic studies of CR have not been performed because of the high degree of sensitivity of human tissues to CR. The maximum tolerated dosage is far too low to allow for detection in metabolic studies (Olajos, 2004). The lactam derivative dibenz[*b*, *f*]1:4-oxazepin-11-(10H)-one is a primary metabolic product of metabolism and a direct precursor of the urinary hydroxylated metabolites. In rats, the lactam, a dihydro-CR metabolite, an amino alcohol of CR, and an arene oxide are metabolites in CR degradation. In the rat, the major mechanism for elimination is sulfate conjugation and biliary excretion to a limited extent. Phase I metabolism by microsomal mixed-function oxidases involves reduction of CR to the amino alcohol, oxidation to form the lactam ring, and hydroxylation to form the hydroxylactams. Phase II conjugation reactions sulfate the hydroxylactam intermediates for renal elimination. Amino alcohol intermediates are conjugated with glucuronide for biliary secretion.

Uptake, Distribution, and Metabolism of CN

The uptake, distribution, and fate of CN have been poorly characterized despite numerous investigations reporting its toxicity. Inhalation of lethal CN, which does

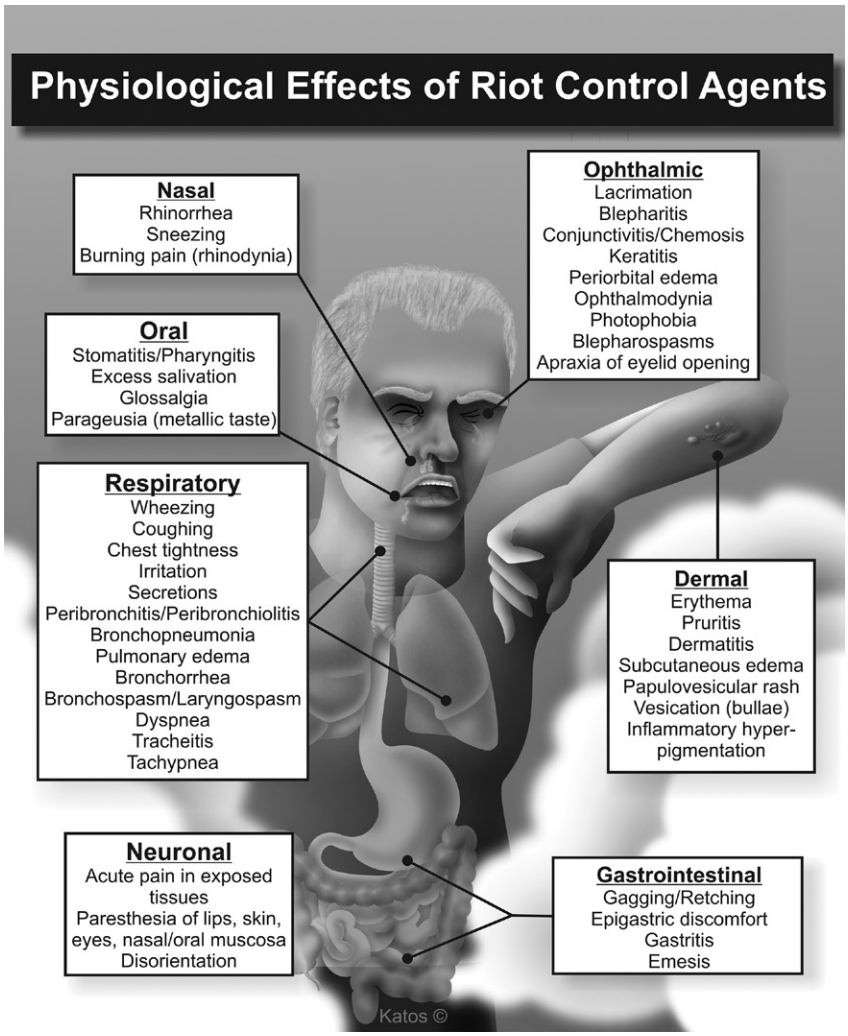


FIGURE 11.11 Physiological effects of RCAs. *Source: Illustrated, copyright protected, and printed with permission by Alexandre M. Katos.*

not metabolize to liberate cyanide, also causes death secondary to effects on the pulmonary system (pulmonary congestion, edema, bronchopneumonia, cellular degeneration in the bronchiole epithelium, and alveolar thickening) in mice, rats, guinea pigs, and dogs (Olajos and Salem, 2001). CN presumably reacts irreversibly with the free sulfhydryl groups of proteins and enzymes. It is thought that CN metabolically converts to an alkylating agent with this affinity for SH groups and nucleophilic sites in tissues (Mackworth, 1948; Olajos, 2004).

Uptake, Distribution, and Metabolism of Capsaicins

Capsaicin and capsaicinoids undergo phase I metabolic bioconversion to catechol metabolites via hydroxylation of the vanillyl ring moiety (Miller et al., 1983). Metabolism involves oxidative, in addition to nonoxidative, mechanisms. An example of oxidative conversion involves the liver mixed-function oxidase system to convert capsaicin to an electrophilic epoxide, a reactive metabolite (Olajos, 2004). Surh and Lee (1995) have also demonstrated the formation of a phenoxy radical and quinine product; the quinine pathway leads to formation of a highly reactive methyl radical (Reilly et al., 2003). The alkyl side chain of capsaicin also undergoes rapid oxidative deamination or hydroxylation (Reilly et al., 2003) to hydroxycapsaicin as a detoxification pathway. An example of nonoxidative metabolism of capsaicin is hydrolysis of the acid–amide bond to yield vanillylamide and fatty acyl groups.

TOXICITY

RCAs produce a wide variety of physiological effects in humans. Figure 11.11 illustrates these generalized toxic signs and symptoms of exposure. The clinical effects in

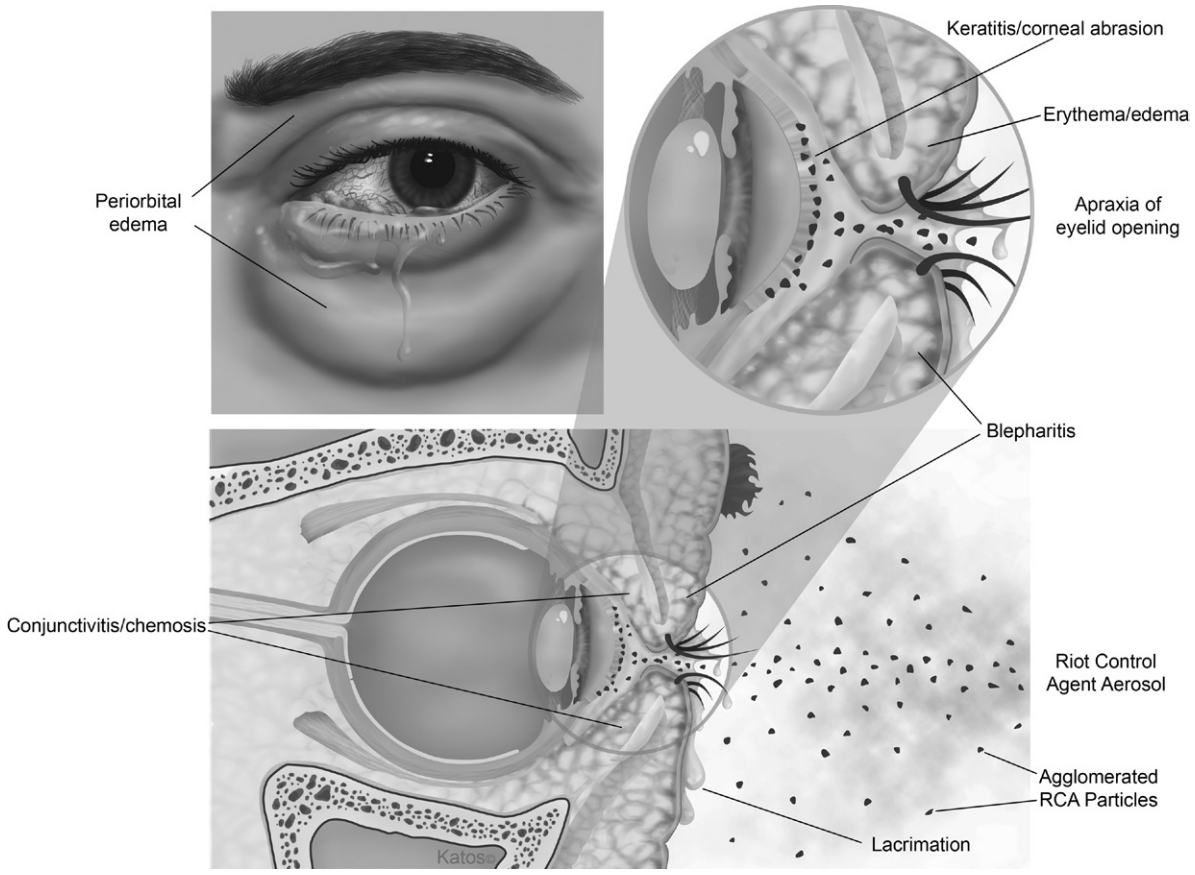


FIGURE 11.12 Exposure of the eye to CS aerosol. Top left panel: External view of left eye immediately after exposure to CS aerosol, showing scleral injection, periorbital edema, and lacrimation. Bottom panel: Penetration of CS aerosol into the eye, sagittal view. Following exposure to CS, the eye responds with inflammation, edema (chemosis), lacrimation, erythema, eye pain, and eyelid closure. Top right panel: Close-up of the eye and eyelids, sagittal view. Inflammation of the eyelids (blepharitis), conjunctivae (conjunctivitis), and cornea (keratitis) are apparent. The eye, in turn, responds with spasms of eyelid closure (blepharospasms) followed by an inability to open the eyelids (apraxia of eyelid opening). Agglomerated CS particles can penetrate the eye on initial contact and cause corneal abrasions. *Source: Illustrated, copyright protected, and printed with permission by Alexandre M. Katos.*

the figure are representative of those encountered after CN or CS exposure. CR causes effects qualitatively similar to those caused by CS, except it has greater potency. The predominant anatomical regions affected include eye, lung, and skin. RCAs also cause nasal, oral, neuronal, and gastrointestinal effects.

Ophthalmological Effects

CN and CS

The eyes are a major target for the short-lived toxic effects of RCAs. Eye findings from RCA toxicity can range in severity from conjunctival erythema to ocular necrosis. Lacrimation, conjunctival erythema/edema, blepharitis, and erythema are the most typical findings after exposure to all RCAs. Toxic signs may further include periorbital edema (Yih 1995), blepharospasm or spasms during eyelid closure (Grant, 1986; Blain, 2003), apraxia of eyelid opening, ophthalmodynia, corneal injury, and ocular necrosis (Grant, 1986). Figure 11.12 illustrates and summarizes the common toxic ophthalmological signs and symptoms associated with RCA aerosol exposure. It is important to note that eye findings tend to be more severe in RCA exposure victims if they are wearing contact lenses (Solomon et al., 2003).

Erythema and edema may last up to 48 h and vascularizing keratitis is not uncommon (Ballantyne et al., 1974), but symptoms generally subside after 30 min (Beswick, 1983), depending on the concentration and duration of exposure (Blain, 2003). Recovery is typically complete within 15–30 min after exposure, but a few signs such as erythema of the lid margins and photophobia may persist slightly longer. The conjunctivae may appear injected or even progress to fulminant conjunctivitis and blurred vision after some RCAs, including CS (Euripidou et al., 2004). Toxic signs in the conjunctivae from CN Mace exposure can include conjunctivitis, sloughing, limbal

ischemia, and symblepharon formation (adhesion of the eyelids to the eyeball) (Scott, 1995). Permanent eye injury is unlikely, except after exposure to high concentrations of CN Mace (Grant, 1986). Although permanent eye damage is uncommon, increased intraocular pressure from edema may precipitate acute angle closure glaucoma if left untreated. Long-term sequelae may include cataracts, vitreous hemorrhage, and traumatic optic neuropathy (Gray and Murray, 1995).

In studies involving human exposure (Rengstorff and Mershon, 1969a,b), CS (0.1% or 0.25% in water; 1.0% in triocyl phosphate) sprayed or administered as ophthalmic drops onto the eyes caused apraxia of eyelid opening with blepharospasm on eyelid closure for 10–135 s. It also caused a transient conjunctivitis but no corneal damage on further inspection with a slit lamp. Rabbit eyes contaminated with CS as a solution (0.5–10% in polyethylene glycol), as a solid, or thermally dispersed as a smoke (15 min at 6,000 mg/m^3) showed greater toxicity with solution. CS in solution caused profuse lacrimation, conjunctivitis, iritis, chemosis, keratitis, and corneal vascularization at concentrations of 1% or more. The lesions tended to be more severe and have a greater duration at higher doses. Histologically, the cornea appeared with patchy denudation of the epithelium and infiltration of neutrophils to the site of injury (Ballantyne et al., 1974). In general, more severe eye exposures have resulted from CN compared with CS agent.

CN causes a similar constellation of ocular signs and symptoms as CS, but CN toxicity is likely to be more severe in the eyes and skin. CN sprayed into the eyes from a distance causes lacrimation, edema of the corneal epithelium and conjunctivae, and reversible epithelial defects of the cornea (Leopold and Lieberman, 1971). At close range, CN can cause long-lasting and permanent damage to the eye. Because RCAs are solids, it is possible for a particle to clump or agglomerate, causing penetration into corneal or conjunctival tissues (Figure 11.12). Agglomerated CN particles can penetrate eye tissue as a result of tear gas cartridge discharge (Levine and Stahl, 1968). In addition to large powder CN agglomerates, traumatic effects from the propellant charge, wadding, or foreign pieces from the cartridge should also be suspected when evaluating eye damage from CN.

Although RCAs produce short-lived effects, rabbits exposed to 10% CN solution caused iritis and conjunctivitis for more than 7 days and caused corneal opacity (Grant, 1986) lasting longer than 2 months (Gaskins et al., 1972). In comparison, CS at the same concentration produced moderate conjunctivitis without iritis or corneal opacity, and eyes returned to normal by the end of 1 week. Another difference between the two agents is that CN produces a more severe reaction than CS when applied to the eye in powder form or as a spray at close range (McNamara et al., 1968).

In addition to opacification, additional corneal effects from particulate CN exposure include possible penetration of the corneal stroma, severe scarring and ulceration, and deficits in the corneal reflex (Scott, 1995; Blain, 2003). Penetration of the corneal stroma may lead to stromal edema and later vascularization, resulting in further ocular complications. These may include pseudopterygium, infective keratitis, symblepharon, trophic keratopathy, cataracts, hyphema, posterior synechia, secondary glaucoma, vitreous hemorrhage, and traumatic optic neuropathy (Hoffman, 1967). Furthermore, a 4% CN formulation produced permanent corneal injury but a 10% CS product did not (Gaskins et al., 1972). In animal studies, high concentrations of CN produce ocular necrosis (Grant, 1986).

The eyes are also affected by a CS agent without direct contact between the agent and the eye. In one report, seven patients were exposed to oral ingestion of a juice drink contaminated by CS pellets (Solomon et al., 2003). In addition to mild headache and gastrointestinal irritation, patients reported ocular irritation and lacrimation. The majority of symptoms resolved within 24 h of exposure.

CR

Higginbottom and Suschitzky (1962), the chemists who discovered CR, first noted the intense lacrimal response to this compound. A splash of CR (0.01–0.1% range solution) causes immediate ophthalmodynia, lacrimation, and blepharospasm, similar to CS and CN (Sidell, 1997). These effects can last 15–30 min before subsiding. Blepharitis (edema of the eyelids), periorbital edema, and injected conjunctivae can last for up to 6 h. In rabbits and monkeys, CR (0.1% solution) causes mild transient erythema, chemosis, and keratitis in the eye. Moderate conjunctivitis has been demonstrated with higher CR concentrations (5% solution) applied directly to the rabbit eye (Rengstorff et al., 1975). Ballantyne et al. (1975) showed that increasing CR concentrations as a solution caused dose-dependent corneal thickening but minor eye effects (mild conjunctivitis and lacrimation) as an aerosol. In animal studies, the effects of CR on the eye are very transient as they are cleared in 1 h, and it produces far less toxicity to the eye than CN (Salem et al., 2006).

Capsaicin

Capsaicin causes conjunctivitis, periorbital edema/erythema, ophthalmodynia, blepharospasm, blepharitis, corneal abrasions, and lacrimation. In a retrospective study of 81 patients who presented to the emergency department after aerosol exposure from law enforcement use of OC, 56% of individuals developed ophthalmodynia, 44% developed conjunctivitis, 40% developed conjunctival erythema, 13% developed lacrimation, and 9% developed corneal abrasions (Watson et al., 1996). Another study involved exposure of 47 human

volunteers to OC for evaluating effects on the cornea and conjunctivae (Zollman et al., 2000). All subjects reported significant eye pain, blurred vision, and lacrimation 10 min after exposure to OC pepper spray, but symptoms improved by 1 h later. Corneal abrasions were not apparent, but 21% of subjects showed evidence of punctate epithelial erosions and reduced corneal sensitivity. Corneal abnormalities were absent 1 week after exposure. Another human study identified 23% of subjects (7 of 30) with corneal abrasions after aerosol exposure to OC spray (Watson et al., 1996). In mice, a single subcutaneous injection of 12.5, 25, or 50 mg/kg capsaicin causes corneal changes characterized by neuronal axon degeneration in the corneal epithelium (Fujita et al., 1984).

Nasal/Pharyngeal Toxicity

RCAs produce oral and nasal symptoms immediately after exposure. Inhalation exposure to CN and CS causes rhinorrhea, sneezing, and burning pain within seconds (Beswick, 1983); a similar burning sensation with increased salivation occurs after oral contact with aerosolized powder or solution. The salivation, pharyngitis, and glossalgia occur within minutes after exposure (Thorburn, 1982; Beswick, 1983). A CR solution (0.01–0.1% range) splashed in the mouth causes salivation and burning of the tongue and palate for several minutes. Splashes of the solution can cause nasal irritation and rhinorrhea (Sidell, 1997). Fumes from burned *Capsicum* plants or capsaicin-containing pepper sprays are highly irritating to the nasal mucosa and cause immediate rhinorrhea (Geppetti et al., 1988).

Cardiovascular Toxicity

Although the evidence is not overwhelmingly impressive, RCAs have apparent effects on the cardiovascular system. Tachycardia and mild hypertension have been noted after exposure (Beswick, 1983). This response may result from anxiety or may be a reaction to the pain as opposed to any toxicological effect. The initial response to aerosolized CS is hypertension and irregular respiration, suggestive of the Sherrington pseudoeffective response. Bypassing the pain receptors of the nose and upper airway by endotracheal administration of CS leads to a decrease in blood pressure and bradypnea, effects also seen after intravenous injection. This suggests the initial pressor effect and irregular respiration are generalized responses to noxious stimuli rather than pharmacological effects of CS. Splash contamination of the face or whole-body drenching with dilute CR solution (0.0010% and 0.0025%) causes an immediate increase in blood pressure and bradycardia (Ballantyne et al., 1976). Intravenous administration of CR in cats causes transient but dose-dependent tachycardia. These pressor effects are postulated to be secondary to CR effects on sympathetic tone to the cardiovascular system or the result of stress and discomfort from the irritation (Ballantyne, 1977a,b).

RCAs have been shown to have a direct effect on the heart. One report linked exposure of high CS concentrations to the development of congestive heart failure (Hu et al., 1989). Furthermore, underlying cardiac disease has been shown to exacerbate toxicity from CS (Worthington and Nee, 1999).

Respiratory Toxicity

CS and CN are disseminated as an aerosol powder or solution. Therefore, the most common route of CN or CS absorption is by inhalation. Inhalation of RCAs causes burning and irritation of the airways, leading to cough, chest tightness, dyspnea (Beswick, 1983; Hu et al., 1989; Blain, 2003), shortness of breath (Euripidou et al., 2004), bronchospasm, and bronchorrhea (Folb and Talmud, 1989). Estimates of the minimal irritant exposure and ICt50 are 0.004 and 5 mg min/m^3, respectively, for CS (Olajos and Salem, 2001). Similar estimates have been made for CN (0.3–1 and 20–50 mg min/m^3) (Olajos and Salem, 2001). Other estimates report that 31 mg/m^3 CN vapor is intolerable to humans after 3 min (Punte et al., 1962).

Laryngospasm can occur immediately or can be delayed for 1–2 days after CN or CS exposure. Delayed-onset laryngotracheobronchitis 1–2 days after exposure, characterized by wheezing, dyspnea, tachypnea, hoarseness, fever, and purulent sputum, was reported in three of eight patients exposed to high concentrations of CN (Thorburn, 1982). Long-term bronchodilator therapy was required in one patient with preexisting pulmonary disease. Reactive airways are associated with high-level exposure to CS and CR (Blain, 2003). Paroxysmal cough, shortness of breath, and chest tightness, characteristic of reactive airway disease, have been demonstrated to last up to several weeks. Pulmonary effects typically resolve by 12 weeks after exposure.

Pulmonary edema may occur up to 24 h after exposure (Stein and Kirwan, 1964; Gonmori et al., 1987). Gonmori et al. (1987) reported a fatality from chloropicrin spray intoxication. The patient, an 18-year-old female, developed pulmonary edema 3 h after exposure. Furthermore, a 43-year-old man developed pulmonary edema complicated by pneumonia, heart failure, and hepatocellular damage after CS intoxication (Krapf and Thalmann, 1981). Delayed-onset bronchopneumonia may occur from prolonged exposure to some RCAs in enclosed spaces (Beswick, 1983).

There is no evidence that CS causes permanent lung damage after one or several exposures to field concentrations (Blain, 2003). Inhalation of an irritant might be expected to exacerbate underlying pulmonary disease such as asthma, emphysema, or bronchitis. Histories of

asthma and chronic obstructive pulmonary disease may exacerbate effects from CS (Worthington and Nee, 1999) or CN (Thorburn, 1982). CS may exacerbate chronic bronchitis or precipitate an attack in individuals with known asthma (Anonymous, 1971).

CN and CS Toxicity in Animals

In animal studies, the cause of death from CN inhalation is the result of toxicity in the pulmonary system. Postmortem examination from acute toxicity lethality studies in animals exposed to CN aerosols reveal pulmonary congestion, edema, emphysema, tracheitis, bronchitis, and bronchopneumonia in dogs, and pulmonary congestion, edema, and bronchopneumonia in mice, rats, and guinea pigs (Olajos and Salem, 2001). Sublethal CN aerosol exposure (62.6 mg/m^3, 0.1 LC50) for 60 min causes cellular degeneration in the bronchiole epithelium and alveolar septal wall thickening attributable to infiltration of mononucleocytes (Kumar et al., 1995).

Exposure to aerosol CS (unreported concentration) in male Wistar rats for 20 min can cause decreased minute ventilation and induce histological lesions of the trachea (cytoplasmic vacuoles in epithelial cells) and lung (emphysema) (Debarre et al., 1999). Lower respiratory tract injury, including fibrosing peribronchitis and peribronchiolitis, can be produced by chloropicrin (Buckley et al., 1984).

CR

CR does not produce any significant respiratory toxicity (Sidell, 1997). CR causes tachypnea and labored breathing in multiple animal species. In humans, aerosol exposure to CR causes respiratory irritation, choking, and dyspnea. One human study involving aerosol exposure to CR (0.25 mg/m^3) in volunteers for 60 min noted decreased expiratory flow rate minutes after exposure. CR was thought to stimulate irritant receptors in the conducting portion of the pulmonary system, causing bronchoconstriction (Ashton et al., 1978). Additionally, CR increased blood volume in the lungs by driving sympathetic tone. Two animal studies evaluated the effect of CR aerosol exposure on the physical and ultrastructural changes in rat lungs (Pattle et al., 1974; Colgrave et al., 1983). Even high CR aerosol doses did not produce significant pulmonary damage. Gross examination of the lungs was normal in both studies. Microscopic examination showed mild congestion, lobar hyperinflation characteristic of emphysema, and hemorrhage. Further pulmonary damage was evident on electron microscopy. CR-exposed lungs showed capillary damage of the endothelium and a thickened, swollen epithelial layer (Colgrave et al., 1983).

Capsaicin

In children, capsaicin spray was demonstrated to cause severe bronchospasm and pulmonary edema (Billmire et al., 1996). In the Billmire study, a 4-week-old infant was exposed to 5% pepper spray after discharge from a self-defense device. The infant suffered respiratory failure and hypoxemia requiring immediate extracorporeal membrane oxygenation. Inhaled capsaicin causes an immediate increase in airway resistance (Fuller, 1991). This dose-dependent bronchoconstriction after capsaicin inhalation in humans is the same as that demonstrated in asthmatics and smokers (Fuller et al., 1985). The capsaicin-induced bronchoconstriction and release of substance P is attributable to stimulation of nonmyelinated afferent C-fibers.

Neurologic Toxicity

RCAs are irritants to the peripheral nervous system (Anonymous, 1999). CN and CS interact with receptors on sensory nerves in the eyes, other mucous membranes, and skin, resulting in discomfort and burning pain. Their neurologic toxicity can range from paresthesias of the lips to burning pain of the eyes (ophthalmodynia), tongue (glossalgia), nose (rhinodynia), throat (pharyngodynia), and skin (dermatalgia). The reaction of CN with sulfhydryl (SH)-containing proteins and enzymes is the cause of denaturation associated with sensory nerve activity (Chung and Giles, 1972). Because RCAs affect the senses, the feeling can become disorienting after exposure, which may explain why some experience temporary loss of balance and orientation after exposure (Thorburn, 1982).

Agitation and panic may develop in those not previously exposed to CN (Stein and Kirwan, 1964; Beswick, 1983). Syncope has also been reported (Thorburn, 1982; Athanaselis et al., 1990), but this is likely attributed to panic. Headaches have been reported in 48% of symptomatic persons exposed to chloropicrin (Goldman et al., 1987). When CN was released into 44 prisoner cells, eight inmates experienced malaise and lethargy; among those hospitalized, one experienced syncope and a severe systemic illness (Thorburn, 1982).

A clinical case report of hand injuries caused by accidental discharges from tear gas pens (Adams et al., 1966) revealed specific neuronal toxicological findings. In each case, CN penetrated into the skin to cause a wound. Neurological examination indicated hyperesthesia of select digits in all cases. Biopsies of digital neurons performed for pathology showed thickened epineurium and tendon sheaths. Some of the patients reported paresthesias months after exposure. The study suggests a possible link between direct chemical injury and nerve damage. The same investigators exposed the sciatic nerves of rabbits to the agent by discharge of a CN pen or by dusting the exposed nerve with 0.2 g CN powder. These animal studies suggested CN can cause inflammation and necrosis in skeletal muscle,

loss of axon cylinders, and replacement of neural elements with granulation tissue and fibroblasts (Adams et al., 1966). Animals exposed to CR exhibit fasciculations, tremors, convulsions, and ataxia; intraperitoneal administration of CR can also cause muscle weakness (Salem et al., 2006).

Capsaicin activates receptors in trigeminal (cranial nerve V) and intestinal neurons. These include pain receptors located in the mouth, nose, stomach, and mucous membranes. Trigeminal neurons utilize substance P as their primary pain neurotransmitter. Capsaicin first induces the release of substance P from the neuron and then blocks the synthesis and transport of substance P to the effector side (Bernstein et al., 1981; Tominack and Spyker, 1987). Substance P depolarizes neurons to produce dilation of blood vessels, stimulation of smooth muscle, and activation of sensory nerve endings (Tominack and Spyker, 1987). Jancso characterized the effects of capsaicin as an initial intense excitation of sensory neurons followed by a lengthy period of insensitivity to physicochemical stimuli (Jancso et al., 1968, 1987). Substance P is associated with sensory (pain) or skin inflammation afferents. It is also a peripheral mediator of neurogenic inflammation and smooth muscle contraction (Lembeck and Holzer, 1979). It contributes to contraction of the esophagus, trachea, respiratory tract, and levator palpebrae muscles (blepharospasm and apraxia of eyelid opening). Capsaicin directly applied to the eye causes a neurogenic inflammation involving vasodilatation and extravasation of fluid and unresponsiveness to chemical stimuli. Capsaicin renders the skin of humans and animals insensitive to various types of painful chemical stimuli (Bernstein et al., 1981). In humans, OC exposure eventually causes loss of the corneal blink reflex (Olajos and Salem, 2001), which is mediated by sensory input from cranial nerve V and motor output via cranial nerve VII.

Gastrointestinal Toxicity

Many reviews state that gastrointestinal effects do not occur on inhalational exposure to RCAs, with the exception of DM; however, nausea, vomiting, and alterations in taste are commonplace in clinical case reports of exposure to CS (Athanaselis et al., 1990; Solomon et al., 2003) and CN (Thorburn, 1982; Blain, 2003). The involvement of retching and emesis tends to occur if the individual is sensitive, the concentration is sufficiently high, the exposure is prolonged, the range is close, or the event occurs in a confined space. Vomiting was reported in 25% of patients with severe reactions to CN in a confined area (Thorburn, 1982). Emesis did not resolve until the next week in one patient. Inhalation of RCAs often leads to parageusias or altered taste of the tongue. In particular, a metallic or burning sensation is often reported (Folb and Talmud, 1989).

Ingestion of CS can also produce episodes of nausea, vomiting, crampy abdominal pain (Blain, 2003), and diarrhea (Blain, 2003; Solomon, 2003). Seven patients in the Solomon study drank juice contaminated with CS pellets and primarily developed gastrointestinal symptoms. Two of the seven patients reported emesis and diarrhea; all patients reported abdominal pain, epigastric discomfort, and burning gastroesophageal reflux. Symptoms resolved 24 h later. Surprisingly, they did not develop parageusia or burning of the tongue after CS ingestion, which is often the case after inhalational CS exposure. Another study designed for patients to taste an admixture of sugar and CS (5–10 pellets, 500 mg each, dissolved in 10 L of water) indicated that patients experienced a 30 s delay in onset of altered taste (Kemp and Willder, 1972); this was most likely because of a masking effect from the sugar. In animal studies, rabbits and rats develop gastroenteritis on CN or CS exposure by ingestion (Gaskins et al., 1972).

Biting and ingesting *Capsicum* plants can cause nausea and vomiting (Tominack and Spyker, 1987). Nausea has also been reported in individuals exposed to pepperball tactical powder containing capsaicin (Hay et al., 2006). Capsaicin causes effects on gastric mucosa, including mild erythema, edema, epithelial cell damage, and gastric hemorrhage (Desai et al., 1977).

Dermatological Toxicity

CN and CS are primary irritants of the integumentary system able to cause first-degree and second-degree burns (Stein and Kirwan, 1964; Weigand, 1969; Hu et al., 1989). Low concentrations of either agent cause erythema, pruritis, subcutaneous edema, paresthesias, and/or burning sensations in exposed areas of the skin within minutes. Erythema is often the first sign of contact dermatitis, occurring minutes after exposure and subsiding approximately 1 h after exposure. These agents follow a time course of skin damage similar to that of mustard agent. Further, if the skin is wet or abraded, then the toxic effects on the skin are more prominent (Holland and White, 1972; Thorburn, 1982; Sidell, 1997). Exposure to higher doses leads to worsening erythema, edema, vesication with bullae (observed hours later), and fever. The extent of toxic effects also depends on thickness of the stratum corneum and time of exposure. Furthermore, contact with water up to 48 h after exposure can exacerbate the painful symptoms (Pinkus, 1978; Blain, 2003). High humidity, diaphoretic subjects, and warm temperatures can all exacerbate the contact dermatitis from RCAs (Hellreich et al., 1969). Areas of occlusive dress over the skin may also result in worse reactions.

Higher concentrations of CS or longer exposures will result in more than erythema, pruritis, and burning pain. Papulovesicular rashes are not uncommon with high

concentrations of RCAs. Typically, edema and vesiculation (bullae dermatitis) occur 24 h after CS or CN exposure (Sidell, 1997). Common sites of bullae are areas under the cuff of a shirt or glove and just under the collar. One study examined the effect of high CS concentrations (300 mg/m^3) tested on the arms of volunteer study patients for exposure times ranging between 15 and 60 min (Hellreich et al., 1967). All participants experienced burning pain approximately 5 min after exposure onset. A Ct range of 4,440–9,480 mg min/m^3 caused immediate patchy erythema, which subsided after 30 min. A Ct range of 14,040–17,700 mg min/m^3 led to greater dermal toxicity and required several hours to subside. Bullous dermatitis occurred in 50% of subjects as a delayed reaction. These bullous lesions resolved in 2 weeks, but an inflammatory hyperpigmentation of the skin remained by 6 weeks after exposure. Differences in individual sensitivities are attributable to skin pigmentation, complexion, and susceptibility to sunburns (Hellreich et al., 1969).

Exposure to other RCAs causes similar dermal effects. CN is a more potent irritant than CS. In a human study involving dermal application, CN (0.5 mg) powder caused irritation and erythema when on the skin for 60 min (Holland and White, 1972). It took 20 mg CS to cause similar effects for the same duration of exposure. Exposure to 5% capsaicin pepper spray causes immediate and severe erythema and edema in the skin (Herman et al., 1998). Similarly, pepperball pellets fired at individuals will cause erythema, pain, and edema at the site of impact. The initial point of contact may become infected, scar, or heal with hyperpigmentation (Hay et al., 2006).

Dermal exposure to CN or CS may lead to an allergic contact dermatitis (Madden, 1951; Penneys, 1971), a delayed hypersensitivity reaction that develops from a previous exposure to RCAs. CS and CN are both skin sensitizers, but CN is more potent (Chung and Giles, 1972). Initial exposure to either may not cause significant toxic signs or symptoms. Exposure to small amounts of the same agent years later, however, may produce a severe allergic erythematous, patchy rash with edema, bullae, purpura, and necrosis. Sensitization is likely to occur after dermal exposure to high concentrations of RCAs (Holland and White, 1972). Hypersensitivity reactions can persist for up to 4 weeks. This phenomenon has been demonstrated so far by CN (Ingram, 1942; Frazier, 1976) and CS (Ro and Lee, 1991), but not CR.

Dermal exposure to CR causes a burning sensation and erythema several minutes later. Burning pain goes away after 15–30 min, but the erythema lasts up to 2 h (Holland, 1974). CR does not induce inflammatory cell migration to the site of injury, bullous dermatitis, or contact sensitization (Ballantyne, 1977a). Repeated application of CR to the skin (applied 5 days/week for 12 weeks) has little effect (Marrs et al., 1982). Similar to the eye and lungs, CR does not demonstrate significant toxicity to the skin.

Hours after dissemination, CS, CN, or CR can pose a toxic danger because they are persistent in the environment. During the riots of the late 1960s, CS was frequently used to control crowds. Inadvertently, firefighters in those metropolitan areas sometimes were exposed as they entered buildings where CS had been disseminated. The force of water from fire hoses and movement within the buildings reaerosolized enough agent toxic enough to cause erythema and edema around the eyes and other areas of exposed skin (Rengstorff and Mershon, 1969a).

Although capsaicinoids may have a vesicant effect, depending on length of exposure, in most cases it produces a burning sensation and mild erythema. Capsaicins cause erythema and burning pain without vesiculation when applied topically to human skin (Watson et al., 1996; Herman et al., 1998). Skin blistering and rash may occur after chronic or prolonged capsaicin exposures.

Other Toxicity

One report noted renal tubular nephritis in a worker killed after an explosion inside a plant manufacturing CS agent (Cookson and Nottingham, 1969). Hepatocellular injury has been linked to serious CS inhalation (Krapf and Thalmann, 1981). To date, animal studies have not documented any relationship between RCA exposure and teratogenicity (Folb and Talmud, 1989). CS did not demonstrate mutagenic potential with the Ames assay (Rietveld et al., 1983). Similarly, CR did not have carcinogenic effects in mice or hamsters (Blain, 2003); CS lacks mutagenicity in several test systems (Wild et al., 1983).

Lethality

Human deaths have been reported from RCA exposure (Thorburn, 1982). Death is usually the result of excessive concentrations used, confined spaces, and prolonged exposures. Death occurs hours after initial exposure, and postmortem findings are consistent with severe airway damage seen in animals. Deichmann and Gerarde (1969) reported a fatality after exposure to high CN vapor concentrations (5.4 g in a 34 m^3 room) for less than 20 min, which equates to approximately 160 mg/m^3. Estimates of the human LCt50 range between 25,000 and 150,000 mg min/m^3 for CS and between 8,500 and 22,500 mg min/m^3 for CN (Olajos and Salem, 2001). High doses of CR aerosol do not produce lethality in animals; CR aerosols of 68,000 mg min/m^3 are not lethal in mice, guinea pigs, or rabbits. The large safety ratio for CR is clearly evident as compared with the other agents.

Although OC is widely regarded as a safe substance with low toxicity, more research should be conducted in light of recent deaths involving pepper spray use by law enforcement agencies. One case involving an inmate who died in custody implicated pepper spray as a direct

contributor to death (Steffe et al., 1995). Billmire et al. (1996) reported the life-threatening effects in a 4-week-old infant exposed to OC spray as a result of an accidental discharge.

RISK ASSESSMENT

The ideal process in RCA risk assessment is to characterize the effectiveness and risk from exposures to situations in which RCAs may be used (National Academy of Sciences/National Research Council, 1994; TERA, 2001; Patterson et al., 2004). To do that, one must identify all pertinent effects of the RCA in question, develop a dose–response assessment, consider an exposure assessment, and finally characterize the risk. When used as intended, RCAs are thought to be safe and of sufficiently low toxicity. They are designed with the purpose of disabling a targeted individual through sensory irritation of the eyes, respiratory tract, and skin. As discussed previously, they are not without additional unwanted effects, especially in circumstances in which high concentrations are used or exposure is prolonged. The previous sections have provided sufficient discussion regarding the potential toxicity to humans as a result of exposure to RCAs, including case reports.

Identification of Intended and Unintended Effects

By providing a minimal force alternative for controlling and managing individuals, RCAs are a desired public health and safety tool for military, domestic law enforcement, and civilian use. As with any chemical intended to benefit the public, it is important first to identify the compounds, their potential adverse impact (unintended effects), and their beneficial impact (intended effects). There are a number of chemicals designed and used as RCAs. In general, they are compounds with low vapor pressures and dispersed as fine particles or in solution from a variety of devices. These dispersal methods can include the gamut from aerial spray (Figure 11.6) to large spray tanks (see Figures 11.2 and 11.4) and small hand-held devices for self-protection. The modern RCAs used today include CN, CS, CR, OC, and PAVA. Their major adverse effects are summarized in Table 11.2. The intended effect for all RCAs is a change in behavioral response of the target. A better measure of this intended effect would be the actual physiological effects produced by RCAs on the eyes, skin, and respiratory tract (Patterson et al., 2004). These are the target organs designed for irritation by RCAs (see Figure 11.11 for review).

Each physiological effect can be evaluated and categorized on a broad spectral continuum from mild to severe. At lower aerosol dosages, the effects from RCAs will be reversible with no serious injury. For instance, typical mild ophthalmic effects include lacrimation (tearing) and transient burning pain (ophthalmodynia). When used at higher levels, in confined spaces, and/or for prolonged duration, there is a greater potential for the toxicity to escalate. Moderate effects would include conjunctivitis, keratitis, blepharitis, chemosis, and periorbital edema. Severe effects result from significantly prolonged duration or high concentrations leading to irreversible damage in the tissues (i.e., loss of vision). These include corneal abrasions, scarring, or opacification. Very serious effects on the eye include symblepharon, pseudopterygium, cataracts, hyphema, posterior synechia, secondary glaucoma, vitreous hemorrhage, and traumatic optic neuropathy. The same analysis can be applied to effects on the skin, respiratory tracts, and additional organ systems affected for each agent.

Dose Response

Dose–response assessment involves evaluating the dose required to produce a particular effect of interest. Ideally, quantitative data on specific doses and their corresponding responses are desired. In reality, threshold data for a particular target organ or effect in a target organ are often available as a substitute. The ophthalmic threshold levels and toxicity estimate for human responses to CN, CS, and CR are shown in Table 11.2. If empirical dose–response data are available, then a dose–response evaluation for a given RCA might include plotting the percent of individuals responding as a function of dose for each toxicological sign or symptom and target organ of interest. Dose–response curves can then be used in modeling studies to estimate the probabilities of intended and unintended effects for a particular risk assessment scenario (Patterson et al., 2004).

Exposure Assessment

The crux of exposure assessment is creating a scenario for human exposure to a given RCA and identifying the exposure factors. This would involve describing the intended targets, environmental conditions (windy, rainy weather, etc.), crowd size and characteristics, delivery device (tear gas canisters or grenades, powder or aerosol), hazards associated with the delivery system such as blunt trauma, the nature of the agent selected (physicochemical properties, solvents, concentration/dose), and duration of exposure. An exposure assessment might include estimation of the amount of systemic exposure through RCA inhalation, absorption through the skin from dermal contact, or intestinal uptake after ingestion. Availability of quality data for each of the aforementioned exposure factors will estimate exposure

with high confidence and a minimal uncertainty level. Unavailability of data is a major limitation if models are used to estimate exposure.

Characterization of the Risk and Risk Management

Estimating or developing probabilities of toxic effects within a population is at the heart of risk characterization. It integrates dose–response and exposure assessments. It is designed to provide the probability of occurrence for effects induced by a given RCA given a particular exposure scenario. For example, a decision-maker will use risk characterization to estimate the probability of a group of effects occurring as a result of clearing a confined space with CS. The probability can be derived as a function of the number of tear gas grenades used. Unfortunately, there is a dearth of specific federal risk assessment and risk management guidance or mandates on RCAs. Therefore, the potential for risk management or mitigation of concerns is not optimized for the health and benefit of the public good (Hauschild, 2004). This is partly attributable to the fact that the process for assessing risk of toxic chemicals has yet to be standardized among federal programs (Burke et al., 1993). Computer modeling to aid risk assessment without empirical data to feed the model can be an academic exercise. The two in combination can be a powerful predictor for risk assessment of any toxic chemical.

TREATMENT

Exposure to RCAs leads to a generalized stress reaction, causing leukocytosis (Thorburn, 1982), hypokalemia, elevated total protein, increased globulin, and high bicarbonate levels (Beswick et al., 1972). Treatment for RCA toxicity is not often required because the course of intoxication is self-limiting for the most part. Serum toxicological testing is not available to detect RCAs (Sidell, 1997). Clinical signs and symptoms from RCA exposure subside in less than an hour. Initial care involves removing the victim from a potentially crowded area of dispersal immediately to minimize exposure time. It is important to note that these victims may require additional assistance during evacuation because of their reduced vision and disorientation. In circumstances in which the concentration of the agent is substantially elevated or the area of release is confined, increased complications and risks of morbidity may arise in the eyes, skin, airways, and lungs.

Eyes

If the eyes are involved to any degree, then a protective mechanism to close the eyelids will be initiated as a result of conjunctivitis, iritis, or keratitis. Photophobia, blepharospasm, and apraxia of eyelid opening prevent the clinician from evaluating the damage. However, a local anesthetic applied to the eye will help with eye pain and allow for further evaluation of the eye by slit lamp. Contact lenses should be immediately removed and the eyes flushed of any dusting or agglomerated solid particles (see Figure 11.12). Eyes should be irrigated with copious volumes of water or saline for at least 15 min to adequately flush the irritant. Diphoterine has also been used to decontaminate eyes and skin after CS tear gas exposure (Viala et al., 2005). If symptoms or signs of eye toxicity persist, then consultation with an ophthalmologist is critical. Elderly patients should be monitored for evidence of possible acute glaucoma (Yih, 1995).

Skin

Early signs of skin toxicity at the time of clinical presentation will often be contact or allergic dermatitis because blisters form hours later. Removal of clothing should be the first step in decontamination. Placement of contaminated clothes in sealed plastic bags by first responders will prevent secondary contamination as a result of reaerosolized agent (Horton et al., 2005). Early studies of CS indicated that mixing CS with sodium hypochlorite (or household bleach) produced a greater reaction than CS alone in patch testing (Punte et al., 1963). Despite its usefulness as a decontaminant for many chemical agents, hypochlorite should never be used to decontaminate RCAs on skin. Use of water for decontamination of skin may result in an initial worsening of the burning sensation (described previously). A solution of 6% sodium bicarbonate, 3% sodium carbonate, and 1% benzalkonium chloride has been shown to provide immediate relief from CS dermatitis as the alkaline solution hydrolyzes the agent (Weigand, 1969; Sidell, 1997). Consultation with a burn unit should be considered when large areas of skin are involved or when children are affected. Medical treatment for dermatitis may include topical steroids such as triamcinolone acetonide (Hellreich et al., 1967; Sidell, 1997), oral antihistamines for pruritis, and topical antibiotics such as silver sulfadiazine (Hellreich et al., 1967). Systemic antibiotics can be administered for secondary infection. Oozing lesions from bullae dermatitis should be treated with wet dressings and changed daily. Deroofing closed vesicles is controversial (Carvajal and Stewart, 1987). Tetanus prophylaxis should be considered.

Respiratory

Removing an exposed patient from the source of intoxication to fresh air will provide immediate improvement.

Patients should be evaluated for hypoxia with pulse oximetry and arterial blood gases. Pulmonary function tests may be helpful in patients with prolonged pulmonary symptoms and followed-up until symptoms resolve. Chest radiography might be useful if concentration was sufficiently high, exposure was prolonged, or dispersal occurred in a confined space. Pulmonary edema may be delayed for 12–24 h after exposure, suggesting a need for follow-up radiographs (Stein and Kirwan, 1964; Solomon, 2003). Laryngospasm is a serious complication that may require tracheal intubation to secure a patent airway. Bronchospasm may be treated with inhaled beta-2 agonists, steroids (methylprednisolone), and aminophilline (Ballantyne and Swanston, 1978; Folb and Talmud, 1989). Arterial blood gas and pulse oximetry should be continued if patients are symptomatic hours after exposure.

CONCLUDING REMARKS AND FUTURE DIRECTION

The goal of RCAs is to irritate or produce temporary incapacitation. Use of irritants to harass enemies dates back several thousand years. Today, law enforcement agencies and military personnel use RCAs for quelling protestors, controlling crowds, subduing combatants, clearing buildings, training in chemical warfare, and area denial. Individuals use hand-held devices for self-protection against an assailant. RCAs are dispersed as aerosols or sprays, causing irritation of mucous membranes of the eyes, respiratory tract, and skin. Symptoms and signs of toxicity typically subside by 30–60 min.

Several lines of evidence suggest that RCAs are safe if used as they were originally intended. Even though RCAs are considered safe, nonlethal, temporary incapacitating agents, they are not without risk. Some of the adverse clinical effects from RCA exposure reported in the literature have involved indiscriminate use (excessive concentrations), prolonged exposure, and dissemination of compound in a confined space. In summary, these nonlethal agents can pose a serious health hazard in their intended targets. Some RCAs have such a poor safety profile that they have been abandoned long ago (DM and CA). CN and CS have a large body of literature from which to compare and contrast their safety, toxicity, and potency. As the data clearly suggest, CS is a safer compound compared with CN. The latest newcomers to the RCA scene are the inflammatory capsaicinoids. OC and PAVA are highly effective irritants that cause similar symptoms as CN and CS. Capsaicinoids gained considerable attention in the 1990s from police departments and the public at large for safe and effective chemical incapacitation of individuals. These compounds are primarily used as defensive sprays by law enforcement to subdue a combative suspect or by individuals for self-protection. Although OC, PAVA, and related capsaicinoids produce a similar constellation of toxic signs and symptoms, they are not currently used to control crowds at the level of a riot. If OC-containing pepper spray is preferred for riot control, then more research will be required to determine whether it is safe for humans. Finally, risk assessment is a process that can identify gaps in the literature and therefore serves to highlight research needs.

The toxicology of RCAs has been the focus of numerous reviews, publications, and textbooks. RCAs comprise a diverse array of chemical compounds with similar toxicities since their introduction on the battlefield in the early part of the past century. They are all designed with the purpose of disabling a targeted group or individual through sensory irritation of the eyes, respiratory tract, and/or skin. By providing a minimal force alternative for controlling and managing individuals, RCAs are a desired public health and safety tool for military, domestic law enforcement, and personal use.

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CHAPTER

12

Psychotomimetic Agent BZ (3-Quinuclidinyl Benzilate)

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INTRODUCTION

The psychotomimetic effect of anticholinergic drugs has been known since ancient times. The first mentioning of deadly nightshade (*Atropa belladonna*) and its effect comes from a Greek philosopher and naturalist, Theophrastus (331–287 BC), known as the "father of botany." During ancient times into the Middle Ages, atropine and other natural alkaloids were commonly used for religious ceremonies, Sabbaths, or as a tool of poisoners. The beginnings of medical use for these substances date back to the seventeenth century. In the 1950s, a number of anticholinergic drugs, including glycolic acid, were investigated for potential military and industrial use. The origin of 3-quinuclidinyl benzilate (BZ; codenamed Agent BZ) can be attributed to the increasing interest of the US military in exploring chemical warfare agents (CWAs) which were nonlethal but incapacitating. Experience with the use of CWAs in World War II has led to an attempt to synthesize new chemicals for military use, but without lethal effects (Ketchum and Sidell, 1997). The research was focused on contemporary incapacitants influencing behavior but without substantial effects on important vital functions. Incapacitating agents include the esters of glycolic acid (i.e., atropine-like anticholinergic compounds). BZ (also known as *3-quinuclidinyl benzilate, QB, QNB, BZ agent, Buzz agent, Ro 2-3308*, or *EA 2277*) is a prototype of the central nervous system (CNS) depressants; and a prototype of CNS stimulants is LSD-25 (D-lysergic acid diethylamide) or cannabinol. LSD-25 was the first candidate for this group of CWAs. However, its physical and chemical properties, as well as unpredictable behavior after exposure, have led to its exclusion from military research. From many tested compounds, anticholinergic drugs were chosen for further research. The anticholinergics are generally "glycolates" (substituted glycolic and tropic acid esters) of which the representative and best-known member is atropine. Major symptoms of low-level atropinization include dry mouth, dilatation of pupils, and tachycardia. With toxic doses of atropine, central excitation becomes more prominent, leading to restlessness, irritability, disorientation, and hallucinations or delirium. With still larger doses of atropine, stimulation is followed by depression, coma, and medullary paralysis. A number of anticholinergic compounds influencing psychotic stage and higher nervous functions were tested (Table 12.1).

This group of compounds has both peripheral and central properties. It is known from ancient literature that atropine has psychotomimetic effects (extracts from *A. belladonna* were used to induce hallucinations in "wizards"). Central effects increase when the compounds are considered in the following rank: atropine, scopolamine, benactyzine, ditrane, and, finally, BZ and other esters of glycolic acid (Albanus, 1970). BZ was originally studied for the therapy of gastrointestinal diseases. But even in small doses, it produces side effects such as confusion and hallucinations. Therefore, BZ was withdrawn from commercial studies and turned over to the US army as a possible candidate for incapacitating agents (Sidell, 1982). BZ is known to neuropharmacologists as the standard drug for testing the central activity of muscarinic cholinergic receptors. Currently, BZ is used in the research of cholinergic neurons in the peripheral nervous system (PNS) and CNS, non-neuromuscular cells and tissues for identification of muscarinic receptors via several laboratory techniques, mainly radio-ligand binding assays (Soukup et al., 2011; Sherin et al., 2012; Arrighid et al., 2013). BZ has also been implicated in several experimental epilepsy models (Schneider and de Lores Arnaiz, 2013), animal models in Parkinson's disease (Knol et al., 2014), animal behavioral studies on the

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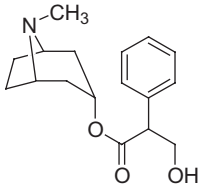
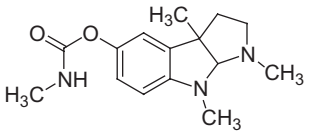
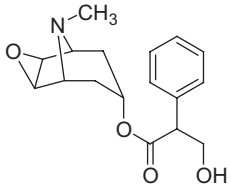
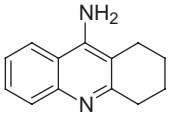
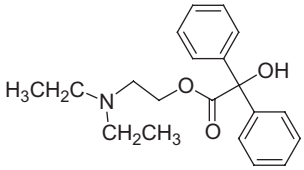
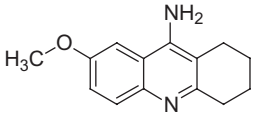
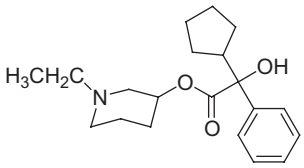
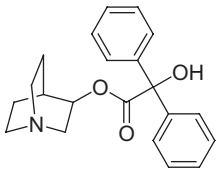
TABLE 12.1 Structural Formulae of Some Anticholinergic Psychotomimetics and Their Antidotes

Compound		Compound	
Atropine		Physostigmine	
Scopolamine		Tacrine	
Benactyzine		7-Methoxytacrine	
Ditrane			
BZ, 3-quinuclidinyl benzilate			

impact on learning and memory (Misík, 2013), neurodegenerative disorders, such as Alzheimer's disease, and other types of dementia (Wyper et al., 1993; Parkasi et al., 2007; Schliebs and Arendt, 2011). In the 1960s, a number of similar compounds, including BZ, were studied in the framework of the military, through industrial liaison programs (Pearson, 2006). These compounds, by politico-military goals and requirements, must contain the following criteria:

- High potency for physiological action (acting in μg/kg or less)
- Rapid onset (min)
- Defined duration (optimally min–h) and reversible effect
- Stability at storage and delivery
- Significant and predictable effect
- Capability of rapid dissemination in defined conditions
- High safety ratio

BACKGROUND

BZ is a white crystalline powder with a bitter taste. It has good solubility in water and organic solvents and is stable at field conditions at a minimum of 1–2 days without loss of its incapacitating activity. BZ is effective by all routes of administration and is metabolized primarily in the liver and excreted by the kidneys. After exposure to an effective dose (ED), mild peripheral effects occur within 1 h and central effects occur after about 4 h. The central effects peak at 8–10 h and last 24–48 h. Physical and chemical properties of BZ are summarized in Table 12.2.

TOXICOKINETICS AND MECHANISM OF ACTION

BZ is absorbed by all the usual routes of administration (oral, parenteral, and inhalation). When administered

TABLE 12.2 Physical and Chemical Properties of 3-Quinuclidinyl Benzilate (BZ, QNB)

		Reference
Appearance	White crystals	
Molecular weight	337.39	
Melting point	190°C (racemic 168°C)	Aleksandrov and Emelyanov (1990)
Boiling point	412°C	Aleksandrov and Emelyanov (1990)
Volatility	c_{max}=0.0005 mg/L (70°C)	Aleksandrov and Emelyanov (1990)
Solubility of hydrochloride	Very good	
Stability in substance	Compound is stable	
Stability in solution	Compound is stable in the water solution	
Persistence in terrain	Air oxidation proof (half-time 3–4 weeks) at 25°C and pH 7	Aleksandrov and Emelyanov (1990)
Method of determination of BZ in field conditions	In Czech army: **a.** indicative tube type PT-51, (producer Oritest Ltd, Prague) **b.** in field laboratory PPCHL-90	
Method of determination of BZ in biological agents in laboratory conditions	Determination: extract spectrophotometry of ion pairsDetection: TLC	Halamek and Kobliha (1993); Skalican et al. (1997)

TABLE 12.3 Pharmacological Activity of the Compounds Tested on Isolated Rat Jejunum

Compound	i.a.	$pD_2 \pm P95$	$pA_2 \pm P95$
Acetylcholine	$\alpha = 1$	6.79 ± 0.06	
Muscarine	$\alpha = 1$	6.59 ± 0.05	
Atropine	$\beta = 0$		8.92 ± 0.05
Benactyzine	$\beta = 0$		7.64 ± 0.07
Scopolamine	$\beta = 0$		8.83 ± 0.06
Ditrane	$\beta = 0$		8.36 ± 0.07
BZ	$\beta = 0$		8.55 ± 0.09

i.a., intrinsic activity; α, intrinsic activity of agonist; β, intrinsic activity of antagonist; pD_2, negative decadic logarithm of agonist ED_{50} ($-\log ED_{50}$ agonist); pA_2, negative decadic logarithm of antagonist ED_{50} ($-\log ED_{50}$ antagonist); P95, 95% confidence limits.

by inhalation, the absorption into the transport system (bloodstream) is more pronounced than with oral administration. Experimental studies were performed with parenteral administration and intravenous (i.v.) administration occur with no loss because of direct involvement of BZ in the transport system. In the bloodstream, the compound is bound to the plasma proteins (particularly albumin), and it is transported to the sites of toxic effect—namely, the CNS and PNS. BZ interferes with cholinergic nerve transmission at the muscarinic sites, both in the peripheral autonomic nervous system and in the brain and spinal cord. Because of the wide distribution of these sites, measurable effects upon almost each phase of neural regulation may be observed. BZ readily crosses the blood–brain barrier (BBB), is distributed to all areas of the brain and spinal cord, and interacts with cholinergic receptors as a competitor with the physiologically active transmitter acetylcholine (ACh). In the PNS, BZ binds to muscarinic ACh receptors in smooth muscles, and like atropine, it has a very high affinity but without intrinsic activity, as shown in Table 12.3 (Fusek et al., 1971).

At the CNS, BZ binds to all subtypes of muscarinic receptors, each of them with different functions in the brain (Lefkowitz, 2004). The particularly long duration of the central action of BZ may be related to its greater affinity for nervous tissue, especially the strong adsorption effect on mitochondria, the subcellular organelles concerned principally with the energy supply to the cell. BZ has been found to reduce the oxygen consumption of nerve cells stimulated in various ways (Jovic and Zupanc, 1973).

TOXICITY

The acute toxicity of BZ (expressed as LD_{50}) in different species following different routes of administration is shown in Table 12.4. It is clear that acute toxicity is relatively low. However, the dose that causes incapacitation is much lower. Therefore, the ratio between the lethal and incapacitant dose levels is in the range of the logarithmic scale.

The EDs of some psychotomimetic drugs for incapacitation in humans were assessed, as presented in Table 12.5. The dose required to produce incapacitation is roughly 40 times lower than the lethal dose. For humans, the ED_{50} for incapacitation by BZ under field conditions is about 60 mg min/m^3 with a body weight of 75 kg, volume of respiration of 15 L/min, and LD_{50} value about 200 μg/kg, p.o. administration.

Symptoms

BZ is active when it is given by the i.v., intramuscular, inhalation, or oral route. The fragmentary data available indicate that higher doses caused the greatest effects and longest durations. From 1960 to 1969, field tests were conducted with BZ by the US Army.

TABLE 12.4 Lethal Doses of 3-Quinuclidinyl Benzilate and Other Anticholinergic Drugs for Different Species

Animal	Route of Administration	LD_{50} (mg/kg) 3-Quinuclidinyl Benzilate (BZ)	Atropine	Scopolamine
Mouse	i.v.	22	74	163
	i.p.	110	256	119
	i.m.	42		
	p.o.	460	693	
Rat	i.v.	20	41	
	i.p.		256	
	i.m.	281		
	p.o.		733	1,270
Guinea pig	i.v.	14	163	
	i.p.		277	
	p.o.		1,100	
Rabbit	i.v.	10		
	i.m.		588	
Cat	i.v.	12		
Dog	i.v.	12		
Pig	i.v.	5		

TABLE 12.5 The EDs of Some Psychotomimetic Drugs for Incapacitation in Man

Drug	Route of Administration	ED (mg/kg)	Reference
LSD-25	i.m.	0.0005–0.001	Hoffman (1960)
	p.o.	0.001	Hollister (1968)
BZ	i.m.	0.006	Sidell et al. (1973)
	i.m.	0.01	Spivak and Milstejn (1973)
	i.v.	0.005	Spivak and Milstejn (1973)
Scopolamine	i.m.	0.024	Ketchum et al. (1973)
Atropine	i.m.	0.175	Ketchum et al. (1973)
Ditrane	i.m.	0.1–0.3	Gershon and Olariu (1960)
	i.m.	0.15	Ketchum et al. (1973)

Sidell's (1982) description of the effects induced by BZ (4.5–17.1 μg/kg) is as follows.

At low doses, the effects include dry mouth, decreased gastric motility, inhibition of sweating, increase in heart rate, papillary dilatation and loss of accommodation, mild sedation, and mental slowing. At high doses these effects are severe. There are marked disturbances of function at all levels of the CNS, motor coordination, attentiveness, and control of thought and the learning process all decline. Confusion, restlessness, impairment of perception and interpretation, and memory span are observed. The first symptoms occur depending on the route of administration, judgment and deficient insight are all features of this syndrome. True hallucinations are present and if the dose is quite high the subject may become stuporous or even comatose for several hours.

After a single injection of BZ (5.0–6.4 μg/kg, i.m.), the following symptoms are observed at various time intervals:

- 10 min: lightheadedness and giggling
- 30 min: dry mouth, blurred vision, nausea, chilly sensations, and twitching
- 1 h: flushed skin, lack of coordination, fatigue, unsteadiness, sleepiness, and quivering legs
- 2 h: many of the above symptoms, plus poor concentration, restlessness or sleepiness, hallucinations, slurred speech, and muscle fasciculation
- 3 h: the above symptoms, plus tremors
- 4 h: the above symptoms, plus difficulty in handling the subject and an increased pulse rate to 130
- 8 h: the above symptoms, plus delirium and hallucinations
- 24 h: persistent delirium, hallucinations, restlessness, unsteadiness, and increased pulse rate in some (but not all) subjects
- 48 h: persistent impairment of functions

Mydriasis and disturbed accommodation remain unchanged (depending on the dose) after 2 or 3 days. BZ has a mean duration of incapacitation of about 70 h. In real-life situations, wide variations in dosage would occur, so the results will also vary over the large time interval.

RISK ASSESSMENT

BZ was stockpiled by US military forces in 1980. However, in 1992, a US delegation to the Conference on Disarmament in Geneva declared that their stocks of BZ were destroyed. It is not known if stocks of BZ are held by any other armed forces. The military use of BZ was limited to special operations, and at present, Agent BZ can be considered a potential agent dedicated for special military operations. There exist a group of other incapacitants (not anticholinergic hallucinogens) known as nonlethal chemicals with very strong effects

(e.g., fentanyl derivatives, as demonstrated in Moscow in 2002). Use of these incapacitants by terrorists and other small, nonstate groups cannot be excluded either. Today, BZ is considered one of a number of nonlethal incapacitating chemical weapons. These compounds are designed and primarily employed to incapacitate personnel or material while minimizing fatalities, permanent injury to personnel, and undesired damage to property and the environment (Pearson, 2006).

TREATMENT

Based on the knowledge of its mechanism of action (simply described as a lack of neurotransmitter ACh), the therapeutic principle of BZ intoxication is to increase the ACh level by inhibitors of cholinesterases. However, their use is limited to their ratio between therapeutic and toxic dose levels, and therefore, reversible inhibitors are preferred, although some experiments with interaction of highly toxic nerve agents (VX and sarin) and BZ on healthy volunteers were described (Sidell et al., 1973). Physostigmine was the first antidote proposed for BZ intoxication. Its antidotal effect against intoxication with extract from *A. belladonna* has been known for more than 150 years. It is known that physostigmine acts as a good antidote for scopolamine intoxication, as it is able to treat all kinds of symptoms, such as electrophysiological, psychiatric, and biochemical changes caused by this anticholinergic drug. Physostigmine was considered one of the potential antidotes against anticholinergics, including esters of glycolic acid. Physostigmine is an antidote against BZ intoxication. An injection of 2–3 mg i.m. is required to alleviate symptoms. Repeated injections at intervals of approximately 15 min to 1 h may be required to build sufficient levels. Once a desirable effect is achieved, it should be maintained by slow intravenous injection or infusion. Doses of 2–4 mg every 1–2 h may be required. Oral dosing (2–5 mg every 1–2 h) should replace i.v. therapy as soon as possible. Peripherally acting anticholinesterase drugs that do not cross the BBB (e.g., pyridostigmine, neostigmine, and pilocarpine) are ineffective antagonists of the central effect of BZ (Greaves, 2002). The side effects of physostigmine (such as bradycardia, arrhythmia, excessive secretion, and convulsion) and its short duration of action, led to use of new inhibitors having lower toxicity in comparison with physostigmine. Acridine derivatives are of great interest (Albert, 1966). From these compounds, 1,2,3,4-tetrahydro-9-aminoacridine (tacrine) was found to be very effective (Fusek et al., 1974). It was found to be an antagonist against morphine and curare overdoses. It is an inhibitor of cholinesterases comparable to neostigmine or physostigmine, and it also inhibits antihistaminic activity. Its effect is more prolonged than that of physostigmine. During the studies of antidotes against anticholinergic hallucinogens, it was recognized as the most promising antidote at the time (Gershon and Angrist, 1973). The antidotal effect of tacrine against ditrane intoxication, including improvement of the electroencephalogram (EEG) changes, was approved by Itil and Fink (1966) in 74 patients. Atropine, scopolamine, and ditrane intoxications were successfully treated using physostigmine and tacrine, as well as low amounts of sarin (Ketchum et al., 1973). However, the toxicity of tacrine was the reason for its limited use (Table 12.6). Moreover, it has other limitations, such as the fact that it caused temporary changes in hepatic function tests. Therefore, it was abandoned (Marx, 1987) (Figure 12.1).

TABLE 12.6 Lethal Doses of Tacrine, 7-Methoxytacrine and Physostigmine in Laboratory Animals

Compound	Animal	Route of Administration	LD_{50} (mg/kg)
Tacrine	Mouse	i.m.	28.9 (24.2–35.8)
	Rat	i.m.	33.8 (28.8–40.6)
	Rat	p.o.	103.7 (84.6–135.8)
	Rat	i.p.	20.2 (16.4–24.8)
	Rat	i.v.	12.0 (10.7–13.6)
	Rabbit	i.n.	13.3 (8.0–18.8)
	Dog	i.m.	12.6 (10.1–14.7)
7-Methoxytacrine	Mouse	i.m.	125 (110–143)
	Rat	i.m.	258 (224–313)
	Rat	p.o.	793 (662–950)
	Rat	i.p.	73.3 (61–90)
	Rat	i.v.	22.2 (19.9–25.2)
	Rabbit	i.m.	75.3 (60–89)
	Dog	i.m.	18.9 (15.2–22.1)
Physostigmine	Mouse	i.m.	0.86 (0.7–1.0)
	Rat	i.m.	2.2 (1.9–2.4)
	Dog	i.m.	0.83 (0.68–0.95)

The 7-methoxy derivative of tacrine (7-methoxytacrine, or 7-MEOTA) was synthesized and tested (Fusek et al., 1986). 7-MEOTA inhibited more butyrylcholinesterase (BuChE) ($I_{50} = 3.5.10^{-7}$ M) than acetylcholinesterase (AChE) ($I_{50} = 3.5.10^{-6}$ M) *in vitro*. The inhibition is a competitive–noncompetitive reversible type. The characteristics of AChE inhibition indicate that 7-MEOTA binds to the active surface of AChE in the gamma-anionic site, like galantamine or coumarine. Inhibition of blood AChE by *O*-ethyl-*S*-(2-dimethylaminoethyl) methylphosphonothioate (0.042 mg/kg = 1.5 × LD_{50}, i.m.) after premedication with 7-MEOTA (100 mg/kg, i.m.) was substantially smaller than in intoxicated animals pretreated with

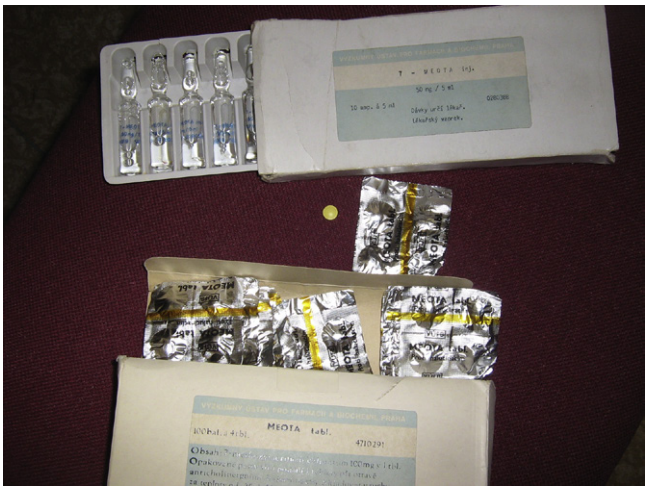


FIGURE 12.1 Antidotes (7-MEOTA) for BZ intoxication; they can be administered by tablets (100 mg) or injections (50 mg/5 mL).

saline, which highlights its possible use for preventing intoxication by organophosphorus anticholinesterase agents. 7-MEOTA enhanced the contraction response of the guinea pig atria (ED_{50} positive inotropic effect = 1.7×10^{-6} M), reduced the frequency of contractions (by binding the compound to the effectors of the cholinergic system of the heart tissue), increased the contraction response of the isolated rat diaphragm, and antagonized the effect of d-tubocurarine. The dose of 3×10^{-6} M of 7-MEOTA entirely suppressed the effect of a dose of 2×10^{-6} M d-tubocurarine. 7-MEOTA elicited a contraction response (1×10^{-7} M) and intensified the response of the isolated rat jejunum to the applied concentration of cholinomimetics. The long-term increase of intestinal peristalsis after low concentrations of 7-MEOTA (1×10^{-7} M) bears witness to the inhibitory effect of the compound on tissue cholinesterases. In a dose-dependent manner, 7-MEOTA effectively antagonized symptoms of intoxication elicited by anticholinergics in dogs. A dose of 5 mg/kg, i.m. of 7-MEOTA shortened the time needed for a fall in the score of the intensity of symptoms elicited by BZ (0.05 mg/kg, i.m.) from 210 min in untreated intoxication to 54 min, respectively (Fusek et al., 1979).

The authors' results show that contributing to the effect of 7-MEOTA (besides the direct influence on effectors of the cholinergic system) can also be mediated through induced cholinesterase inhibition in the CNS and PNS. The more beneficial properties of 7-MEOTA in comparison with physostigmine suggests the possible therapeutic use of this compound in cases where physostigmine was applied previously. In contrast to physostigmine, the application of 7-MEOTA does not require repeated doses for maintaining the therapeutic effect at low dosages and has minimal side effects.

7-MEOTA is a potent, centrally active cholinesterase inhibitor. Therefore, this new drug has applications not only as an antidote against BZ intoxication, but also for the treatment of cholinergic deficit neurological disorders, such as Alzheimer's disease. In isolated rat jejunum, 7-MEOTA increased muscle contractility, and in isolated guinea pig ventricular myocytes, this compound was found to prolong transmembrane action potential and decrease the amplitude of the plateau. In isolated rat diaphragm, 7-MEOTA increased muscle contractility after electric stimulation of the phrenic nerve.

7-MEOTA was found to antagonize convulsive action of pentamethylenetetrazole and significantly decreased the number of surviving animals following administration of this drug. Results from behavioral studies indicated that 7-MEOTA antagonized the anticholinergic syndrome evoked by scopolamine, ditrane, and BZ.

The antidotal effect of 7-MEOTA on the anticholinergic syndrome was potentiated by nootropics, by diazepam, and especially by opioid peptides (Fusek, 1977). 7-MEOTA also antagonized the side effects of tricyclic antidepressants and protected AChE against inhibition by some organophosphate anticholinesterase compounds. Acute toxicity of 7-MEOTA was low: LD_{50} (i.m.) was 125 mg/kg in mice and 258 mg/kg in rats. The oral LD_{50} in rats was found to be 793 mg/kg. Analogous values for THA were 29, 34, and 104 mg/kg (Fusek, 1977). Also, 7-MEOTA was found to be markedly less toxic than THA in dogs and rabbits. A subacute toxicity study of 7-MEOTA (with 3 months of administration) demonstrated that the compound was well tolerated at doses of 25 mg/kg (i.m.) and 50 mg/kg (p.o.) in rats and 2.5 mg/kg (i.m. and i.v.) in beagles. No pathological changes were observed in biochemical, hematological, and morphological investigations.

ANALYTICAL METHODS

The fluorometric method for 7-MEOTA determination in biological material was developed, and blood concentration profiles of 7-MEOTA in rats and healthy volunteers were estimated (Filip et al., 1991). Maximal concentrations of 7-MEOTA in human blood were observed approximately 0.5–1 h after i.m. and 4 h after p.o. application of the drug. The half-life was 5 h, and an effective level was maintained for 12 h. Similar results in rats with radiolabeled [H3]7-MEOTA were obtained. The main part of 7-MEOTA was eliminated by both urine and feces within 6 h after administration. The majority of the compound was eliminated in an unchanged form, and the remaining portion was metabolized. Among the metabolites, 7-MEOTA and its conjugate with glucuronic acid were demonstrated; further metabolites were likely compounds with the OH group in position 1 or 2 (Patocka et al., 1996).

In healthy volunteers, 7-MEOTA was well tolerated at a single dose of 2 mg/kg (p.o.) or 1 mg/kg (i.m.) following

daily administration (for 7 days). The compound did not influence the cognitive functions of healthy persons. The blood concentration profiles of volunteers corresponded well with those in rats.

On the basis of the results obtained, it was decided that 7-MEOTA would be studied in more detail, including preclinical testing. The first and second phases of clinical testing on healthy volunteers were performed. It was found that 7-MEOTA is well tolerated after oral or intramuscular administration (2 mg/kg, p.o. and 1 mg/kg, i.m.) (Filip et al., 1991). Clinical testing of this drug was performed on patients with tardive dyskinesias occurring after lengthy administration of neuroleptics with good therapeutic efficacy (Zapletalek et al., 1989). As a result, it was introduced in the form of tablets (7-MEOTA, 100 mg) and injections (50 mg in 2 mL) to the Czech army as an antidote against psychotomimetic agents.

CONCLUDING REMARKS AND FUTURE DIRECTION

BZ was recognized as an incapacitant agent for military use. It was stockpiled and stored as a CWA by the US army, but their stocks were destroyed in 1992. The use of this agent is not excluded. Its mechanism of action is based on the interaction with cholinergic receptors. The symptomatology is characterized by peripheral action (vegetative symptoms) and influence on the CNS, including hallucinations. The antidotes and effective antidotal therapy are of vital interest and are possible using commonly available physostigmine or 7-MEOTA, respectively. The use of BZ in the research of the cholinergic nervous system cannot be neglected.

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CHAPTER

13

Neurological Effects and Mechanisms of Blast Overpressure Injury

Dorothy L. Dobbins and Xiaoping Pan

INTRODUCTION

Blast-induced neurotrauma is one of the most serious health concerns of soldiers returning from war. Blast-induced traumatic brain injuries (bTBIs) are considered the signature wound of the war in Iraq (Cernak and Noble-Haeusslein, 2010; Hicks et al., 2010; Balakathiresan et al., 2012; Tate et al., 2013). Approximately 20% of returning veterans sustain a traumatic brain injury (TBI) (Brenner et al., 2010; James et al., 2014) and 50% of all injuries involve explosive devices (Cernak et al., 2001). Generally, bTBIs are induced through exposure to oscillating overpressure airwaves generated by blasts from explosives. These pressured waves propagate and transfer kinetic energy to individuals, causing a variety of damages. Injuries sustained from blast exposures are graded based on the size and proximity to the blast (Cernak and Noble-Haeusslein, 2010; Hicks et al., 2010). Typically the initial neural tissue damage is short-lived and quickly overcome (McCrea et al., 2009; Balakathiresan et al., 2012). Unfortunately, in warfare, the possibility of repetitive exposure to blast overpressure is essentially certain. Repetitive bTBIs are problematic given their more complicated nature and the ability to cause extensive and prolonged damage in the brain (Comper et al., 2005; Hicks et al., 2010; Balakathiresan et al., 2012; Mouzon et al., 2014; Stein et al., 2014).

The bTBIs generally manifest through a sequence of events. Effects from the blast itself cause shearing of brain tissues, stretching and rupturing of axons, diffuse axonal injury, neuronal cell death, and neurodegeneration. Neuroinflammatory responses are generated soon after blast exposure (Saljo et al., 2000; Cernak et al., 2001, 2004; Comper et al., 2005; Hoge et al., 2008; Blyth and Bazarian, 2010; Brenner et al., 2010; Hicks et al., 2010; Balakathiresan et al., 2012; Cornelius et al., 2013). The cascade of biochemical changes, which occur after bTBIs, can serve as a mechanism for repairing tissue damage and neuron loss from inflammatory responses. These biochemical responses cause changes to neuronal circuitry and the density of neuronal populations through increased neurogenesis and axonal sprouting (Cernak et al., 2004; Blyth and Bazarian, 2010; Hu et al., 2012; Wang et al., 2012; Cornelius et al., 2013).

Cellular and molecular changes that result from bTBIs induce a sequela of behavioral and cognitive deficits as well. Individuals present with symptomology similar to that of posttraumatic stress disorder (PTSD) and exhibit changes in mood and sleep patterns (Cernak et al., 2001; Comper et al., 2005; Hoge et al., 2008; Blyth and Bazarian, 2010; Brenner et al., 2010; Hicks et al., 2010; Cornelius et al., 2013). These mental status changes may persist for a long time after the blast overpressure exposure. It is likely that the changes to the overall connectivity of the brain could account for the prolonged effects of bTBIs given the changes in neuronal populations after bTBI (Blyth and Bazarian, 2010; Hu et al., 2012; Cornelius et al., 2013).

Because of the transient nature of primary injuries, diagnosing bTBIs is difficult, especially in mild bTBIs, which lack visible characteristics for imaging. The damage to brain tissue is often short-lived and the delay in the generation of secondary effects allows many bTBI cases to remain undiagnosed (Comper et al., 2005; Ruff et al., 2009; Sivanandam and Thakur, 2012). Research focusing on advancing diagnostic approaches is active, especially in the fields of advanced imaging techniques and development of relevant clinical biomarkers. In addition to body fluid–based protein markers, further investigations have focused on identifying circulating

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micro RNAs (miRNAs) as biomarkers and assessing its diagnostic value in clinical settings. Additionally, more research is needed to further investigate the mechanisms by which secondary injury occurs after bTBI. A greater understanding of bTBI pathological mechanism will enhance the development of intervention strategies to treat cognitive and behavioral deficits.

BLAST WAVES AND INJURY PHYSICS

The use of explosive devices in warfare has escalated within the past few decades. Physics of blast waves are well understood. Generally, a detonation from an explosive device transfers chemical energy to surrounding molecules. Medium receiving the transferred energy, generally air or water, will display a surge in density, temperature, and pressure. The changes lead to collisions of molecules and cause them to move at a speed that exceeds the speed of ambient pressure waves. The need to alleviate disturbances and compression cannot be achieved normally and the medium will form a transient blast wave to disperse molecules and restore the previous ambient state (Stuhmiller and Richmond, 1991). As a result, blast waves will spherically radiate outward at supersonic speeds from the detonation. Blast waves have a peak overpressure front that is followed by a negative pressure wave caused by the overexpansion of gases as ambient pressure is surpassed. Eventually, the energy dissipates and previous normal pressures are achieved. This description represents an ideal open field blast wave, which is highly predictable through the Friedlander equation. Unfortunately, contact with solid surfaces causes pressure waves to be reflected and rebounded with amplified force. When blast waves reflect off of solid surfaces, air molecules are compressed even further, allowing for incident wave fronts to propagate in different directions and move at various temperatures and pressures. This type of blast wave propagation is more typical to warfare explosions and is known as complex blast waves. Additionally, in warfare, crudely constructed explosive devices can issue multiple blast waves from a single device. These can impact initial waves, complicating blast wave propagation and its effects (Stuhmiller and Richmond, 1991; Cernak and Noble-Haeusslein, 2010; Hicks et al., 2010; Bass et al., 2012; Magnuson et al., 2012).

Exposure to blast overpressure waves can cause extensive damage to an individual. There are a variety of mechanisms through which injuries can be sustained. The transfer of kinetic energy from blast overpressure exposure typically generates primary injuries, such as the shearing of tissues. Shrapnel and other debris can impact an individual and cause secondary injury. Tertiary injuries are sustained from impact with surrounding objects and quaternary injuries are those sustained from intensive thermal changes caused by the primary explosion as well as any other injuries not previously described (Cernak and Noble-Haeusslein, 2010; Hicks et al., 2010; Balakathiresan et al., 2012; Bass et al., 2012; Magnuson et al., 2012; Nakagawa et al., 2011; Tate et al., 2013).

The primary effects of blast waves can often be overlooked and are under-represented when examining effects of overpressure exposure (Kocsis and Tessler, 2009). The means by which primary injuries impact the brain are summarized in Figure 13.1. The primary blast wave transfers kinetic energy to an individual. The transfer of energy primarily affects pressure-sensitive organs of the respiratory and circulatory systems, thorax, abdomen, and middle ear (Stuhmiller and Richmond, 1991; Kocsis and Tessler, 2009; Cernak and Noble-Haeusslein, 2010; Magnuson et al., 2012). This energy transfer causes substantial damage to the body, generally through shearing of tissues. Once kinetic energy has transferred to an individual, it can travel to the brain through vasculature and up through the foramen magnum via cerebrospinal fluid (Hicks et al., 2010). The transfer of kinetic energy can also be transcranial, impacting the brain with blunt force. Once inside an individual, blast waves can continue to rebound off of dense tissues such as bone. This makes the brain particularly vulnerable to impairment. The initial force from changes in pressure can cause a rotational movement of the brain on the brain stem, damaging medial temporal lobe structures, areas vital to learning and memory. Additionally, the pressure wave continues to rebound off the skull and further impacts the brain (Balakathiresan et al., 2012). It is important to note that, in a war situation, soldiers are often geared with heavy protective equipment, including helmets and body armor. Cernak and Noble-Haeusslein (2010) have stated that the protective equipment can provide an enhanced point of contact for the transfer of kinetic energy and can trap additional energy, allowing it to rebound further onto an individual.

DEFINING AND DIAGNOSING bTBI

Neurotrauma caused by overpressure exposure to the brain is described as bTBI. bTBIs vary in nature and are dependent on the force of the blast and proximity of the individual when exposed. Defining and diagnosing bTBIs present much difficulty. It is well-established that such injuries have both transient primary effects and secondary effects that can manifest some time after exposure. Generally, secondary effects of bTBIs occur approximately 3 months after injury and can persist for years (Busch and Alpern, 1998; Hoge et al., 2008; Blyth and Bazarian, 2010; Mouzon et al., 2014). Conventional imaging techniques such as computed tomography and

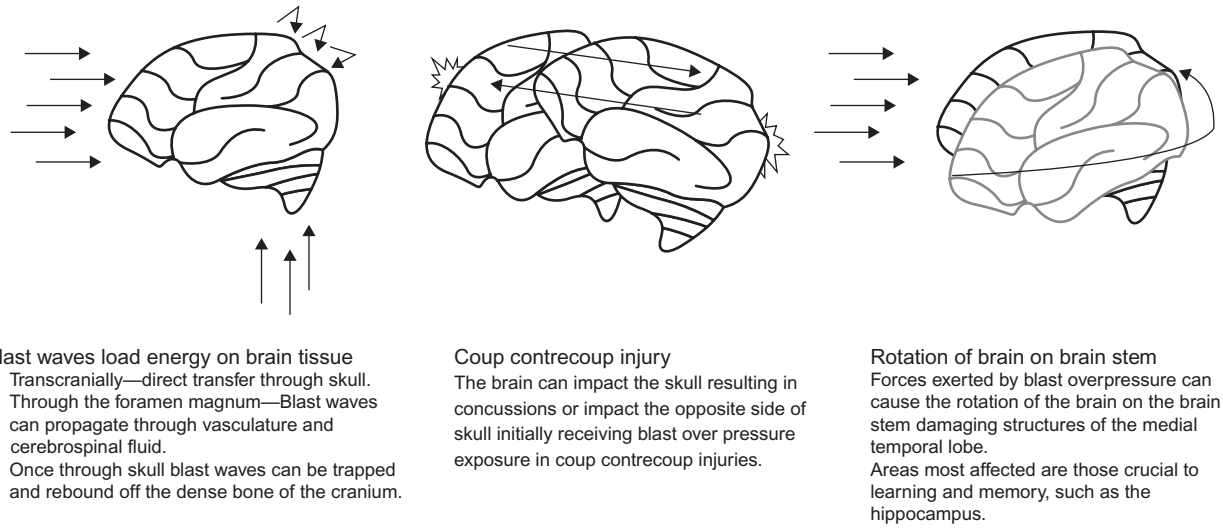

FIGURE 13.1 Loading and mechanical effects of blast over exposure on the brain. Blast overpressure waves issued from the detonation of explosive devices impact the brain in a variety of ways. Overpressure energy can load onto the brain transcranially or via the foramen magnum as it travels through cerebrospinal fluid or blood vessels. Once trapped within the skull, blast waves can further rebound off of the skull and impact the brain multiple times. Additionally, the sheer force of blast waves can cause the brain to impact the skull, resulting in a concussion. When such impacts occur on the opposite side from where the initial force was received, coup contrecoup injuries occur. Finally, torsional strain from blast waves can cause the rotational movement of the brain on the brain stem, damaging medial temporal lobe structures crucial to learning and memory.

magnetic resonance imaging are routinely used for TBI diagnostics; however, it is difficult to detect subtle or no observable pathological changes associated with mild TBI (Redell et al., 2010; Balakathiresan et al., 2012). Unfortunately, the majority of TBIs (approximately 70%) are classified as mild (mTBI) and it is estimated that three-quarters of mTBIs are undiagnosed (Busch and Alpern, 1998; Comper et al., 2005; Snell et al., 2009; Blyth and Bazarian, 2010). The World Health Organization identifies mTBIs as nonpenetrating damage to the brain caused by mechanical energy or physical force which results in at least one of the following: a loss of consciousness lasting less than 30 min; postconcussive amnesia lasting less than 24 h; confusion; disorientation and any other neurological abnormalities; or lesions that do not require surgery. This criterion is combined with a Glasgow Coma Scale score of 13–15 (Comper et al., 2005; Rao et al., 2009; Ruff et al., 2009). Mild TBIs often result in transient or little to no gross deformation to brain structures; therefore, they often go undiagnosed. In a war setting, soldiers without life-threatening injuries are often dismissed or are examined less. This is especially problematic with mTBIs, given that the focal injuries are transient and behavioral and cognitive symptomology does not occur until much later (Kobeissy et al., 2013). In a military setting, repeated exposure to blast overpressure waves is common as soldiers are quickly returned to duty because of the lack of obvious identifiable injury. The prognosis for an mTBI is generally good; symptomology and full recovery should occur within months after exposure (McCrea et al., 2009; Kobeissy et al., 2013; Wortzel et al., 2013). However, repeated exposure complicates bTBIs and causes long-lived and detrimental effects (McCrea et al., 2009; Balakathiresan et al., 2012; Wortzel et al., 2013; James et al., 2014; Mouzon et al., 2014).

Currently, mild bTBI diagnostics generally occur much later after blast exposure and are heavily reliant on recalled events from patients and those who witnessed the initial injury (Blyth and Bazarian, 2010). Individuals suspected of sustaining a bTBI will be interviewed about loss of consciousness and how the injury occurred. However, the postconcussive amnesia and loss of consciousness directly after injury make such diagnostic approaches unreliable (Ruff et al., 2009). Further, subsequent reports of headache, sleep disruptions, mental status changes, and other symptoms mimic other neurological disorders, which make diagnostics even more difficult. The cognitive and behavioral changes after bTBIs can manifest months after the initial injury, propagating this problem.

EXPERIMENTAL ANIMAL MODELS OF bTBI

A multitude of bTBI research has generated the current knowledge about such injuries. Generally, bTBI mechanisms are studied in animal models. There are many different methods for generating animal models

for investigating various aspects of bTBIs. Initially, open field exposure was used, which mimics the effects of a simple blast wave described. Animals were placed at various proximities to explosive devices to determine the lethality of blast exposure and generate dose–response curves. Some studies currently make use of this model when examining mild bTBIs (Rubovitch et al., 2011; Risling and Davidsson, 2012).

Cernak (2005) and Xiong et al. (2013) have examined the variety of TBI models currently used. Classic direct methods include fluid percussion injuries (FPIs) and the controlled cortical impact (CCI) in which craniotomies are performed, and a force is applied to the intact Dura. FPI uses a cylindrical reservoir containing saline. One end of the cylinder is attached to a cap adhered to the skull of the subject and the other end contains a pendulum. When the pendulum is released, a surge of pressure rushes down and impacts the skull. This model produces more severe injuries, such as swelling and intracranial hemorrhaging. The injury is also variable depending on the craniotomy. When pressure is applied between bregma and lambda, centrally, resulting injuries are more severe. For the majority of studies, a lateral approach is favored. Unfortunately, FPIs are sometimes difficult to replicate and the craniotomy methods vary and influence results. Recently, advances have been made to modify this model to increase reproducibility. Similar to FPI, CCI delivers force to the skull through the use of a piston, which is driven by the compressed air. This model is generally used in rodents and produces a more controlled and reproducible injury. Various mechanical factors such as the degree of impact and the depth of disturbance to the brain can be regulated. This model can be used to mimic a milder TBI and has been used in the examination of cognitive deficits as well as genetic and molecular mechanisms.

Other models have been developed to observe the brain injury and its progression *in vivo*. Roth et al. (2014) have demonstrated the benefits of skull thinning and compression injuries. The skull was shaved to a thickness of less than 50 μm and then compressed with surgical tools until it concaved slightly. This technique does not directly mimic blast injuries. However, it allows for a better understanding of the immediate response of brain tissue to damage because the brain can be observed through the thinned skull with a laser-scanning microscope.

There is a variety of weight-drop methods that are more popular. Feeney's model uses a craniotomy and allows for a metallic weight to be dropped on the exposed skull. This model does not directly mimic human injuries and causes focal damage, resulting in hemorrhaging and other tissue damages. Weight-drop models without the use of craniotomies are more common and are typically referred to as Marmarou's model or impact acceleration models. This method is much more controlled and better mimics blast injuries. Graded injuries result in response to the force impacting the brain, as is seen in bTBIs. This model uses a Plexiglas tube to drop the weight from a fixed height to the subject's head. The animal's head rests on a thick piece of foam, which cushions but does not impede the impact. Intense neuroinflammation and diffuse axonal injuries are observed with this model, making it a better representative of human bTBIs (Cernak, 2005; Xiong et al., 2013). Despites many advantages, animal models generated by direct impact methods are less preferable when studying complicated systemic responses as modeled by the open field exposure and the blast simulation techniques.

Finally, recent advancements have given rise to the development blast or shock tubes. These models place animals in a chamber some distance away from an explosive device. However, the explosion can be difficult to replicate and injuries may vary depending on the explosives used and impact angle at which blast waves hit the subject. A similar method uses compressed air instead of explosives. In this case, a diaphragm separates two sections, a compression chamber and a main section. The compression chamber is loaded with compressed air until the diaphragm ruptures, which projects an overpressure wave down the length of the main section. Subjects are usually placed at the end of the main section, where they can be impacted by the overpressure wave. This method produces a blast pressure profile that mimics the open field explosion. The injury in subjects is reproducible and directly mimics effects seen by primary blast injuries. Unfortunately, this method does not allow for the complex blast waves seen in warfare but does closely represent bTBIs exhibited by humans. Rodents are primarily used in these models (Cernak, 2005; Cernak et al., 2011; Risling and Davidsson, 2012; Xiong et al., 2013). The indirect methods of inducing bTBIs seem to be the most similar to human injuries and are the most preferable models currently available.

The selection of an appropriate model is crucial when studying bTBIs. There are many more models of TBIs that have been used. The ones detailed here are methods commonly used when investigating bTBIs. Although each of the methods has its benefits and limitations, all methods further the development of diagnostics, therapeutics, and mechanisms for understandings bTBIs.

NEUROLOGICAL EFFECTS

The sequence of neurological effects after blast exposure is outlined in Figure 13.2. Primary neurotruama after blast exposure presents as edema, embolisms, hematomas, and increased permeability of the blood–brain barrier (BBB). These are accompanied with shearing of

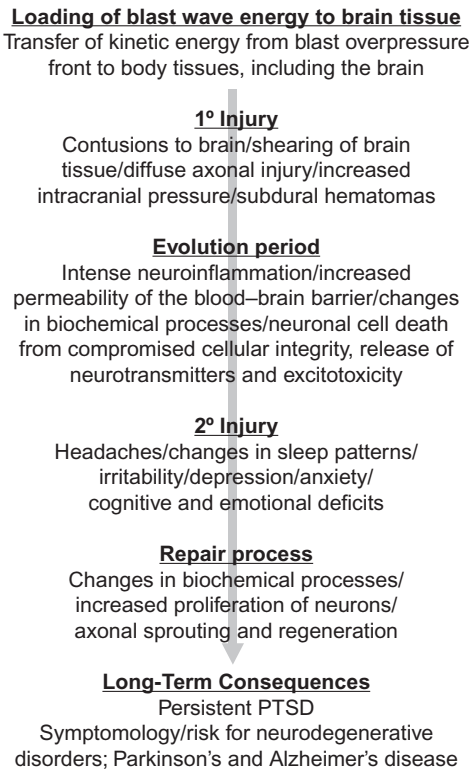

FIGURE 13.2 Progression of bTBI. The sequela of events after blast overpressure exposure. Blast-induced traumatic brain injuries follow a sequence of events. Initial tissue damage from the force of blast waves lead to an evolutionary period of changes in biochemical processes in the brain. These changes result in neurodegeneration and neurological dysfunction. Repair processes may lead to permanent changes to the neural networks through increased neuronal proliferation and regeneration. Long-term consequences can persist after bTBIs. Generally, permanent effects are increased risks for neurodegenerative disorders.

tissues, damage to axons (often seen as diffuse axonal injury), changes to the neuronal cytoskeleton, and other cellular damage and cell death through both apoptotic and necrotic pathways (Busch and Alpern, 1998; Saljo et al., 2000; Cernak et al., 2004; Kocsis and Tessler, 2009; McCrea et al., 2009; Blyth and Bazarian, 2010; Cernak and Noble-Haeusslein, 2010; Hicks et al., 2010; Balakathiresan et al., 2012; Wang et al., 2012). This damage will allow for an evolution period during which intense neuroinflammation occurs, further changes to the BBB will allow for the exchange of various molecules to and from the brain, and metabolic and energy dysregulation will manifest (Kocsis and Tessler, 2009; Cernak and Noble-Haeusslein, 2010; Cornelius et al., 2013). The tissue damage and compromised cellular integrity can cause excessive release of neurotransmitters and other molecules, contributing to excitability of neurons, neuronal death, and neurodegeneration (Cernak et al., 2001). This evolution period will lead to what is known as the secondary injury commonly called postconcussive syndrome (Busch and Alpern, 1998; Blyth and Bazarian, 2010; Hicks et al., 2010). These include a myriad of symptoms similar to PTSD and other neuropsychological effects, which include depression, anxiety, increased irritability and aggression, disruption to sleep patterns/ insomnia, and cognition and memory deficits. In addition, individuals suffering from mTBIs most often report headaches, dizziness, and fatigue (Comper et al., 2005; Hoge et al., 2008; Rao et al., 2009; Blyth and Bazarian, 2010; Brenner et al., 2010; Hicks et al., 2010; Sivanandam and Thakur, 2012; Bramoweth and Germain, 2013; Cornelius et al., 2013; Mouzon et al., 2014). There have even been reports linking mTBIs to increased substance abuse (Blyth and Bazarian, 2010; James et al., 2014) and suicidal ideations (Kang and Bullman, 2008; Bramoweth and Germain, 2013; James et al., 2014). Further, some long-term neurodegeneration risks are associated with the blast overpressure injury. Several recent studies have also suggested a relationship between bTBI and neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (Sivanandam and Thakur, 2012; Cornelius et al., 2013).

INJURY AND RECOVERY MECHANISMS

The mechanisms of bTBI-induced neurological effects have not been fully understood. Fortunately, this has been a rapidly growing area of research recently. Some studies focus on the initial injury, examining the role of oxidative stress in generating secondary effects. Cernak et al. (2001) suggested reactive oxygen species (ROS) would compromise the integrity of cell membranes, making them more susceptible to hyperexcitability. It was suggested that oxidative stress could play a role in the reduced hippocampal volume as seen in TBIs and contribute to cognitive deficits. The increased permeability of the BBB also plays a role in changes of metabolic functioning and decreases in neuronal density soon after blast exposure (Cernak et al., 2004). Cornelius et al. (2013) and Hicks et al. (2010) stated metabolic changes could lead to intense neuroinflammation after initial injuries. Several studies continue the idea that neuroinflammation mediates changes in neuronal populations, specifically within the hippocampus. Recently, a surge of interest regarding microRNAs has identified their role in the pathogenesis of bTBIs, given the ability of short noncoding RNAs, such as miRNAs, to regulate the destruction or downregulation of target genes. Several miRNAs are found to be upregulated soon after bTBI (Redell et al., 2010; Hu et al., 2012), and after bTBI several miRNAs have been found to mediate a decrease in neuroinflammation and an increase in cell survival in the hippocampus (Redell et al., 2010, 2011). Further studies

examining the functional outcomes of bTBIs have examined behavioral effects and found behavioral deficits to be persistent up to 1 year after bTBI in animal models (Mouzon et al., 2014; Snell et al., 2009).

It is plausible that the behavioral and cognitive deficits seen with PTSD symptomology could be mediated by changes in neuronal networks. After neuroinflammation, regeneration processes (neurogenesis, synaptogenesis, and cell proliferation) are prevalent, especially in the hippocampus (Blyth and Bazarian, 2010; Redell et al., 2010, 2011; Balakathiresan et al., 2012; Hu et al., 2012; Wang et al., 2012; Cornelius et al., 2013), and play a role in injury repair. This would also indicate behavioral and cognitive deficits may be attributed to the neurodegeneration and neuronal cell death after blast injury. It is important to note that the repair processes, changes in neuronal network, and an increased proliferation could contribute to further PTSD symptomology and its persistence. In addition, aberrant regulation of various proteins (Saljo et al., 2000; Blyth and Bazarian, 2010; Sivanandam and Thakur, 2012; Mouzon et al., 2014) can contribute to the pathogenesis of neurodegenerative disorders that may manifest much later after blast overpressure exposure.

Debates exist regarding whether bTBIs, PTSD, and other symptoms are comorbid or if bTBIs generate the symptomology. Many investigations have not found prolonged effects after mTBIs and, on the contrary, have seen complete reversal of mTBI symptomology (McCrea et al., 2009; Snell et al., 2009; Stein et al., 2014). Some studies agree with a comorbid stance between PTSD and bTBI but suggest the comorbidity amplifies and extends the effects of bTBI (Busch and Alpern, 1998; Brenner et al., 2010; Hicks et al., 2010). However, changes to neuronal tissue and therapeutic approaches would suggest against comorbidity of PTSD and bTBIs. There are conventional therapeutic techniques such as therapy sessions and exercise practices that have been shown to be beneficial for bTBI recovery (Comper et al., 2005; McCrea et al., 2009). However, pharmaceuticals targeting PTSD symptomology do not show the same improvement in individuals with PTSD or depression in the absence of bTBI when compared with individuals experiencing both (Comper et al., 2005).

Symptomology induced after bTBI is likely dependent on secondary effects of blast trauma. After exposure to blast overpressure, extensive tissue damage initiates neuroinflammation and neuronal cell loss. To combat such effects, biochemical processes are activated to mitigate neuroinflammation, promote cell survival, and enhance proliferation. Because of their anatomic locations, the medial temporal lobe structures are extremely vulnerable to impacts of blast overpressure. Therefore, studies of histological and biochemical changes in these areas will help to reveal mechanisms underlying the prolonged bTBI-induced behavioral and cognitive symptomology after such injuries.

BODY FLUID–BASED BIOMARKERS OF INJURY

Currently, there are studies investigating the use of protein and small RNA molecules in the brain and body fluids as biomarkers. Some promising protein markers are S100b, glial fibrillary acidic protein, and the ubiquitin carboxy-terminal hydrolase-1 (Svetlov et al., 2009). Tate et al. (2013) additionally described two proteins identified in TBIs that have the potential for biomarkers. The spectrin breakdown products are involved in the deterioration of the cytoskeleton in the central nervous system. Additionally, they identify the deregulation in ubiquitin carboxy-terminal hydroxylase–L1, a substance known to indicate axonal degeneration. One of the most significant recent findings has been with miRNAs circulating in the blood. These molecules represent a window to our status of health. Mitchell et al. (2008) reported that a set of miRNAs are measurable and highly stable in blood plasma of human subjects. They also demonstrated that although synthetic exogenous miRNA (naked) were rapidly degraded in the plasma, endogenous plasma miRNAs are resistant to RNase activity and remain stable. For neurological dysfunctions, blood miRNAs have been proposed as biomarkers for Alzheimer's disease (Maciotta et al., 2013) and predictor of brain aging (Li et al., 2011). Recent studies have suggested that TBI alters serum/plasma miRNA expressions, representing a rich source of molecular biomarkers. Several miRNAs markers (miR-16, miR-92a, and miR-765) have been identified as biomarkers for TBI diagnostics within the 24 h after injury, and the combined use of these miRNA markers has gained satisfactory diagnostic specificity and sensitivity for both severe and mild TBIs classified using the GCS score (Redell et al., 2010). Notably, the expression level of plasma miRNA can be used to indicate injury severity and discriminate mild and severe TBI (Redell et al., 2010), suggesting the value of miRNA markers as indicators for prognosis of TBI. Recently, Balakathiresan et al. (2012) have investigated the miRNA signature after repeated blast overpressure injury using a rat mode. Findings suggested that the miRNA let-7 is a promising biomarker for bTBI with high neurophysiological significance. However, more work needs to be performed to verify and assess the diagnostics value of body fluid–based biomarkers in human bTBI studies. The temporal and dynamic expressions of protein and small RNA markers are another area of importance requiring further investigation. It is clear that more reliable and effective means of diagnosis are essential for bTBIs, especially in a warfare setting.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In warfare, blast-induced neurotrauma is common and many bTBIs are classified as mild and go undiagnosed. Repeated exposure amplifies effects of blast overpressure exposure that can result in long-lasting neurological deficits. The initial effects of bTBIs are gradient and dependent on exposure and proximity to the explosion. However, secondary biochemical responses alter neuronal networks and seem to be responsible for the sequela of behavioral and cognitive deficits that follow bTBI. The transient nature of initial injury and the delayed manifestation of secondary injury make diagnosis difficult when injuries are not immediately addressed, especially in warfare. Further advances in diagnostic techniques, especially for mild bTBI, are urgently needed. Emerging body fluid–based protein and small RNA biomarkers must be verified in human bTBI populations. More research is needed to examine bTBI and its relation to behavioral and cognitive deficits and to identify mechanisms of symptomology. Finally, the need for therapeutic interventions is high. Targeting the evolution of secondary injuries is of key importance and several research efforts are trending toward this method. Advances in understanding the biochemical nature of secondary injury of bTBIs have led to the identification of aberrantly regulated proteins and small RNAs that may serve as diagnostic markers and therapeutic targets that would halt the pathogenesis of bTBI. There have been several advancements made in recent years to further the understanding of bTBIs. However, the complex nature of blast waves in warfare makes exposure to overpressure waves a serious problem requiring continuous investigations for improved understanding and management of bTBIs.

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CHAPTER

14

Thallium

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INTRODUCTION

Thallium (Tl) is a soft, bluish-white metal that occurs naturally in the Earth's crust. It was discovered by Sir William Crookes in 1861 while making spectroscopic determinations of tellurium in residue material from a sulfuric acid plant. The name comes from the Greek word *thallos*, which means "green shoot" or "twig," which refers to the green spectral emission lines originally used to identify the element. It is a heavy metal (with density 11.83 g/cm^3 and atomic number 81) whose use is mainly in the electronics industry (e.g., infrared detectors and semiconductor materials), with smaller quantities of the substance used in glass manufacturing and the pharmaceutical industry (in which the radioactive isotope Tl-201 is utilized). Thallium can be released into the environment by cement manufacturing, the burning of certain coal deposits, and the production of nonferrous metals (Kazantzis, 2000; Peter and Viraraghavan, 2005). Normally, Tl is found in the environment in low concentrations, commonly in the Tl (+1) or Tl (+3) oxidation state. The maximum contaminant level (MCL) for Tl in drinking water is 0.002 mg/L (US EPA, 2009), a level at which no adverse human health effects are anticipated. Commercial Tl is usually obtained during the refining process for iron, cadmium, lead, or zinc (USGS, 2013). Thallium is a highly toxic element, and salts of Tl are colorless, odorless, and tasteless. Thallium has no known biological function and has been the least studied of the toxic metals; lead, mercury, and cadmium have attracted more attention. Thallium salts were introduced as pesticides in Germany in 1920. The sulfate salt is most common and has been widely used as a rodenticide and ant killer. Tl has been associated with intentional and accidental poisonings since that time, although problems decreased greatly after its use was banned in major parts of the world (Saddique and Peterson, 1983).

BACKGROUND

Thallium has two important oxidation states, Tl (+1) and Tl (+3). The trivalent form more closely resembles aluminum, and the monovalent form more closely resembles alkali metals such as potassium. The toxic nature of the monovalent Tl is due to its similarity to potassium in ionic radius and electrical charge. Thallium sulfate use as a pesticide was restricted in 1965 in the United States, and the World Health Organization (WHO) recommended in 1973 against its use as a rodenticide due to its toxicity (WHO, 1973). From 1912 to 1930, thallium compounds were used extensively for medicinal purposes; for example, in the treatment of ringworm (because of the depilatory effects), dysentery, and tuberculosis. The narrow margin between toxicity and therapeutic benefit, however, eventually eliminated the practical use of these compounds. Due to its highly toxic nature, delayed symptoms and lack of taste or odor, Tl salts have been used for suicide attempts and in the intentional poisoning of individuals or small groups of people. Although the reported symptoms of Tl poisoning are diverse, the classic syndrome involves gastroenteritis, polyneuropathy, and alopecia. Fictional accounts of Tl as the agent of an intentional poisoning include Agatha Christie's mystery novel *The Pale Horse.* More recent accounts or suspicions of Tl use include medical case reports and reports in the mainstream press. Chronic Tl exposure has been reported in the industrial setting and exposure limits have been established (Peter and Viraraghavan, 2005). However, there currently is insufficient epidemiological data concerning the chronic effects of Tl on humans, as well as a lack of data concerning the mutagenic effects and the effect of Tl on genetic material (Rodriguez-Mercado and Altamirano-Lozano, 2013). The radioactive isotope Tl-201 is a gamma emitter used in cardiac imaging, similar to technetium-99, and it

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has a half-life of approximately 3 days. Tl-201 is the most common isotope in use. Tl-204 decays by beta particle emission and has a half-life of 3.8 years.

TOXICOKINETICS

Thallium is rapidly absorbed from the gastrointestinal tract and is well absorbed through the skin. There is little information concerning absorption from the respiratory tract. Once absorbed, Tl is rapidly distributed throughout the body to all organs, with the highest concentrations occurring in the kidney following an acute exposure. Both monovalent and trivalent Tl appear to distribute in a similar manner, and it is not known if metabolic processes can change the valence state. Thallium can pass the placental barrier and the blood–brain barrier (Sullivan, 1992; Leonard and Gerber, 1997).

Elimination of Tl is mainly through the gastrointestinal tract, but elimination also occurs through the kidneys, saliva, hair, skin, sweat, and breast milk. Relative amounts excreted by each route vary by species. Thallium is likely excreted through intestinal and gastric secretions associated with potassium loss or excretion. Likewise, reabsorption of Tl also occurs, mainly from the colon. The estimated biological half-life of Tl is 10 days, but values up to 1 month have been reported (WHO, 1996).

MECHANISM OF ACTION

Although the precise mechanism of action of Tl is unknown, its similarity to potassium has been shown to play a significant role. Thallium has an atomic radius similar to potassium and has shown a 10-fold affinity over potassium in Na/K-ATPase, resulting in lower activity of the enzyme. Tl will inhibit the influx and efflux of potassium in mitochondria without affecting the movement of sodium. In addition to disturbing mitochondrial function, Tl has been shown to increase the levels of hydrogen peroxide and increase lipid peroxidation and oxidative stress (Hanzel and Verstraeten, 2005). The metabolism of glutathione can be disrupted by Tl, increasing the susceptibility to reactive oxygen species (Cvjetko et al., 2010). Thallium can also inactivate sulfhydryl groups, including those that affect the permeability of the outer mitochondrial membrane. Thallium can act as a Lewis acid, with an affinity for organosulfur compounds, which may account for its causing hair loss. The binding of cysteine by Tl may inhibit the keratinization of hair by preventing the cross-linking of proteins (Mulkey and Oehme, 1993).

TOXICITY

The available human literature on Tl mainly consists of case reports from the results of acute poisoning, accidental ingestion, or suicide attempts (Hammouri et al., 2011). Although the acute classic syndrome of Tl poisoning involves gastroenteritis, polyneuropathy, and alopecia, not all these effects are observed in every case. The onset and sequence of symptoms will vary with the dose and duration of exposure. The lowest-known toxic dose in the human is 0.31 g, which was reported to cause symptoms but not death (Cavanagh et al., 1974). Oral doses of 6–40 mg/kg have been lethal within 10–12 days. Other toxic doses delivered by mouth to humans fall into the range of 10–15 mg/kg for adults (WHO, 1996; Moore et al., 1993). Children have been poisoned with Tl at dose levels of 4–8 mg/kg.

Several hours following an acute exposure, initial symptoms may include gastroenteritis, including nausea, vomiting, and diarrhea. With a relatively small dose, these symptoms may be relatively mild and diffuse, with little progression for 2–5 days. Gastrointestinal bleeding or constipation may then develop, along with central nervous system and peripheral nervous system effects. These include paresthesia, with reports of the feeling of "burning feet." Additional neurological symptoms can include lethargy, delirium, seizures, and coma (Tsai et al., 2006). An initial presentation resembling Guillain–Barré syndrome has been reported (Misra et al., 2003). Nonspecific kidney and liver damage can develop as well. In severe exposures, circulatory symptoms may result, including hypertension, tachycardia, and cardiac failure. Initial dermatological involvement may include anhydrosis (which can cause fever), and this can be followed some time later by diaphoresis. In the second week following exposure, additional dermatologic symptoms appear. These include an increased darkening of the hair papillae, followed in several days by a developing alopecia. By three weeks after exposure, almost complete alopecia may manifest. At this time in the syndrome, there may be ataxia, tremors, and a painful neuritis in the lower extremities that may be severe. Following a lethal dose, death by renal or cardiac failure commonly occurs within 10–12 days. Recovery from Tl poisoning can require several months and residual neurological problems may remain, including weakness, memory impairment, and psychological disturbances (Pau, 2000; Tsai et al., 2006).

RISK ASSESSMENT

Although reported as an agent of intentional poisoning for an individual or small group of people, the broad

use of Tl in chemical warfare or terrorism has not been reported (Salem et al., 2008). The most commonly available radioisotope of Tl-201 is a gamma emitter with a short half-life, making it a poor candidate for a radiological dispersion device (Burnham and Franco, 2005; Chin, 2007). The Tl-204 isotope is a beta emitter with a half-life of 3.8 years, and its commercial use is limited. It is really not used in any significant amount (and thus there is not enough raw material to make a decent radioactive device). The chelating agent used to treat Tl exposure, ferric-hexacyanoferrate (II), known as Prussian Blue, is also used to treat radiocesium exposure; therefore, it is included in many antidote stockpiles (Ansari, 2004).

TREATMENT

The diagnosis of Tl poisoning is based upon exposure history (if available), compatible clinical time course, and symptoms, as well as the finding of above-background levels of Tl in urine, serum, or other clinical specimens. The appropriate methodology for urine or blood Tl includes atomic absorption spectroscopy (flame or flameless) and evolving methodology such as inductively coupled plasma atomic emission spectroscopy (ICP-AES). Use of colorimetric analyses of these specimens can lead to false positives (CDC, 1987). Normal or background concentration of Tl in urine is given as up to a level of <10 μg/L, with concentrations elevating several hundred- to several thousand-fold following acute exposure. Treatment should be initiated when 24 h urinary Tl excretion exceeds 0.5 mg. Additionally, a toxic level of Tl in the urine of >300 μg/L has been suggested (Sullivan, 1992). Blood Tl levels in exposure situations are less well characterized, and values above 5 μg/L are considered evidence of excess Tl exposure (CDC, 1987). Prussian Blue is the treatment of choice for Tl exposure, in that it acts by binding Tl in the gastrointestinal tract, making it unavailable for reabsorption (Altagracia-Martínez et al., 2012; Sun et al., 2012; Riyaz et al., 2013). This will increase the fecal excretion of Tl and decrease the half-life. Prussian Blue is also used in the treatment of radiocesium and acts by the same mechanism (Yang et al., 2008). The suggested dosage regime for adults and adolescents is 3 g given orally three times a day. Children between the ages of 2 and 12 years can be given 1 g orally three times a day. The clinician should check for proper gut functioning before starting treatment because constipation is a common symptom of Tl intoxication. Fluid diuresis and other symptomatic and supportive care should also be provided. Recently, a workgroup composed of international experts assembled to review and provide recommendations on the use of extracorporeal treatment for poisonings. In the case of severe Tl poisoning, the workgroup strongly recommended extracorporeal removal for Tl, although it was acknowledged that limited evidence exists (Ghannoum et al., 2012).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Thallium remains a toxic metal of concern for both accidental and intentional exposure. Its historical use as an intentional poison against individuals or small groups is based on its delayed onset of symptoms and the fact that its salts are tasteless and odorless. Although the incidence of intoxication with Tl has decreased with its decreasing availability, clinicians should remain familiar with clinical features, diagnostic considerations, and treatment regimes. History has shown that Tl is not an agent of choice for chemical warfare or terrorism, and the use of radiological Tl for these purposes is also unlikely. However, preparations for such an unlikely event are relatively straightforward; major considerations include the stockpiling of Prussian Blue (which is handy to have because it is also needed for radiocesium treatment) and the retention of analytical capabilities for Tl in biological and other samples.

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CHAPTER

15

Arsenicals: Toxicity, Their Use as Chemical Warfare Agents, and Possible Remedial Measures

Swaran J.S. Flora

INTRODUCTION

Arsenic is a metalloid (semi-metal) member of group V elements of the periodic table with oxidation states of +3 (As III) and +5 (As V), in which the former (As III) is more than the latter (As V) (Aposhian et al., 2003). Both species occur in organic as well as inorganic compounds. Arsenic combines chemically with most nonmetals to form a variety of both inorganic and organic compounds. Organoarsenic compounds with trivalent arsenic were produced as pesticides as well as chemical warfare (CW) agents in the first half of the twentieth century (Aposhian et al., 2003; Li et al., 2005). A number of organic arsenicals have been developed for use as CW agents. These arsenic-containing substances in chemical weapons programs are of human and ecotoxicologic relevance. The story of arsine and lewisite encapsulates the key elements of the history of chemical weapons and their continuing power (Sugden, 2008). Although, information about arsenic and its inorganic and organic derivatives are well-documented, there is little literature available about their role as CW agents. This chapter provides readers with updated information about the organic arsenicals as CW agents and also gives a comprehensive account of toxicity due to inorganic arsenicals. Arsenic poisoning has recently assumed an alarming proportion in approximately nine districts of West Bengal, India, so much so that it has been earmarked as "the biggest arsenic calamity in the world." In view of rapid spread of various diseases arising out of arsenic contamination (e.g., arsenical dermatosis, melanosis, keratosis, edema, gangrene) in different areas of West Bengal and also in adjoining areas of Bangladesh, there is a need to provide readers with information about the modes and sites of action after exposure to environmentally relevant levels of arsenicals and to determine the effects of arsenicals in susceptible populations. The most toxicologically potent arsenic compounds are in the trivalent oxidation state. This is because of their reactivity with sulfur-containing compounds and generation of reactive oxygen species (ROS). However, humans are exposed to both trivalent and pentavalent arsenicals.

BACKGROUND

Arsenic (As) occurs in a variety of different chemical forms, among them volatile (gaseous) species, usually referred to as arsine and methylarsines (Mestrot et al., 2011). It is also well-known as an insecticide in the form of lead arsenate and arsenic acid, and in pharmacy, especially in the forms of salvarsan and neosalvarsan. Arsenic derivatives are also of value from the point of view of a CW agent (Wexler, 2004).

Arsine, the most toxic form of arsenic, exhibits some characteristics that may make it useful as a CW agent. Arsine is a colorless, odorless, nonirritating gas that is 2.5-times denser than air (Thomas and Young, 2001; James and Woods, 2006). At concentrations higher than 0.5 ppm, a garlic-like odor may be noted, but arsine is toxic at much lower concentrations. Acute arsine poisoning due to inhalation of arsine gas (AsH_3) is rare but has no known antidote. It is the most acutely toxic form of arsenic, causing rapid and severe hemolysis immediately on exposure. The mechanisms of hemolysis are not completely understood. Arsine has a short

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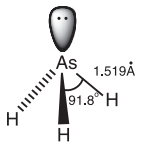
half-life (27–96 h) and is converted to various arsenic derivatives. A number of other arsine-derived organoarsenic compounds have been developed and used as CW agents, including lewisite (L), methyldichloroarsine (MD), diphenylchloroarsine (DA), and ethyldichloroarsine (ED) (Ishizaki et al., 2005). Arsines family includes compounds with various toxicities; however, arsenic trihydride or arsine is the most toxic form. It is a powerful hemolytic gas but has never been used as a chemical weapon because it exerts nonimmediate and nonpersistent toxicity. However, cases of occupational exposure are still reported despite strict regulation. This agent, whose mechanism of action is still not well-defined, is poorly recognized because intoxication is rare. Fast detection means are available, but health professionals need to learn to recognize arsine intoxication to provide early, specific treatment and avoid damages (Plantamura et al., 2011).

Arsenicals are considered a threat, not so much from large nation states but from smaller, less developed nations and/or by terrorist organizations. The relative ease of production coupled with their effectiveness against an unprotected population make organic arsenicals a continued threat in the twenty-first century. This chapter describes the human health aspects of arsine, some of the important organic arsenicals including inorganic arsenic, and the current status of development of suitable therapeutic measures.

ARSINE



Arsine (AsH_3) is a colorless, extremely flammable gas with a garlic-like odor. The most common synonyms for arsine are arsenic hydride, arsenic trihydride, hydrogen arsenide, and arsenous hydride. The relative molecular mass of arsine is 77.95. Its boiling point is −62°C and vapor pressure at 20°C is 1,043 kPa. Arsine is a strong reducing agent, deposits arsenic on exposure to light and moisture, and is easily transformed into other oxidized arsenic forms (e.g., As (III) and As (V)). The arsine gas is colorless, odorless, and 2.5-times denser than air (Table 15.1). Arsine is a class of organoarsenic compounds of the formula $AsH_{3-x}R_x$, where R = aryl or alkyl (Franke 1967; Fowler and Weissber, 1974).

Possible sources of occupational exposure are many and include the semiconductor industry during microchip production and other industries in which workers are involved in galvanizing, soldering, etching, and lead plating. It also can be produced inadvertently by mixing arsenic-containing insecticides and acids (James and Woods, 2006). In humans and animals, arsine is metabolized to trivalent arsenic as well as pentavalent arsenic. Arsenic (III) is methylated to monomethylarsonate (MMA) and dimethylarsinate (DMA). Arsine metabolites are mainly excreted via urine (Apostoli et al., 1997; James and Woods, 2006).

TABLE 15.1 Table Showing Physical and Chemical Properties of Arsine

Properties of Arsine	
Molecular formula	AsH_3
Molar mass	77.95 g/mol
Appearance	Colorless gas
Density	4.93 g/L, gas; 1.640 g/mL (− 64°C)
Melting point	−117°C (157 K)
Boiling point	−62.5°C (210 K)
Solubility in water	0.07 g/100 mL (25°C)
Molecular weight	77.95

Arsine is supposed to be the most toxic form of arsenic. The acute toxicity of arsine in different species, including human, is high. The target organ of arsine is the hematopoietic system and, in particular, the erythrocytes. Inhaled arsine gas is distributed rapidly and causes massive red blood cell (RBC) hemolysis that can potentially lead to cellular hypoxia (Hatlelid et al., 1995; Hatlelid et al., 1996; Apostoli et al., 1997) The mechanisms involved in hemolysis are not elucidated fully, but studies reveal that arsine causes a nonspecific disruption of ion gradients, leading to cell membrane instability, that sulfhydryl group in cell membrane are probable targets of arsine toxicity, and that hemoglobin is an important subcellular target of arsine toxicity (Winski et al., 1997).

Arsine poisoning can lead to acute renal tubular necrosis and ultimately to oliguric/anuric renal failure (Rogge et al., 1983). Renal failure can be attributed to direct effect of arsine on renal tissue, heme-pigment nephropathy, or renal hypoxia secondary to massive hemolysis and decreased oxygen carrying capacity of the blood.

Exposure to other arsenic compounds to which arsine is metabolized can induce lung, bladder, kidney, and skin cancer in humans (Lenza, 2006).

Synthesis of Arsine

Arsine is formed whenever nascent hydrogen is released in the presence of arsenic or by action of water on a metallic arsenide. The formation of arsine

can be described with the help of the following reaction (Anthonis et al., 1968; Coles et al., 1969):

$$3H_2 + HAsO_2 \rightarrow AsH_3 + 2H_2O$$

AsH_3 is also prepared by the reaction of As^{3+} sources with H^- equivalents (Bellama and Macdiarm, 1968).

$$4AsCl_3 + 3NaBH_4 \rightarrow 4AsH_3 + 3NaCl + 3BCl_3$$

Alternatively, sources of As^{3-} react with protonic reagents to produce arsine:

$$Zn_3As_2 + 6H^+ \rightarrow 2AsH_3 + 3Zn^{2+}$$

Sources of Exposure

The main anthropogenic sources of arsine include its accidental formation, particularly in the chemical and nonferrous (zinc, copper, and cadmium) metallurgical industries, production or use of the gas itself during manufacture of semiconductors as a doping agent, and in battery production as an alloy with lead (Aposhian, 1997; Winski et al., 1997).

Arsine is extensively used in the semiconductor industry for epitaxial growth of GaAs and as a dopant for silicon-based electronic devices. Arsine is also used in organic synthesis and as an agent in the manufacture of light-emitting diodes, and for manufacturing certain glass dyes (HSDB, 1999).

Human Arsine Exposure

Arsine might be a potential CW agent (Lenza, 2006). Occupational sources where exposures to arsine at levels sufficient to cause acute arsine intoxication have occurred include copper smelting and refinery, bronzing process, chemical companies (cleaning a clogged drain), transistor industry, and burnishing of metals (Romeo et al., 1997). Many processes including electrolyte refining, galvanizing, soldering, etching, lead plating, metal smelting, and extraction may expose workers to toxic concentrations of arsine (Chein et al., 2006).

Metabolism of Arsine

Animal Studies

The main route of arsine excretion is via urine after metabolism. Levvy (1947) studied the elimination of arsenic in arsine-exposed mice and compared it with the animals exposed to sodium arsenite. Arsenic was excreted exponentially in mice intravenously administered arsenite; after 24h, less than 10% of the dose remained. However, arsenic arising from inhalation exposure to arsine 180mg/m^3 for 120min was excreted more slowly; after 24h, approximately 45% of arsenic remained in the exposed mice.

Human Studies

Apostoli et al. (1997) detected the presence of arsenic in tissues, blood, and urine of workers in the petroleum industry who were poisoned with arsine. In a fatal case of arsine poisoning, arsenic was found in the liver at a concentration of 11.8mg/g, in the spleen at 7.9mg/g, in kidneys at 3.2mg/g, in the brain at 0.6mg/g, and in the urine at 0.6mg/m. Trace amounts were also found in the blood (Apostoli et al., 1997). Inhaled arsine was rapidly dissolved in body fluids and oxidized to As(III) (Apostoli et al., 1997). Part of As(III) is further oxidized to As(V), as indicated by the appearance of As(V) in urine of humans exposed to arsine 1–2 days after exposure. Trivalent arsenic is methylated to MMA and DMA (Romeo et al., 1997).

Mechanism of Toxicity

The mechanism of arsine (AsH_3) toxicity is not completely understood, but hemoglobin (Hb) has long been recognized as a necessary component of the overall mechanism of AsH_3-induced hemolysis (Rael et al., 2006). After inhalation of arsine gas, it causes rapid destruction of RBCs, leading to hypoxia and renal failure. Two mechanisms have been proposed for arsine poisoning: reaction with sulfhydryl groups (Winski et al., 1997; Waters et al., 2004) and oxidative stress (Blair et al., 1990; Hatlelid et al., 1996; Hatlelid and Carter, 1997). Besides these two mechanisms, nonspecific disruption of ion gradient leading to cell membrane instability and lysis of RBCs has also been proposed as mechanism for the toxic effects of arsine (James and Woods, 2006). Hatlelid and Carter (1997) postulated that the hemolytic activity of arsine is related to oxidative stress through the formation of hydrogen peroxide and arsine adducts with hemoglobin.

Oxyhemoglobin (oxyHb) has been recognized as a necessary component for overall mechanism of AsH_3-induced hemolysis, as conversion of oxyHb to carboxyHb prevents hemolysis in erythrocytes exposed to AsH_3 (Rael et al., 2000).

Some studies have suggested that the sulfhydryl groups of glutathione (GSH) prevented hemoglobin oxidation and in this manner are essential for the maintenance of intact erythrocyte structure. In an *in vitro* study, a decrease in reduced GSH concentration in human RBCs was found to correlate with the hemolytic action of arsine (Rael et al., 2000). Blair et al. (1990) recorded a 60% decrease in reduced GSH level in erythrocytes exposed to arsine *in vitro*. However, Hatlelid et al. (1995) showed that the depletion of reduced GSH in RBCs in dogs neither preceded nor coincided with hemolysis.

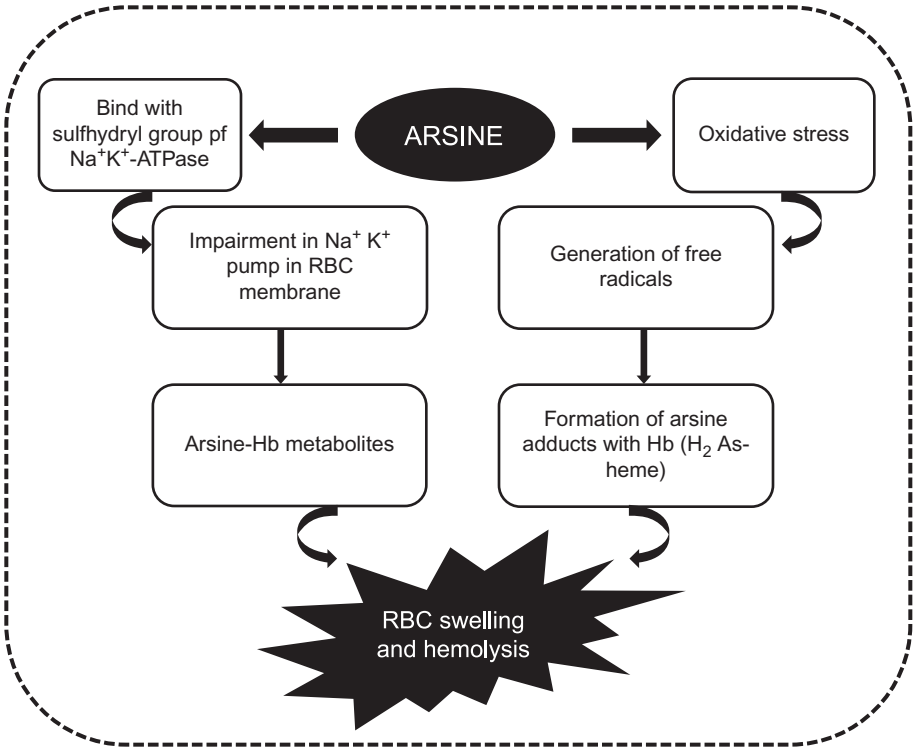


FIGURE 15.1 Schematic diagram showing hemolytic activity of arsine. Figure shows that arsine reacts with the sulfhydryl group of Na^+K^+-ATPase, leading to the impairment in the sodium-potassium pump which subsequently causes red cell swelling and hemolysis.

According to the hypothesis of the sulfhydryl-dependent mechanism of arsine toxicity, arsine reacts with the sulfhydryl group of Na^+K^+-ATPase, causing an impairment in the sodium-potassium pump that subsequently causes red cell swelling and hemolysis (Figure 15.1). The affinity of trivalent arsenic for the sulfhydryl group is well-known. Winski et al. (1997) reported profound abnormalities in membrane ultrastructure and in RBC volume, which were manifested by potassium leakage, sodium influx, and increases in hematocrit in arsine-exposed red cells. However, no change in ATP and ATPase was observed after exposure to arsine. Hemolysis in arsine-exposed RBCs depended on membrane disruption caused by arsine–hemoglobin metabolites, the ultimate toxic species (Winski et al., 1997).

Effects on Humans

Toxicity of arsine to humans was first demonstrated in 1815, when a German chemist accidentally inhaled arsine vapor during an experiment (Romeo et al., 1997).

Acute Arsine Poisoning

Persons exposed to arsine gas are often unaware because there are no symptoms at the time of exposure (James and Woods, 2006). The majority of arsine exposures are clinically acute; however, lower levels of chronic exposure have been reported in literature (Casarett and Doull, 2001). Most patients report little or no discomfort at the time of exposure. Although a garlic-like odor may be noted with higher ambient arsine concentrations, serious toxicity may result from clinically nondetectable exposures (Levvy, 1947; Apostoli et al., 1997; Thomas and Young, 2001; Young et al., 2003; Lenza, 2006; Song et al., 2006). Symptoms after sub-lethal arsine exposure may include abdominal pain, hematuria, and jaundice (Figure 15.2).

Physical Signs

Physical signs and their severity depend on the concentration of arsine gas and the duration of the exposure and have been mentioned in Figure 15.2 (Levvy, 1947; Apostoli et al., 1997; Thomas and Young, 2001; Young et al., 2003; Lenza, 2006; Song et al., 2006).

Immediate Effects

Clinical manifestation of arsine intoxication appears within 24 h of exposure (usually within a few hours). Renal failure due to tubular destruction is an important consequence of arsine exposure. The urine is frequently discolored (brown, red, or black), and hemoglobin in the urine is thought to be the major cause. Urinalysis

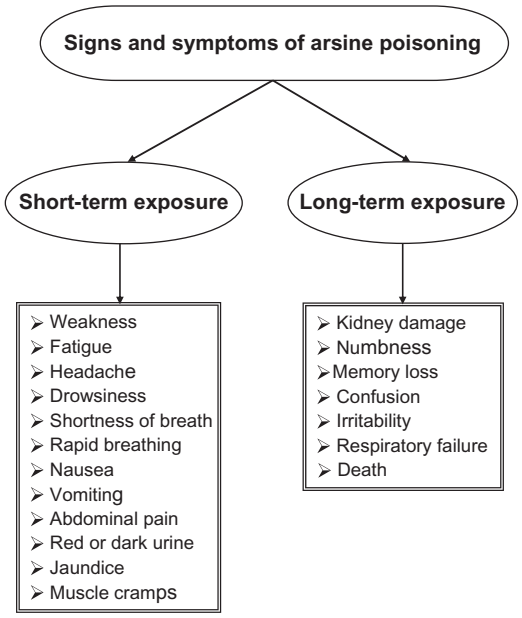


FIGURE 15.2 Figure showing signs and symptoms of arsine poisoning. Signs and symptoms following short-term and long-term sub-lethal exposure to arsine.

typically shows large amounts of protein and free hemoglobin with only a few RBCs. The characteristic red/bronze tint of the skin is induced by hemolysis and may be caused by hemoglobin deposits (James and Woods, 2006). In some cases, hepatomegaly and splenomegaly with tenderness of costovertebral angle, fever, tachycardia, and tachypnea occur. Hemolytic anemia is the most consistent clinical finding in humans. Massive hemoglobinuria may lead to anuria, which, if untreated, is often the cause of death (Hatlelid et al., 1996; Winski et al., 1997).

Late Effects

Late consequences of acute arsine poisoning include chronic renal damage, hematological changes, polyneuritis, and neuropsychological symptoms (e.g., irritation, confusion, memory losses, agitation, and disorientation). Peripheral neuropathy was still present 6 months after exposure (Gosselin et al., 1982). Extreme restlessness, loss of memory, agitation, and disorientation occurred several days after exposure and lasted approximately 10 days in two patients heavily exposed to arsine. An increase in total cell count and macrophages in bronchoalveolar lavage was observed in an arsine-exposed worker. Progressive improvement in diffusing capacity of lungs was observed only after 2 months of treatment. Hepatitis in an arsine-poisoned patient on day 20 after the acute hemolysis also has been reported (Mora et al., 1992).

Long-Term Exposure

Long-term exposure may cause symptoms similar to those observed in acutely poisoned individuals. The main differences from acute poisoning were in a delay in onset and development of peripheral neuritis, development of gastrointestinal track involvement, and development of hemolysis and renal impairment. Lowered hemoglobin levels were found in zinc ore smelting workers exposed to arsine for long periods and who had urinary arsenic concentration less than 0.2 mg/L (Watson and Griffin, 1992).

Diagnostic Tests

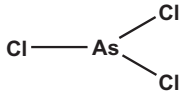
Laboratory Studies

Complete blood count, methemoglobinemia, urinalysis, arsenic levels, electrocardiogram, and imaging studies were performed.

Complications

Complications include hemolytic anemia, renal failure, hyperkalemia, and death. Overwhelming exposures cause rapid death from massive hemolysis. Most deaths occur from renal failure in patients who survive acute exposure. Patients surviving acute arsine exposure may develop chronic arsenic toxicity, including anemia and peripheral neuropathy.

ORGANIC ARSENICALS



Organic arsenicals are a series of blister agents based around a chloroarsine ($AsCl_3$) molecule in which one of the chlorine atoms is replaced by an organic radical. These chloroarsine are effective cytochrome oxidase destroyers, or blood agents. Many organic radicals penetrate human skin, carrying their compounds with them (Cohen et al., 2006).

Background

Interests in organic arsenicals date back to the mid nineteenth century. Chemists discovered that arsenic–chloride compounds (chloroarsines), in which one of the chlorine atoms is replaced by an organic radical, tends to be harmful both to insects and to human tissue (McManus and Huebner, 2005; Bartelt-Hunt et al., 2008). The trench warfare stalemate during World War I created a tactical need for a chemical weapon that was

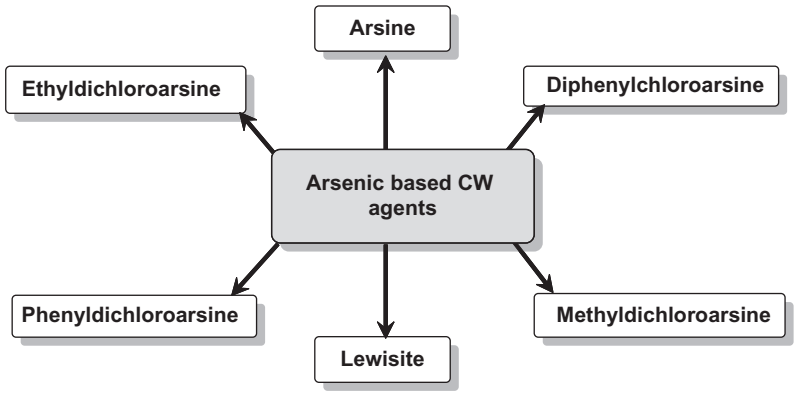


FIGURE 15.3 Figure showing arsenic based CW agents.

both short-acting (e.g., nonpersistent, volatile) and lethal (Stephenson, 2006). To fill this need, the first weaponized organic arsenical, MD, was delivered. Two additional organic arsenicals, DA and ED, soon augmented MD. A fourth organic arsenical named lewisite was discovered by US Army Medical Corps but was never deployed in World War I (Boronin et al. 1996; McManus and Huebner, 2005; Bartelt-Hunt et al.,2008) (Figure 15.3).

Mechanism of Toxicity

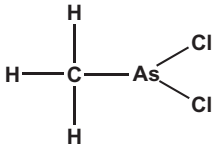
The exact mechanism for the toxic effects of organic arsenicals is unknown. DNA alkylation and/or inhibition of GSH-scavenging pathways are two postulated mechanisms (Nesnow et al., 2002). On contact with arsenicals, a blistering reaction occurs on skin, eye, or pulmonary tissues (Cohen et al., 2006; Devesa et al., 2006; Kojima et al., 2006). Animal data and limited human trials suggest that organic arsenicals readily penetrate the skin. Within seconds of contact, the chemical fixes itself to the epidermis and dermis. Pain is immediate, followed by destruction of subcutaneous tissue. The separation of dermis from epidermis and capillary leakage cause fluid-filled vesicles (McManus and Huebner, 2005; Naranmandura and Suzuki, 2008). Intravascular hemolysis of erythrocytes with subsequent hemolytic anemia may result (Wu et al., 2003).

Symptoms

Vapor contact with the conjunctiva may be the victim's first symptom. Severe conjunctival irritation and blepharospasm may lead to loosening of corneal epithelial cells and swelling and edema of the cornea. Mucosal damage starts in the nose and descends down the respiratory mucosa in a dose-dependent fashion. Immediate pain, lacrimation, and irritation accompany the damage. DA vapor causes vomiting that develops within 1–2 min after exposure to DA.

The immediate onset of symptoms after exposure makes severe or systemic toxicity to organic arsenical unlikely. However, prolonged contact may lead to involvement of multiple organs (Kojima et al., 2006; Kinoshita et al., 2007).

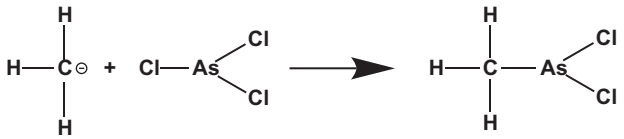
Methyldichloroarsine



MD was apparently used by Germans in 1917. MD is a colorless liquid with a powerful burning odor that boils at 132°C. It is somewhat soluble in water and is soluble in organic solvents. The specific gravity is 1.838 at 20°C. The vapor pressure at 25°C was found to be 10.83 mmHg. The material is not only toxic but also has remarkable vesicant properties, comparing favorably with mustard gas in this respect (Bennett and Dill, 1994).

Structure of MD

The structure of MD consists of a trichloroarsine ($AsCl_3$) molecule combined through catalysation with a methyl (CH_3) group.

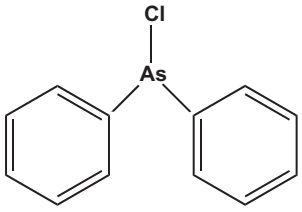


Pathology

Chlorine bonds in MD give it its blistering qualities. The methyl group simply aids in its assimilation into the

human body. Chlorine reactivity causes severe respiratory pain and damage to the membranes of the lungs. "Dry-land drowning" can occur as the lungs flood with water and mucous and the victim dies of a combination of blood poisoning and asphyxiation (Pitten et al., 1999).

Diphenylchloroarsine

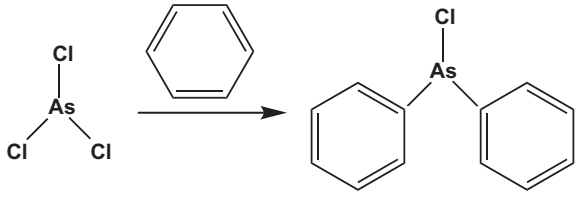


DA is an irritating substance that was developed in 1918 for use in a smoke generator known as the M-device. The researchers described DA as having "a very powerful irritant action on the mucous membranes of the eyes and nose, causes painful blistering of the skin, and is very dangerous for those working with it, since its vapor causes respiratory embarrassment, faintness, and long-lasting paralysis and anesthesia of the extremities" (Ishii et al., 2004; Kato et al., 2007).

Because DA and related compounds cause intense effects on the nasal and upper respiratory passages, they are referred to as "sneeze gas" (sternutators). Like other arsenicals, DA is a white solid and was originally produced as both a casualty gas and a mask breaker during World War I.

Structure

DA is prepared from trichloroarsine ($AsCl_3$):

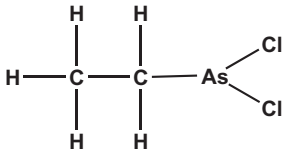


Two chlorine ions are replaced by benzene groups, forming a stable compound that may be safely stored under all field conditions.

Effects of DA

The immediate effects of DA are those associated with tear gas compounds: severe irritation to the eyes, nose, and throat. Severe headache and the feeling of tightness of the chest and of the bowels occur within 1 min of inhalation of this compound. Headache rapidly develops into a general nausea that results in vomiting within 3 min. In closed or confined spaces, DA can produce fatalities by first causing unconsciousness and then asphyxiation (Ochi et al., 2004).

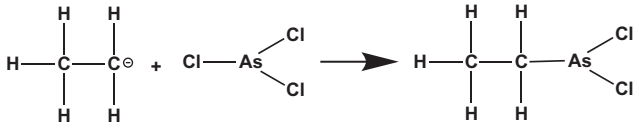
Ethyldichloroarsine



ED was the third, and last, of the three blistering arsenicals developed in late 1917–1918. Despite serious efforts made to weaponize this compound, few literature exists on the effectiveness and history of its use. Fast-acting compared with mustard or phosgene, ED is a colorless liquid that smells like rotting fruit and has multiple effects on the body.

Structure

The structure of ED consists of trichloroarsine ($AsCl_3$) molecule combined through catalysation with ethyl (C_2H_5) group.

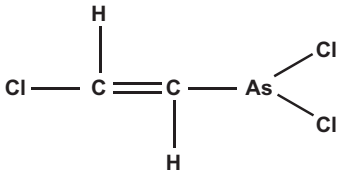


Production of ED is similar to that of MD, involving the ethylation of a chlorinated arsenite or arsenate salt, or in reductions of arsenious oxide, As_4O_6, a naturally occurring compound (Bartelt-Hunt et al., 2006).

Effects of ED

Chlorine bonds in ED cause its blistering, lachrymatory, and harsh respiratory effects. Fluid-filled blisters form only after prolonged exposure. Inhalation can cause pulmonary edema or "dry-land drowning." A lethal exposure, however, depends on the period of exposure. A dose of 3,000–5,000 mg min/m^3 is generally lethal. Dosages as low as 5 mg min/m^3 may cause severe discomfort to the eyes and throat. Sub-lethal dosages are detoxified by the body. The ethyl arsenic group of ED may cause systemic damage to bone marrow and to the digestive and endocrine systems. Blisters may appear within 2–4 h after skin redness or rash. However, like the mustards, ED actively attacks lung tissue. Damage to lung tissue is permanent in its survivors and presents a hazard area for future infections and tumors. ED is highly poisonous to the eyes. It may cause permanent corneal damage.

Lewisite



Lewisite (chlorovinyldichloroarsine), a war gas, is a colorless, oily liquid at room temperature with a faint "geranium-like" odor. Lewisite dissolves slowly in water and hydrolyzes rapidly to hydrochloric acid and lewisite oxide (Daniels et al., 1990). Lewisite produces irritation and blistering of the skin and injury to the eyes and lungs promptly after high exposure; at lower levels the effects resemble exposure to tear gas, with irritation of skin, eyes, and respiratory tract. Chronic exposure may lead to development of chronic bronchitis and predispose to Bowen squamous cell intraepithelial cancer of the skin.

History and Background Information

Lewisite (also known as Agent L), is no longer considered a state-of-the-art CW agent. Lewisite is a significant threat to unprotected personnel and causes prompt incapacitation from eye injuries and respiratory irritation, coupled with long-term incapacitation from skin burns, pulmonary injury, and systemic illness. Its decomposition products are toxic, making decontamination difficult. Munitions containing lewisite may contain toxic stabilizers. Lewisite is effective as vapor, aerosol, or liquid (Sidell et al., 1997).

Toxicity and Mechanism of Action

Trivalent arsenic is considered the component of lewisite that is primarily responsible for its vesicant and systemic toxicity (Aposhian et al., 2003). Trivalent arsenic exerts its toxic effect by binding to sulfhydryl-containing proteins, especially enzymes, thus inhibiting pyruvate oxidation (Black, 2008). Trivalent arsenic readily penetrates skin, exerting its toxic action systemically and causing painful localized blistering. Lewisite produces pulmonary edema, diarrhea, restlessness, weakness, subnormal temperature, and low blood pressure. Edema and hemorrhaging associated with lewisite exposure can lead to shock and death (Flora et al., 2007a).

A no-observed adverse-effect level of 0.016 mg/kg/day in rabbits and 1.5 mg/kg/day in rats was identified (Daniels et al., 1990). Lewisite is capable of producing DNA damage; however, direct tests of its mutagenic potential have been inconclusive (Datta et al., 2007).

Toxicokinetics

The cellular poisoning effects are attributed to the inhibition of cellular enzyme systems (Watson and Griffin, 1992), especially as a result of arsenic complexing with sulfhydryl groups of proteins and enzymes. This agent affects many sulfur-containing enzymes, including amylase, lipase, cholinesterase, some adenosine triphosphate (ATP) enzymes, creatine phosphokinase, and, of central importance, the pyruvate oxidase system. Two mechanisms have been proposed for the aforementioned effects (Sidell et al., 1997): reactions with GSH, leading to loss of protein thiol status, loss of calcium ion homeostasis, oxidative stress, lipid peroxidation (LPO), membrane damage, and cell death, and reactions with sulfhydryl groups on enzymes leading to inhibition of pyruvate dehydrogenase (PDH) complex, inhibition of glycolysis, loss of ATP, and cell death.

Clinical and Pathological Findings

Signs and symptoms of acute lewisite exposure include a rapid onset of irritation to the eyes and mucous membranes of the upper respiratory tract (lachrymation and rhinitis). In more serious cases of vapor intoxication, chest pain, nausea, vomiting, headache, weakness, convulsions, hypothermia, and hypotension occur (Sidell et al., 1997; Katos et al., 2007). Laboratory tests of the blood of persons exposed may show hemoconcentration; animal studies suggest elevated liver enzymes, including lactate dehydrogenase (LDH) (King et al., 1994; Sasser et al., 1999). The following sub-sections describe the effects on specific body sites.

Skin

Exposure of the skin to vapor causes immediate itching or stinging within 1 min, followed by erythema over 10–30 min. More intense exposures, including liquid contact, produce intense stinging and the formation of small vesicles over the next 24 h, with later enlargement of the vesicles with accumulation of a nontoxic fluid (McManus and Huebner, 2005; Kato et al., 2007).

Eye

Immediate eye pain and blepharospasm result from lewisite exposure, followed by conjunctival and lid edema. Severe exposures can produce necrotic injuries of the iris with depigmentation, hypopion, and synechia development (McManus and Huebner, 2005).

Respiratory System

Li et al. (2013) conducted an analysis of the damage of acute arsine poisoning on respiratory system. Mild respiratory exposures resemble upper respiratory infections, and more severe exposures cause lower respiratory effects, with continuous coughing, laryngitis, and aphonia (McManus and Huebner, 2005).

Nervous System

Neurological findings are inconsistent despite reports of convulsions and coma with severe exposures

(McManus and Huebner, 2005). Neurologic complications after mild exposures have not been described. Edema and hemorrhage in the brain are rare, but no reports of degeneration of peripheral nerves were found.

Cardiovascular System

Bradycardia, dyspnea, hypotension, and hemoconcentration have been reported in severe intoxication. These effects are mediated by vasodilatation and increased capillary permeability (Watson and Griffin, 1992). Dilation of the right side of the heart in severe poisoning in animals has been reported.

Other Systems

Human ingestion experience is not documented (NAS, 1997) but would be expected to produce severe abdominal pain and bloody diarrhea. Nausea and vomiting occur from respiratory or dermal exposure. In humans and animals, vomiting is associated with retching (Sidell et al., 1997). There is no documentation of liver effects from chronic lewisite exposure. There are no specific musculoskeletal findings, although weakness has been observed. There is little clinical information about effects on bone marrow and the immune and endocrine systems. Renal disorders, although theoretically possible, are not described. There is also no substantial evidence that lewisite is carcinogenic, teratogenic, or mutagenic (Sidell et al., 1997).

INORGANIC ARSENIC

Arsenic is a metalloid that belongs to group VA of the periodic table. It exists in three oxidation states: metalloid (0), trivalent (−3 or + 3), and pentavalent (+5). The most common inorganic trivalent arsenic compounds are arsenic trioxide, sodium arsenite, and sodium trichloride. Pentavalent inorganic arsenic compounds are arsenic pentaoxide, arsenic acid, and arsenate (e.g., lead arsenate and calcium arsenate). In general, the toxicity of arsenic compounds is in the following order: arsine > arsenites > arsenates > organic > elemental.

Sources and Uses

Uses

Arsenic is used as a bronzing and decolorizing agent in the manufacture of glass, as a desiccant and defoliant in agriculture and in the production of semiconductors, and as a byproduct of the smelting of nonferrous metals, particularly gold and copper, from coal residues (Tanaka, 2004).

Exposure

Arsenic exposure occurs from inhalation, absorption through the skin, and primarily by ingestion of arsenic-contaminated drinking water. The exposure to arsenic may come from natural sources, industrial processes (semiconductor manufacturing), commercial products, food, and medicines (Kosnett, 1990). Acute arsenic poisoning is only limited to homicidal or suicidal attempts.

Absorption, Distribution, and Excretion

Respiratory absorption of arsenic is a two-stage process involving deposition of the particles onto airway and lung surfaces, followed by absorption of arsenic from deposited particulates. Trivalent and pentavalent inorganic arsenic have been reported to cross the placenta in laboratory animals and humans (Bollinger et al., 1992). Recent studies indicate that the main form of arsenic bound to rat hemoglobin is dimethylarsinic acid (DMA), the primary metabolite of inorganic arsenic (Lu et al., 2004).

In humans as well as in most animal species, exposure to either arsenite or arsenate leads to an initial accumulation in the liver, kidneys, and lungs (Fujihara et al., 2004). Arsenic is a terribly poisonous material and there have been many reports of arsine poisoning in workers. Findings indicate induction of acute kidney injury by arsine. However, extensive investigations are required because reports describing ultrastructural findings are rare. Present evidence suggests incidents of acute arsine poisoning complicated by acute kidney injury as revealed by characteristics of the renal pathology (Lee et al., 2013). The clearance from these tissues is, however, rather rapid, and a long-term retention of arsenic is seen in hair, skin, squamous epithelium of the upper gastrointestinal tract, epidymis, thyroid, lens, and skeleton (Hughes et al., 2003). At 2–4 weeks after exposure ceases, most of the arsenic remaining in the body is found in keratin-rich tissues, such as skin, hair, and nails. Arsenic metabolism is characterized by two main types of reaction. First is reduction of the pentavalent arsenic to trivalent arsenic. This conversion of pentavalent arsenic species to trivalent arsenic species is catalyzed by arsenate reductase (Radabaugh and Aposhian, 2000; Radabaugh et al., 2002). Second is oxidative methylation reactions in which trivalent forms of arsenic are sequentially methylated to form monomethylated, dimethylated, and trimethylated products using *S*-adenosyl methionine as the methyl donor and GSH as an essential co-factor (Vahter, 2002). Pentavalent arsenic has been reported to be less toxic than inorganic trivalent arsenic. The metabolic methylation historically had been considered as a detoxification process. Recently, it has been established that trimethylated arsenicals, particularly monomethylarsinous acid (MMA(III)) and dimethylarsinous acid (DMA(III)), exist as intermediates in the metabolic methylation process of inorganic arsenic in humans (Mandal et al., 2001) and

are more active than the parent inorganic arsenic for enzymatic inhibition, cytotoxicity (Petrick et al., 2001), and genotoxicity (Nesnow et al., 2002). The major route of excretion after exposure to inorganic arsenic is via kidneys (Csanaky and Gregus, 2005).

Biochemical and Toxic Effects

Hematopoietic

The hematopoietic system is affected by both short-term and long-term arsenic exposure. Arsenic is known to cause a wide variety of hematological abnormalities like anemia, absolute neutropenia, leucopenia, thrombocytopenia, and relative eosinophilia, which is more common than absolute eosinophilia, basophilic stippling, increased bone marrow vascularity, and rouleaux formation (Rezuke et al., 1991). These effects may be due to a direct hemolytic or cytotoxic effect on the blood cells and a suppression of erythropoiesis. The mechanism of hemolysis involved depletion of intracellular GSH, resulting in the oxidation of hemoglobin (Saha et al., 1999). Arsenic exposure is also known to influence the activity of several enzymes of heme biosynthesis. Arsenic produces a decrease in ferrochelatase and a decrease in COPRO-OX, and an increase in hepatic 5-aminolevelinic acid synthetase activity (Woods and Southern, 1989). Sub-chronic exposure to arsenic has also been reported to inhibit δ-aminolevulinic acid synthetase and ferrochelatase activities, leading to increased uroporphyrin (URO) and coproporphyrin and COPRO urinary excretion. In chronically exposed humans, arsenic alters heme metabolism, as shown by an inversion of the urinary COPRO:URO ratio (Garcia-Vargas et al., 1996). Some recent studies also suggested a significant inhibition of blood δ-aminolevulinic acid dehydratase (ALAD) after sub-chronic and chronic arsenic exposure (Flora, 1999; Kannan et al., 2001; Flora et al., 2002). Anemia and leucopenia are common effects of poisoning and have been reported from acute, intermediate, and chronic exposure. These effects may be due to a direct effect of arsenic on the blood cells and a suppression of erythropoiesis. There was a proposal that the profile of urinary porphyrins could be used as early biomarkers for arsenic toxicity in humans chronically exposed to arsenic via drinking water.

Skin (Dermal)

Dermal changes most frequently reported in arsenic-exposed humans include hyperpigmentation, melanosis, hyperkeratosis, warts, and skin cancer. However, dermal effects appear to be highly dependent on time of exposure (Rossman, 2003). Arsenic-exposed skin cancer occurs mostly in unexposed areas such as trunk, palms, and soles. More than one type of skin cancer is reported, with the most common being Bowen disease, squamous cell carcinomas, basal cell carcinomas, and combined forms. It appears that skin cancer lesions related to arsenic exposure act as early warning signals of the subsequent development of cancers of internal organs many years later (Wong et al., 1998). Brittle nails, the surface of which are marked by transverse bands (leukonychia striata arsenicalis transverses), have been associated with arsenic poisoning; the characteristic bands are known as Reynolds Aldrich–Mees lines.

Hepatic

Arsenic is one of the first chemical agents that liver disease was attributed to in humans. Early symptoms in patients with arsenic-induced hepatic injury include bleeding esophageal varices, ascites, jaundice, or simply an enlarged tender liver (Rahman et al., 1999; Santra et al., 2000). Recent animal studies have shown that hepatic enzyme changes occur after arsenic exposure and these enzyme changes involved in the antioxidant defense system and membrane damage due to lipid per oxidation precede the pathomorphological lesions of arsenic-induced hepatic fibrosis in mice (Santra et al., 2000; Mishra et al., 2008).

Gastrointestinal

Gastrointestinal symptoms are common during acute poisoning. Gastrointestinal effects due to chronic arsenic poisoning are called arsenicosis. The patients report metallic taste and garlic odor. Clinical signs of gastrointestinal irritation due to acute arsenic poisoning include burning lips, painful swallowing, thirst, nausea, and abdominal colic (Goebel et al., 1990). The toxic effects of arsenic on the gastrointestinal mucosal vasculature are vasodilatation, transduction of fluid into the bowel lumen, mucosal vesicle formation, and sloughing of tissue fragments. Rupture of vesicle may cause bleeding, profuse watery stools (“rice-water stools”), and protein-losing enteropathy. The most likely mechanism of gastrointestinal toxicity is damage of the epithelial cells, with resulting irritations.

Respiratory

Studies from West Bengal, India, draw attention to both restrictive and obstructive lung disease. Respiratory disease is more common in patients with the characteristic skin lesions due to chronic arsenic toxicity (Mazumder et al., 2000). Humans exposed to inhalation of arsenic dust or fumes are more likely to be encountered in mining and milling ores and in industrial processing (such as smelting industry); this often produces irritation of mucous membranes, resulting in laryngitis, bronchitis, rhinitis, and tracheobronchitis, causing stuffy nose, sore throat, dyspnea, chest pain, and chronic cough (ATSDR, 2000). Pulmonary edema may occur, especially in cases of inhalation.

Cardiovascular

It has been suggested by several epidemiological studies that chronic inhalation of arsenic trioxide can increase the risk of death attributable to cardiovascular disease in humans (Saha et al., 1999). Both acute and chronic arsenic exposures cause altered myocardial depolarization and cardiac arrhythmias that may lead to heart failure. Arsenic causes direct myocardial injury, cardiac arrhythmias, and cardiomyopathy. Blackfoot disease, which causes gangrene of the foot and is unique to a limited area on the southwestern coast of Taiwan, is due to long-term exposure to high levels of inorganic arsenic in well water (range, 0.01–1.82 mg/L) (Tsai et al., 1999). Epidemiological studies also indicate that excess intake of arsenic leads to a variety of vascular diseases such as Blackfoot disease, Reynaud phenomenon, cardiovascular and cerebrovascular diseases, atherosclerosis, and hypertension (Simeonova and Luster, 2004).

Reproductive and Developmental

Reproductive and developmental effects of inorganic arsenic on humans and animal species have been reported. Limited animal studies suggest that arsenic can produce malformation, intrauterine death, and growth restriction (Gulob et al., 1998). Arsenic readily crosses the placenta. Sarkar et al. (2003) also suggested that spermatogenesis and/or sperm function might be impaired by organic arsenicals. Ahmad et al. (2001) observed pregnancy outcomes in women chronically exposed to arsenic through drinking water. The authors reported that arsenic contamination is a threat to healthy and safe pregnancy outcomes.

Neurological

After initial contradictory reports, it is now established that arsenic could cross the blood–brain barrier and produces alternations in whole rat brain biogenic amine levels in animals chronically exposed to arsenite (Tripathi et al., 1997). The neurological effects are many and varied. Usually, peripheral neuropathy, sensory neuropathy (Hafeman et al., 2005), and encephalopathy are the initial symptoms associated with acute arsenic poisoning. Acute exposure to arsenic in humans has been shown to result in problems of memory, difficulties in concentration, mental confusion, and anxiety (Rodriguez et al., 2003). Other neurological symptoms that arise because of arsenic are primarily those of a peripheral sensory neuritis, predominantly numbness, severe paresthesia of the distal portion of the extremities, diminished sense of touch, pain, heat, and cold, and symmetrically reduced muscle power (Menkes, 1997).

Diabetes Mellitus

Noninsulin-dependent (type 2) diabetes is the prevalent form of diabetes mellitus found in populations chronically exposed to inorganic arsenic from the environment (Rahman et al., 1998; Tseng, 2002). Type 2 diabetes is characterized by insulin resistance of internal organs and peripheral tissues, resulting in impaired glucose utilization and, consequently, in abnormally high blood glucose levels between meals and especially after meals. Insulin resistance and β-cell dysfunction can be induced by chronic arsenic exposure, and these defects may be responsible for arsenic-induced diabetes mellitus (Tseng, 2004).

Mechanisms of Toxicity

Arsenic in its free form generates free radicals, resulting in LPO, depletion of antioxidant enzymes, and DNA damage, thereby establishing oxidative stress as the major mechanism of As-induced toxicity and carcinogenicity (Ratnaike, 2003; Flora, 2011). Trivalent inorganic arsenicals, such as arsenite, readily react with sulfhydryl groups such as GSH and cysteine (Delnomdedieu et al., 1994). The complex between arsenic and vicinal sulfhydryl group is particularly strong. Arsenite inhibits PDH activity (Hu et al., 1998), perhaps by binding to the lipoic acid moiety. Inhibition of PDH ultimately leads to decreased production of ATP. Methylated trivalent arsenicals such as MMA^{III} are potent inhibitors of GSH reductase and thioredoxin reductase (Styblo et al., 1997). The inhibition may be due to the interaction of trivalent arsenic with critical thiol groups in these molecules. A mechanism of toxicity of pentavalent inorganic arsenic, such as arsenate, is its reduction to a trivalent form, such as arsenite. The reduction of arsenate to arsenite occurs *in vivo.* Another potential mechanism is the replacement of phosphate with arsenate. Oxidative injury causing damage to DNA molecules and various cell components, such as polyunsaturated fatty acid residues of phospholipids, amino acids, peptides, and proteins, has been reported as one of the foremost consequences of As exposure because these are susceptible targets of metal-induced ROS attack (Flora, 2011).

Oxidative Stress

Oxidative stress has now been established as one of the major mechanisms involved for arsenic-induced carcinogenesis. A number of recent reports provided direct evidence of inorganic arsenic-induced free radical formation or production of oxidative stress (Flora, 1999; Liu et al., 2000; Sun et al., 2005). ROS that damage DNA *in vitro* are generated from iron released from ferritin. The results suggest that some clastogenic effects of arsenic are mediated via free radicals (e.g., peroxynitrite, superoxide, hydrogen peroxide, and possibly free iron). Vega et al. (2001) suggested that arsenic exposure could increase the production of ROS, activation of transcription factors (e.g., AP-1, *c-fos* and NF-κB), and oversecretion of pro-inflammatory and growth-promoting

cytokines, resulting in increased cell proliferation and, finally, carcinogenesis. Increased ornithine decarboxylase activity is often interpreted as a biomarker for cell proliferation (Kitchin, 2001). Arsenite appears to have an effect on the cell cycle, which may alter cell proliferation. The amplification of the gene codes for the enzyme dihydrofolate reductase enhanced by arsenic.

Diagnosis

Clinical Features

Clinical features like skin lesions and neuropathy are crude and imprecise indicators of the severity of poisoning. White striae in the fingernails are also a useful clue in the diagnosis of arsenic toxicity. These white striae are also known as "Mee's line." Other symptoms are anemia, leucopenia or pancytopenia, gangrene of the feet (Blackfoot disease), hyperpigmentation, hypopigmentation, and hyperkeratosis. Keratosis and pigmentation are the characteristic skin lesions associated with arsenic toxicity. They are typically exhibited by diffuse thickening of palms and soles, alone or in combination with nodules and presence of raindrops in the form of numerous rounded hyperpigmented macules in the body (Tondel et al., 1999). Leucomelanosis is another common skin lesion, consisting of hypopigmented macules with a spotty white appearance. Elevated arsenic content in hair and nail segments, normally less than one part per million (ppm), may persist for months after urinary arsenic values have returned to baseline. They are preferable samples to detect and quantify exposure to arsenic, because absorbed arsenic accumulates in both hair and nails and elevated levels are noted within a few weeks after acute poisoning (Hinwood et al., 2003). The arsenic contents of the fingernails and toenails have also been used as bio-indicators of past arsenic exposure, and fingernail arsenic has been reported to be significantly correlated with hair arsenic content (Lin et al., 1998). The segmental growth of the hair shaft also provides valuable information about duration and type of arsenic poisoning (Kakkar and Jaffery, 2005).

Blood arsenic levels are highly variable. Blood arsenic, normally less than 1 μg/dL, may be elevated on acute intoxication. It is probably the most important diagnostic test for detecting arsenic exposure. Arsenic metabolites (inorganic arsenic + MMA + DMA) in urine have also been used as biomarkers of recent arsenic exposure (Yamauchi et al., 1989). A recent study by Yoshimura et al. (2011) showed that acute arsenic poisoning can also be confirmed by speciation analysis of arsenic compounds in the plasma and urine by HPLC-ICP-MS. Multiple blood purification methods can also be used as a combined treatment for acute arsine poisoning (Wu et al., 2010).

Clouded urine is frequently seen in most severely arsenic-intoxicated patients as absorbed arsenic if primarily excreted through urine (Hughes and Kitchin, 2006). Increased urinary protein content and aberrant excretion of trace elements also signify enhanced renal dysfunction (Xie et al., 2001). Although estimation of urinary arsenic is a consistent biomarker of exposure, it possesses few drawbacks, including accurate sample collection time and volume of urine to be voided.

Other Biomarkers

Essentially all organ systems must be evaluated. Thus, laboratory tests should include complete blood count, liver and renal functional tests, and blood, nail, and urine arsenic levels. Table 15.2 exhibits various biomarkers for effective diagnosis of arsenic intoxication. Other biomarkers of arsenic exposure include nonerythrocyte porphyrin enzyme activities and urine transforming growth factor TNF-α, accompanied by induction of heme oxygenase, mitogen-activated protein kinases, the ubiquitin-dependent proteolytic pathway, and protein kinase C in various tissues. These tests are still being investigated in laboratories and their clinical usefulness remains to be proven (Chapell et al., 2001). Cytogenetic markers such as chromosomal aberrations contribute significantly toward detection of this carcinogen. People with chronic arsenicosis have been shown to have increased chromosomal aberrations (Ghosh et al., 2006) and sister chromatic exchange (SCE). However, it is still not the most interesting biomarker to detect the précoce effects of arsenic and is difficult to use in large-scale epidemiological studies because of its cost and difficulty of implementation (Eslava, 2004). Micronuclei in isolated bladder and buccal cells have been considered possible target tissues from direct exposure to As in drinking water (Bonassi et al., 2007).

TREATMENT

Highly specific treatment is required for poisoning with arsenicals. There is no specific antidote for the treatment of arsine poisoning. Victims may be administered a high flow of oxygen. One of the first strategies for the treatment of arsine poisoning involves stopping the ongoing hemolysis, which may lead to renal dysfunction (Kilmecki and Carter, 1995). Exchange transfusion is currently the treatment of choice (James and Woods, 2006). Chelation therapy generally is not recommended to reduce hemolysis; however, chelating agents are shown to reduce arsenic in arsine-exposed subjects. Since World War II, dimercaprol has been the standard treatment for poisoning by arsenicals. DMPS (unithiol) and DMSA (succimer), dithiol water-soluble analogs of BAL, were developed in the Soviet Union and China in the late 1950s. These three agents were mainly used for the chelation treatment of arsenic and mercury intoxication for more than half a century. Studies indicate that dithiol chelators enhance arsenic excretion and support

TABLE 15.2 Routes of Exposure and Relevant Biomarkers for Various Arsenic Compounds

#	Compounds of Arsenic	Route of Exposure	Symptoms/Biomarkers	References
ARSINE				
		Inhalation	Vertical white lines on nails, discolored brown, red or black urine, free hemoglobin in urine, red/bronze tint in skin	James and Woods (2006)
ORGANIC ARSENICALS				
(a)	Methyldichloroarsine	Dermal exposure	Blistering in skin, "dry-land drowning"	Bennett and Dill (1994); Pitten et al. (1999)
(b)	Diphenylchloroarsine	Inhalation	Irritation in the mucous membranes of eyes and nose, painful blistering in skin	Ochi et al. (2004); Kato et al. (2007)
(c)	Ethyldichloro-arsine	Dermal, inhalation	Fluid-filled blisters form in skin, pulmonary edema or "dry-land drowning"	Bartelt-Hunt et al. (2006)
(d)	Lewisite	Dermal, inhalation,	Irritation and blistering of the skin, Immediate eye pain and blepharospasm	McManus and Huebner (2005)
INORGANIC ARSENIC				
		Inhalation, dermal, ingestion	Hyper pigmentation, melanosis, hyperkeratosis, warts, decrease in ferrochelatase, increase in hepatic 5-aminolevelinic acid synthetase activity, pulmonary edema, peripheral neuropathy, sensory neuropathy and encephalopathy, diabetes mellitus, clouded urine, SCE	Woods and Southern (1989); Mazumder et al. (2000); Rossman (2003); Hafeman et al. (2005); Ghosh et al. (2006); Hughes and Kitchin (2006)

a therapeutic role for these chelators in the prompt treatment of acute poisoning by arsenic (Kosnett, 2013). However, treatment should be initiated as rapidly as possible because delay may result in reduction of efficacy as the time interval between metal exposure and onset of chelation increases. DMPS and DMSA have a higher therapeutic index than BAL and do not redistribute arsenic or mercury to the brain. Although chelation may accelerate metal excretion, potential therapeutic efficacy in terms of decreased morbidity and mortality is largely unestablished in cases of chronic metal intoxication. In the next paragraphs, we discuss the efficacy of chelating agents for treating arsenicals, their drawbacks, and the current advancement in the area.

Chelating Agents and Chelation Therapy

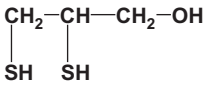
Chelating agents are organic compounds capable of linking together metal ions to form complex ring-like structures called chelates. Chelators act according to a general principle: the chelator forms a complex with the respective (toxic) ion, and these complexes reveal a lower toxicity and are more easily eliminated from the body. This mechanism could be represented as:

$$\text{Metal} - \textit{in vivo}\,\text{site} + \text{chelating agent} \rightarrow \textit{in vivo}\,\text{site} + \text{metal–chelating agent complex}$$

Chelation thus may be defined as the incorporation of a metal ion into a heterocyclic ring structure (Flora and Sekhar, 2004).

Some of the chelating agents listed here have been reported to be useful in the treatment of arsenic:

2,3-Dimercaprol (Dimercaprol; British Anti-Lewisite, BAL)



2,3-Dimercaprol (British anti-lewisite (BAL)) was originally developed to treat the effects of lewisite, namely systemic poisoning and local vesication. The empirical formula of BAL is $C_6H_8OS_2$ and its molecular weight is 124.21. It is an oily, clear, colorless liquid with a pungent, unpleasant smell typical of mercaptans and has a short half-life.

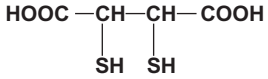
Drawbacks

BAL is unstable and easily oxidized; therefore, it is difficult to store and requires ready-to-use preparation. In addition to rapid mobilization of arsenic from the body, it causes a significant increase in brain arsenic (Hoover and Aposhian, 1983; Flora and Pachauri, 2010). Due to its oily

nature, administration of BAL requires deep intramuscular injection that is extremely painful and can cause an allergic reaction (Flora and Tripathi, 1998).

Two water-soluble analogs of dimercaprol have been studied as lewisite antidotes. They are meso 2,3-dimercaptosuccinic acid (DMSA) and 2,3-dimercapto-1-propane sulfonic acid (DMPS). These two drugs circumvent two major disadvantages associated with treatment with BAL: the need for intramuscular injection and limitation of dose by toxicity.

Meso 2,3-Dimercaptosuccinic Acid

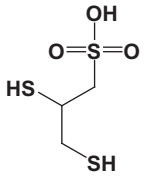


One of the chemical derivatives of dimercaprol (BAL) is DMSA. DMSA is an orally active chelating agent that is much less toxic than BAL and its therapeutic index is approximately 30-times higher. The empirical formula of DMSA is $C_4H_6O_4S_2$ and its molecular weight is 182.21. It is a weak acid that is soluble in water.

Drawbacks

DMSA distribution is predominantly extracellular because it is unable to cross the hepatic cell membrane. Hence, it is able to chelate arsenic from extracellular sites but not from intracellular sites. Adverse reactions of DMSA include gastrointestinal discomfort, skin reaction, mild neutropenia, and elevated liver enzymes. Some evidence of embryo toxicity/fetal toxicity due to DMSA administration was also reported (Domingo, 1998).

Sodium 2,3-Dimercaptopropane-1-Sulfonate



DMPS, first introduced as "Únithiol" as a BAL analogue, has the empirical formula $C_3H_7O_3S_3Na$ and molecular weight of 210.3. DMPS is more effective than BAL for arsenic therapy in mice (Hauser and Weger, 1989). DMPS, being hydrophilic, is distributed in the extracellular space but may enter cells by specific transport mechanisms. DMPS appears to be bio-transformed in humans to acyclic and cyclic disulfides, which are rapidly eliminated from the body through the kidneys. However, it is important to note that this drug does not redistribute arsenic, lead, or inorganic mercury to the brain (Flora and Pachauri, 2010).

There have been a number of published reports available for the treatment of chronic arsenic exposure in animals, but not for the treatment of subjects exposed to arsenic compounds.

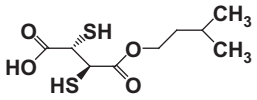
Drawbacks

No major adverse effects after DMPS administration in humans or animals have been reported (Hruby and Donner, 1987). Oral administration of DMPS did not adversely affect late gestation, parturition, or lactation in mature mice, and fetal and neonatal development does not appear to be adversely affected (Domingo, 1998).

Monoesters of DMSA

Recently, some monoesters and diesters of DMSA, especially the higher analogues, have been developed and tried against cases of experimental heavy metal poisoning to address the shortcomings of DMSA, particularly in depleting intracellular arsenic (Aposhian et al., 1992; Mehta et al., 2002; Kalia and Flora, 2005; Flora et al., 2007b,c). Due to its lipophilic nature, it can easily cross the cell membrane and chelate arsenic from intracellular and extracellular sites. This is the best advantage of treatment with DMSA monoesters. Among these monoesters are monoisoamyl DMSA (MiADMSA), mono *n*-amyl DMSA, mono *n*-butyl, and mono iso-butyl DMSA. MiADMSA was the most effective in increasing the survival of mice and in depleting arsenic tissue burden (Kreppel et al., 1995; Flora et al., 2004). Several recent published studies clearly point to the fact that the analogues of DMSA were capable of crossing the membranes and were more effective in reducing the toxic metal burden in acute and sub-chronic arsenic intoxication (Flora et al., 2004a,b, 2003; Flora and Sekhar, 2004; Saxena and Flora, 2004; Saxena et al., 2005). Most of these conclusions are based on experimentally induced metal intoxication, and no clinical data are available so far.

Monoisoamyl DMSA



Monoisoamyl DMSA (MiADMSA) is a C_5-branched chain alkyl monoester of DMSA. Kreppel et al. (1995) also reported the superior efficacy of MiADMSA and mono *n*-amyl DMSA in protecting mice from the lethal effects of arsenic and in reducing body burden from arsenic. These studies thus support that MiADMSA could be a potential drug to be used in the treatment of chronic arsenic poisoning. MiADMSA is new and is one of the most effective of the vicinal class of metal mobilizing agents (Xu et al., 1995; Saxena et al. 2005). Although, the compound is more

toxic than the parent diacid DMSA (Mehta et al., 2002; Flora and Mehta, 2003), its structure features and recent experimental evidence suggest that it might be effective in chelating arsenic (Flora and Kumar, 1993; Flora et al., 2002). More pronounced efficacy of MiADMSA could be attributed to its lipophilic characteristic (Flora et al., 2005). It appears plausible that MiADMSA could decrease the oxidative stress in tissues either by removing arsenic from the target organs or by directly scavenging ROS via its sulfhydryl group (Mishra et al., 2008).

No report is available regarding the therapeutic efficacy of MiADMSA against lewisite toxicity and other arsenicals. However, we reported the effect of MiADMSA on the reversal of gallium arsenide (GaAs)-induced changes in the hepatic tissue (Flora et al., 2002). MiADMSA was found to be better than DMSA in mobilizing arsenic and in the turnover of the GaAs-sensitive biochemical variables. A study was conducted to explore the optimum dose and route of administration for achieving maximum arsenic elimination with minimal side effects. Pharmacokinetic analysis of MiADMSA was conducted, including oxidative stress parameters, identification of arsenic levels in soft tissues, liver function test, and histopathological studies. Plasma kinetics involving MiADMSA (plasma-free drug and total drug) at 50 and 100 mg/kg orally was performed. MiADMSA at a 50 mg/kg dose administered orally provided approximately 45% and 75% protection against oxidative stress and in lowering body arsenic burden, respectively. Also, pharmacokinetic analysis supported prolonged availability of the drug through oral administration. Collectively, these findings led to the conclusion that oral administration of MiADMSA was more effective than intraperitoneal administration and that the minimum effective dose with the least side effects was 50 mg/kg (Flora et al., 2012). Generation of free radicals in case of arsenic toxicity is known to cause cellular apoptosis through a mitochondrial-driven pathway. A study was conducted to investigate the effect of arsenic interactions with various complexes of the electron transport chain and to evaluate if the complex could trigger apoptosis. Another aspect evaluated was chelation with MiADMSA and reversal of detrimental effects. Arsenic induced free radical generation in rat neuronal cells, leading to diminished mitochondrial potential and enzyme activities of all the complexes of the electron transport chain. MiADMSA, however, was able to reverse most of these arsenic-induced altered variables to various extents; still, DNA damage remained unaffected (Dwivedi et al., 2011). Ram Kumar et al. (2013) also reported that MiADMSA significantly reversed the As-induced alterations in behavior and biochemical variables suggestive of oxidative injury. As-exposed rats showed significant differences in behavioral functions and water maze learning. Further, biochemical studies performed also showed significant alteration, which was effectively restored on MiADMSA treatment. Not only in vivo studies but also in vitro studies have revealed the therapeutic efficacy of MiADMSA against arsenic-induced toxic manifestations. Arsenic toxicity leads to various skin manifestations and arsenic accumulation in keratinized tissue. Hence, a study was designed to investigate time-dependent and dose-dependent effects of arsenic using the HaCaT cell line along with evaluation of treatment with MiADMSA. Results interestingly depicted that pretreatment of cells with MiADMSA elicited significant protection against arsenic-induced oxidative stress and apoptotic cell death. The findings are of clinical relevance and suggest MiADMSA to be a promising candidate in protecting skin against arsenic-induced toxic effects (Pachauri et al., 2013).

Despite a few drawbacks/side effects associated with MiADMSA, these results suggest that MiADMSA may be a future drug of choice because of its lipophilic character and the absence of any metal redistribution. However, significant copper loss requires further studies (Mehta et al., 2002; Mehta et al., 2006). Moderate toxicity after repeated administration of MiADMSA may be reversible after the withdrawal of the chelating agent.

Drawbacks

It is reported that the toxicity of DMSA with LD_{50} of 16 mmol/kg is much lower than the toxicity of MiADMSA with LD_{50} of 3 mmol/kg but lesser than BAL (1.1 mmol/kg). Flora and Mehta (2003) reported that administration of MiADMSA led to no major alternations in the heme synthesis pathway except for a slight increase in the zinc protoporphyrin levels, suggesting mild anemia. MiADMSA was seen to be slightly more toxic in terms of copper and zinc loss and some biochemical alterations in the hepatic tissue in females as compared with male rats (Mehta et al., 2006).

Role of Antioxidants

Oxidative stress can be partially implicated in arsenic toxicity, and a therapeutic strategy to increase the antioxidant capacity of cells may fortify the long-term effective treatment of arsenic poisoning. This may be accomplished either by reducing the possibility of metal interacting with critical biomolecules and inducing oxidative damage or by bolstering the antioxidant defenses of the cells through endogenous supplementation of antioxidant molecules (Flora et al., 2007b; Flora et al., 2013). Although arsenic-induced oxidative stress has been well-documented, the usefulness of antioxidants in conjunction with chelation therapy has not yet been extensively investigated. *N*-acetylcysteine (NAC) is a thiol, a mucolytic agent, and a precursor of L-cysteine and reduced GSH. NAC is a source of sulfhydryl-containing antioxidant that has been used to mitigate various conditions

of oxidative stress. Combined administration of NAC and succimer after arsenic exposure led to a significant recovery in biochemical variables indicative of oxidative stress and arsenic depletion from soft organs (Flora, 1999; Kannan and Flora, 2006). Various vitamins have been found to reduce the toxic manifestation of heavy metals (Flora et al., 2002; Flora et al., 2013). Combined administration of vitamin C plus DMSA and vitamin E plus MiADMSA led to a more pronounced depletion of brain arsenic. Also, supplementation of vitamins led to significant restoration of inhibited blood δ-ALAD activity and other oxidative stress parameters in liver, kidneys, and brain. These results suggested that co-administration of vitamin E or vitamin C may be useful in arsenic poisoning, although it has only a limited role in depleting arsenic burden (Kannan and Flora, 2004). In another study, alpha-lipoic acid was shown to have substantial antioxidant properties when administered (70 mg/kg body weight) once daily for 60 days along with arsenic. Effects of alpha-lipoic acid over arsenic-induced oxidant production and LPO level in discrete brain regions of rats were also examined. Cortex, hippocampus, and striatum exhibited a greater increase of LPO levels than did the cerebellum and hypothalamus. Also, simultaneous lipoic acid treatment along with arsenic proved to be sufficient in reducing oxidant production and LPO levels in all rat brain regions. The study demonstrated that arsenic-induced deficits in brain regions can be overcome through simultaneous treatment with lipoic acid (Shila et al., 2005). Garlic is another well-known folk remedy for a variety of ailments; however, very few studies are available suggesting its beneficial role against arsenic toxicity. In a study by Flora et al. (2009), the protective efficacy of aqueous garlic extract on parameters suggestive of hepatic injury, tissue oxidative stress, and mobilization of arsenic was investigated using two different doses. Results suggested that garlic extracts contain strong antioxidant properties that could be beneficial in preventing arsenic-induced toxicity in cells. A study was conducted in rabbits to assess the oxidative injuries caused by arsenic toxicity and to evaluate the detoxifying effects of exogenous antioxidants, vitamins, zinc, selenium (VZS), or a plant polyphenol. Results in studies using rabbits indicated that arsenic induces toxicity associated with an increase in LPO and nitric oxide production in the body. Administration of exogenous antioxidants such as polyphenols and a recipe of vitamins, zinc, and selenium, however, was found to be useful for arsenic detoxification (Rabbani et al., 2003). Taurine (2-aminoethanesulfonic acid) has also been shown to exert a protective effect against arsenic-induced cytotoxicity in murine hepatocytes. The cytoprotective activity of taurine against arsenic poisoning was found to be comparable with that of a known antioxidant, vitamin C. This further suggests that taurine protects mouse hepatocytes against arsenic-induced cytotoxicity (Sinha et al., 2007). Among various herbal extracts, *Moringa oleifera* (*M. oleifera*) seed powder has also been shown to restore arsenic-induced oxidative stress and to reduce body arsenic burden. Hence, it was concluded that concomitant administration of *M. oleifera* seed powder with arsenic could significantly protect against arsenic-induced toxic manifestations and thus could also be beneficial during chelation therapy with a thiol chelator (Gupta et al., 2007). Concomitant oral supplementation of *Centella asiatica* (100, 200, or 300 mg/kg orally once daily) during arsenic exposure (20 ppm in drinking water for 4 weeks) has also been shown to be an effective strategy against arsenic toxicity. More extensive studies are recommended for determining the effect of co-administration of *C. asiatica* during chelation therapy with a thiol chelator (Gupta and Flora, 2006).

Not only altered biochemical variables but also supplementation of antioxidants has led to alleviation of arsenic-induced molecular alterations. Contamination of arsenic in drinking water is associated with several human diseases, including cancer. A significant increase in the levels of protein oxidation, DNA strand breaks, and DNA–protein cross-links was observed in blood, liver, and kidney of rats exposed to arsenic (100 ppm in drinking water) for 30 days. However, co-administration of ascorbic acid and alpha-tocopherol to arsenic-exposed rats showed a substantial reduction in the levels of arsenic-induced oxidative products of protein and DNA. The results support the fact that free radical generation is one of the major mechanisms of arsenic-induced toxic manifestations and suggest that ascorbic acid and alpha-tocopherol supplementation can improve the arsenic-induced molecular alterations (Kadirvel et al., 2007). Cardiac dysfunction has been shown to be associated with arsenic toxicity, which results in reduced cardiomyocyte viability, increased ROS production, and intracellular calcium overload, and has induced apoptotic cell death by mitochondrial-dependent caspase-3 activation and poly-ADP ribose polymerase (PARP) cleavage. All these changes were found to be associated with increased IKK and NF-kappaB (p65) phosphorylation. Arsenic also markedly increased the activity of p38 and JNK MAPKs. Taurine effectively suppressed these apoptotic actions, suggesting its protective role by attenuation of p38 and JNK MAPK signaling pathways. Results suggest that taurine effectively prevented arsenic-induced myocardial pathophysiology, attenuated NF-kappaB activation via IKK, p38, and JNK MAPK signaling pathways, and hence could be an effective therapy against As-induced cardiovascular burden (Ghosh et al., 2009).

Combination Treatment

As discussed, metal chelators are administered to increase the excretion of arsenic but, unfortunately, the

use of these chelators is comprised by a number of drawbacks (Mehta and Flora, 2001). These drawbacks mean that it is necessary to search for new treatments with no side effects. A number of strategies have been discussed (Kalia and Flora, 2005). Among these strategies, combination therapy is a new and a better approach to treat cases of metal poisoning (Flora et al., 2007c; Mishra et al., 2008). We investigated if co-administration of thiol chelators like meso 2,3-dimercaptosuccinic acid (DMSA) or sodium 2,3-dimercaptoprpane 1-sulfonate (DMPS) along with a newly developed thiol chelator, monoisoamyl DMSA, is more beneficial than monotherapy with these chelators in counteracting chronic arsenic toxicity (Flora et al., 2005; Bhadauria and Flora, 2007). It was concluded that concomitant administration of DMSA, a chelator known for its extracellular distribution with a lipophilic chelator, like MiADMSA, could play a significant and important role in abating a number of toxic effects of arsenic in animals compared with treatment with these chelators alone. We suggested that analogues with long carbon chain (MiADMSA and MchDMSA) are better chelators than a chelator with a shorter carbon chain (MmDMSA) or DMSA. It is assumed that analogues of DMSA eliminate arsenic simultaneously from the cell and provide assistance in bringing GSH homeostasis toward normalcy. Further combination therapy with DMSA and MiADMSA or MchDMSA was proven to be more beneficial than combined treatment with MmDMSA and DMSA (Mishra et al., 2008). Generation of ROS is one of the major mechanisms of arsenic-mediated oxidative stress; a study was conducted to explore the kinetic relationship of ROS with calcium and to dissect the calcium ion channels responsible for calcium imbalance after arsenic exposure. Another crucial aspect investigated was whether monotherapy or combinational chelation therapy prevents arsenic-induced neuronal apoptosis in guinea pigs. Results indicated that arsenic caused a significant increase in ROS, followed by NO and calcium influx, which was mainly dependent on L-type voltage-gated channels that disrupt mitochondrial membrane potential and increase bax/bcl2 levels and caspase 3 activity, leading to apoptosis. Another interesting and useful finding was that combination therapy of DMSA and MiADMSA was most effective in reversing arsenic-induced alterations. Results provide strong evidence for the role of L-type calcium channels in regulating arsenic-induced calcium influx and that DMSA plus MiADMSA combination therapy might be a better and much more effective strategy than monotherapy in mitigating chronic arsenicosis (Pachauri et al., 2013).

Not much experimental evidence is available and there is a need for in-depth investigation in this area. Investigating the effects of combination therapy, particularly in case of chronic arsenic poisoning, in which a strong chelating agent is administered along with another structurally different chelating agent, is necessary (Kalia and Flora, 2005) to evaluate if combination treatment is able to promote the elimination of arsenic and restore arsenic-induced biochemical and clinical alterations.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Arsenic is used in industries such as agricultural production and appears in the food chain. Although information about arsenic and its inorganic and organic derivatives is now widely reported, there is very little information about their use as CW agents. The use of arsenicals remains a potential threat because they are relatively easy to manufacture and may cause significant morbidity and mortality. Knowledge about these arsenicals is very important to plan a response in an emergency. Lewisite is one arsenical that has not been studied in detail. There is very little information available regarding the detailed toxic effects of organic arsenicals, particularly carcinogenicity, mutagenicity, and teratogenicity. Lewisite, an organic arsenical war gas that is a vesicant, has been reported for its ability to bind with the thiol group, leading to the possibility of its undesirable effects on a variety of enzymes. Thus, there is a very strong possibility that exposure to lewisite might also lead to carcinogenic effects, but this hypothesis requires experimental and epidemiological evidence. It is not known if lewisite is persistent. However, arsenic is an elemental poison and any residual hydrolysis, combustion, and decontamination product is likely to contain an arsenical compound. Some of the major areas for future research direction include the possibility of delayed or latent effects arising after organic arsenical (particularly lewisite) exposure. These effects have been studied in detail recently after inorganic arsenic exposure; however, there is very little information available after organic arsenical exposure. Development of antidotes is another area that requires immediate attention. Although one approved antidote, BAL, is available for lewisite. However, further investigations are required with other derivatives of BAL, like DMSA and DMPS, or monoesters of DMSA, like MiADMSA. Chelators are generally not of any immediate benefit as far as toxicity of arsine once hemolysis has begun. Some new chelating agents are in experimental stages like MiADMSA, but the effectiveness and safety of these chelating against arsine poisoning is unknown. Toxicodynamics of arsine is also one area that requires exploration because this information will be of immense help in developing a suitable antidote, particularly for impending hemolysis by removing or displacing arsine.

Inorganic arsenic, particularly arsenic (III), is a well-documented potent carcinogen causing cancer of bladder, lung, skin, and possibly liver and kidney. Because of failures in attempts to study the carcinogenic effects of arsenic in animal models, the mechanism of arsenic-induced carcinogenic effects remains unclear. The newly discovered potency of trivalent methylated arsenic metabolites opens new opportunities for mechanistic studies. No treatment of proven benefit is available to treat chronic exposure. Treatment options advocated are vitamins, mineral supplements, and antioxidant therapy. The benefits of these treatment measures need to be evidence-based to receive endorsement and wider application. Further research work is also recommended in the following areas: molecular mechanism of action of clinically important chelators; intracellular and extracellular chelation in relation to mobilization of aged arsenic deposits and the possible redistribution of arsenic to sensitive organs as the brain; effect of metal chelators on biokinetics during continued exposure to arsenicals; combined chelation with lipophilic and hydrophilic chelators; use of antioxidants, micronutrients, or vitamins as complimentary agents or antagonists; minimization of the mobilization of essential trace elements during long-term chelation; and fetotoxic and teratogenic effects of chelators.

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CHAPTER

16

Fluoroacetate

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INTRODUCTION

Fluororganic compounds attracted the attention of researchers more than 70 years ago, when a group of very toxic compounds was isolated from a large class of biologically inert chemicals, having the general formula CH_2FCOOR and the common name *fluoroacetate (FA)*. The toxicological effects of FA do not become apparent immediately, even after exposure to lethal doses, but only after a latent period of half an hour to several hours for animals and humans. The level of FA in some plants can reach up to 5 g/kg dry weight (Hall, 1972) and can cause death of livestock and domestic animals, sometimes with appreciable economic damage (McCosker, 1989; Minnaar et al., 2000a; Lee et al., 2012). FA can be found in fog and raindrops in some industrial regions (Rompp et al., 2001). The best-known representative of FA is its sodium salt (SFA, also known as *compound 1080*). This substance is used in several countries for controlling populations of some vertebrates, sometimes causing the death of farm animals (Giannitti et al., 2013). There are also series of fluorocompounds whose metabolism is connected with the formation of FA. These include the following:

- Antineoplastic drugs (5-fluorouracil and isomers of fluoronitrosourea)
- *N*-(2-fluoroethyl) derivatives of the narcotic analgesics normeperidin and normethazocin
- Pesticides, 1,3-difluoro-propanol and fluoroacetamide (FAA, compound 1081)
- 1-(di)halo-2-fluoroethans
- Fluoroethanol (Reifenrath et al., 1980; Tisdale and Brennan, 1985; Feldwick et al., 1998)

The urgency of the problems associated with FA toxicity and the need for an effective therapy for acute intoxication has greatly increased in connection with a more recent threat—international terrorism (Holstege et al., 2007). Physicochemical features of FA, the lack of taste and odor, delayed manifestation of toxicity, and similarity of clinical signs of intoxication to some natural indispositions—these characteristics necessitate comprehensive studies of mechanisms of action of FA and search for effective therapeutic means for treatment of acute intoxication.

BACKGROUND

FA was initially synthesized in 1896 and only decades after that was found in *Dichapetalum*, *Gastrolobium*, *Oxylobium*, *Acacia*, *Palicourea*, *Mascagnia*, *Tanaecium*, and *Amorimia* plants growing in Australia, South and Central Africa, and South America (Oerlichs and McEwan, 1961; de Oliveira, 1963; McEwan, 1964; Aplin, 1971; Vickery et al., 1973; Camboim et al., 2012a; Lee et al., 2012; Esters et al., 2013). Chemically pure FA is a very stable compound, and the energy of dissociation of the fluorocarbon bond in the molecule is considered one of the highest of the natural compounds (Ichiyama et al., 2004). However, FA is broken down in biological preparations from plants (Minnaar et al., 2000a). In soils, the major degradation pathway for SFA occurs through microbial degradation to the hydroxyl metabolite, hydroxyacetic acid, and microbial mineralization to CO_2; temperature, rather than soil type or moisture content, was the dominant factor affecting the rate of degradation (Northcott et al., 2014). Seven

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bacteria from soil and plant samples able to degrade SFA were identified by 16S ribosomal ribonucleic acid (rRNA) gene sequencing as *Paenibacillus* sp. (ECPB01), *Burkholderia* sp. (ECPB02), *Cupriavidus* sp. (ECPB03), *Staphylococcus* sp. (ECPB04), *Ancylobacter* sp. (ECPB05), *Ralstonia* sp. (ECPB06), and *Stenotrophomonas* sp. (ECPB07) (Camboim et al., 2012a). Also, two SFA-degrading bacteria from caprine rumen, *Pigmentiphaga kullae* (ECPB08) and *Ancylobacter dichloromethanicus* (ECPB09) (Camboim et al., 2012b), and one from bovine rumen belonging to the phylum Synergistetes (Davis et al., 2012) were identified, the latter functioning in anaerobic conditions.

After inhalation or ingestion, FA is easily absorbed by tissues, and its high toxicity is independent of its route of entry into organisms (Chenoweth, 1949). The mechanism of toxic action of FA is widely known as "lethal synthesis" (Peters, 1952; Peters and Wakelin, 1953), the essence of which is conversion of nontoxic FA to toxic fluorocitrate (FC) within the cells of an organism. The main reasons of death are considered to be imbalance of intracellular ions, osmotic imbalance, and deficit of adenosine triphosphate (ATP) as a consequence of aconitase blockade (Buffa et al., 1973). The latent period, from the moment of poisoning with FA to manifestation of clinical signs, is 0.5–3 h (in warm-blooded animals). This period reflects penetration of FA into blood and cells and conversion of FA to FC, with the consequent uncoupling of intracellular metabolism. Death usually occurs within 24–48 h, but can happen later. At autopsy, there are no specific signs of intoxication (Peters et al., 1981). For warm-blooded animals that are the least adapted to FA, the lethal dose is less than 2 mg/kg (Atzert, 1971). However, there is a considerable species-specific difference in clinical signs of intoxication and differences in sensitivity to the poison (Chenoweth, 1949). The mean lethal dose varies from 0.05 mg/kg for dogs to 150 mg/kg for possums. The most common criterion of tolerance or sensitivity of animals to FA is intensity of metabolism. Thus, in the lizard *Tiliqua rugosa*, the level of metabolism of FA is 10 times lower than that of the rat *Rattus norvegicus*, and the lethal dose for the lizard is 100 times higher than that of the rat (Twigg et al., 1986). Low intensity of metabolism means low conversion of FA to FC, which makes possible more effective excretion and detoxication. In the absence of specific clinical, physiological, and morphological signs of intoxication, determination of FA in tissues with citrate and fluoride ions can be a diagnostic confirmation of FA intoxication (Schultz et al., 1982; Koryagina et al., 2006).

TOXICOKINETICS

Detoxication

The main pathway of detoxication of FA is its defluorination via a GSH-dependent mechanism involving nucleophilic attack on the β-carbon atom and formation of fluoride and *S*-carboxymethylglutathion, with subsequent cleavage of the latter into amino acids and *S*-(carboxymethyl) excreted in the urine as a conjugate complex (Mead et al., 1979; Tecle and Casida, 1989). The highest defluorinating activity was found in liver, followed by kidney, lung, heart, and testicles in a descending line. No defluorinating activity was found in the brain. Activity of enzymes responsible for defluorination depends on glutathione (GSH) concentration with a maximum above 5 mmol/L, the apparent Km being 7 mmol/L at saturating concentrations of GSH (Soiefer and Kostyniak, 1983). Defluorination is mainly carried out by anionic proteins having GSH transferase activity, though the anionic fraction contains nearly 10% of proteins without this activity, but it is also capable of defluorination of FA. Moreover, cationic enzymes were shown to be responsible for about 20% of cytosolic defluorination of FA (Wang et al., 1986). The GSH-dependent enzyme defluorinating FA is not identical to GSH-dependent *S*-transferases; it is a FA-specific defluorinase having an acidic isoelectric point (pH = 6.4) and a molecular weight of 41 kD (27 kD for the main subunit; Soiefer and Kostyniak, 1984). Activity and specificity of defluorinase isoenzymes vary markedly and is a subject of research presently (Tu et al., 2006; Nakayama et al., 2012).

Analytical Procedure

Analysis of biological samples for FA is rather problematic because of the high polarity of the fluorine-carbon bond in the molecule. Liquid chromatography has been applied for analysis of FA in different media (Allender, 1990), and analysis of FA in plants and gastric content by high-pressure liquid chromatography with ultraviolet detection has also been described (Minnaar et al., 2000b). Being a nonvolatile substance, FA was commonly analyzed by gas chromatography (GC), as a methyl derivative (Stevens et al., 1976), ethyl or *n*-propyl derivatives (Peterson, 1975) and as pentafluorobenzyl esters (Vartiainen and Kauranen, 1984). Derivatization with 2,4-dichloroaniline in the presence of *N*,*N*-dicyclohexylcarbodiimide was used for GC analysis of SFA in water (Ozawa and Tsukioka, 1987) and blood serum (Demarchi et al., 2001). A modified procedure by Eason et al. (1994) achieved low detection limits for FA at the level of 0.01 µg/g in plasma and urine, and 0.002 µg/g in the tissue and feces of sheep and goats. However, this procedure is labor and time-consuming, and the GC-electron capture detection procedure applied is considered unreliable at this level of sensitivity.

The main problem for GC analysis of FA in biological samples is co-elution of the matrix components. This can be overcome by sampling of the analyte from an

equilibrium vapor phase. Static head-space analysis of SFA as ethyl fluoroacetate, with linear range for SFA in water of 5–200 µg/mL and a detection limit of 0.5 µg/mL has been reported (Mori et al., 1996). Solid-phase microextraction (SPME) from an equilibrium vapor phase has all the advantages of head-space analysis, whilst being a much more sensitive technique. We reported on a novel procedure for determination of FA in water and biological samples, involving ethylation of FA with ethanol in the presence of sulfuric acid, SPME of the ethyl fluoroacetate formed with subsequent analysis by GC-MS (Koryagina et al., 2006). To overcome the problem of the presence of the components co-eluting with FA derivatives, we made use of GC-MS in the Selective Ion Monitoring mode. To avoid a partial overlapping of the internal standard's peak with the sample matrices' components, quantification was performed with the use of two internal standards, carbon tetrachloride and toluene. The calibration plots for the determination of SFA in biological samples was linear in the SFA concentration range 0.01–5.0 µg/mL for both internal standards, and a linear relationship in blood plasma was observed in the range of 0.01–5.0 µg/mL ($r = 0.95$). With toluene as internal standard, the linear regression equation was $Y = 0.014X$ (Y was the ratio S(EthylFA)/S(toluene); X was the concentration of SFA, µg/mL). The relative standard deviation (RSD) for FA quantification at 0.1 µg/mL was 12% ($n = 5$). With carbon tetrachloride as internal standard, a linear relationship in plasma was observed in the range of 0.01–5.0 µg/mL ($r = 0.98$). The linear regression equation was $Y = 0.1656X$ (Y was the ratio S(EthylFA)/S(CCl4); X was the concentration of FA, µg/mL). The RSD for FA quantification at 0.1 µg/mL was 6% ($n = 5$), and the detection limit was 0.01 µg/mL ($S/N = 3$). The calibration characteristics of rat organ homogenates were identical to that of plasma.

Distribution in Tissues and Elimination

The data on toxicokinetics of FA are rather contradictory, apparently depending on analytical procedures and dose of the poison; also, there is evidence for animal species specificity. The first data on toxicokinetics of FA demonstrated its rather uniform distribution between organs, with some predominance in heart, brain, and kidneys (Gal et al., 1961). The half-life was calculated to be not less than 2 days, and this could cause secondary toxicity arising from ingestion of meat from the poisoned animals (Aulerich et al., 1987). The half-life of FA was shown to be 10.8 h for sheep and 5.4 h for goats that were administered 0.1 mg/kg; maximal concentration of FA 2.5 h after the poisoning was revealed in blood plasma (0.098 µg/mL), followed by kidneys (0.057 µg/g), skeletal muscles (0.042 µg/g), and liver (0.021 µg/g). Only traces of FA were found in all the tissues examined 96 h after the poisoning

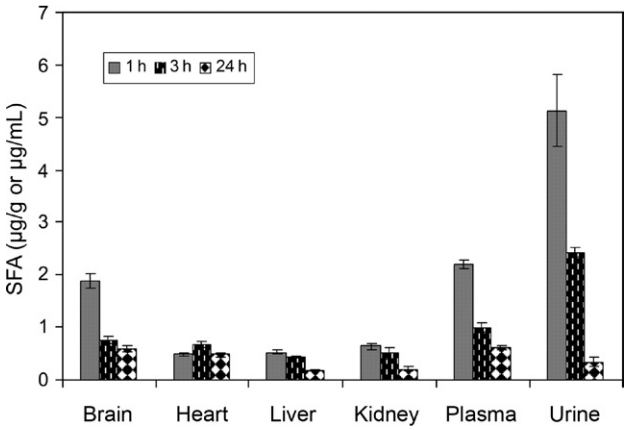

FIGURE 16.1 Data on the determination of FA (recounted as SFA) in rat organ homogenates and body fluids, at times following poisoning with SFA at a peroral dose of 2 mg/kg (1/2 LD_{50}). Standard deviations (shown) were based on 4–6 replicate analyses.

(Eason et al., 1994). At 1 and 12 h after introduction of SFA (0.25 mg/kg) to rats, a similar ratio of FA was found in rat plasma (0.26 and 0.076 µg/mL, correspondingly) (Eason and Turck, 2002), the half-life period being 2.9 h. On the other hand, for rabbits under subacute intoxication with FA, the half-life was found to be 1.1 h, and the level of FA in rabbit muscles, kidneys, and liver was much higher than in blood plasma (Gooneratne et al., 1995).

In our experimental work, the use of the above-mentioned SPME method in combination with GC-MS produced the following results (Figure 16.1): maximal concentrations were found in rats 1 h after the poisoning, 2.2 µg/mL in blood plasma and 1.89 µg/g in brain; there were 3–4 times less FA in rat kidneys, liver, and heart (from 0.64 to 0.50 µg/g). After a further 2 h, the distribution between the tissues was more equal, resulting from a prominent decrease of FA in plasma and brain and a small decrease (or even elevation) of FA in other organs. Further decrease of FA was found in all the tissues except for the heart 24 h after the poisoning. After 72 h, no FA was detected in plasma; we did not measure FA in rat organs at this point. The half-life was calculated to be 3.6 h.

MECHANISM OF ACTION

Molecular Mechanism of Aconitase Inhibition

The mechanism of the inhibitory effect of FA on aconitase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) is one of the most interesting in biochemistry. Upon entering an organism, nontoxic FA undergoes a series of metabolic conversions, the result of which is synthesis of highly toxic FC; this process was termed *lethal synthesis* (Peters, 1952). FC is formed by the enzymatic condensation of fluoroacetyl-CoA with oxaloacetate, catalyzed by citrate

(*si*)-synthase (EC 4.1.3.7) (Eanes and Kun, 1974). FC was initially considered to be a competitive aconitase inhibitor, but in the early 1990s, it was suggested that FC acts as a "suicide substrate" because it has a high affinity for aconitase at any concentration of the competitive citrate (Clarke, 1991). Aconitase affects the conversion of citrate to isocitrate through an intermediate, *cis*-aconitate, which binds with aconitase in two different ways, swung 180° to the $C^{\alpha} - C^{\beta}$ bond (Gawron and Mahajan, 1966). Aconitase includes a [4Fe–4S] cluster, and the catalytic conversion involves substrate coordination to a specific iron atom in this cluster, Fe_a (Lauble et al., 1992). The single inhibitory isomer was shown to be (−)-*erythro*-2-flurocitrate (2R, 3R) (Carrell et al., 1970), from which aconitase removes fluoride ion with a stoichiometry of 1 F^- per enzyme molecule (Tecle and Casida, 1989). The defluorination results in generation of an actual aconitase inhibitor, 4-hydroxy-*trans*-aconitate (HTA), which binds very tightly—though not covalently—with aconitase (Lauble et al., 1996). The natural aconitase substrate isocitrate should be at a 10^6-fold excess in order to slowly displace HTA from its complex with aconitase. The HTA–aconitase complex involves four hydrogen bonds, which hold together HTA, a water molecule, Asp165, and His167 (Lauble et al., 1994, 1996). In contrast, isocitrate has only one such bond.

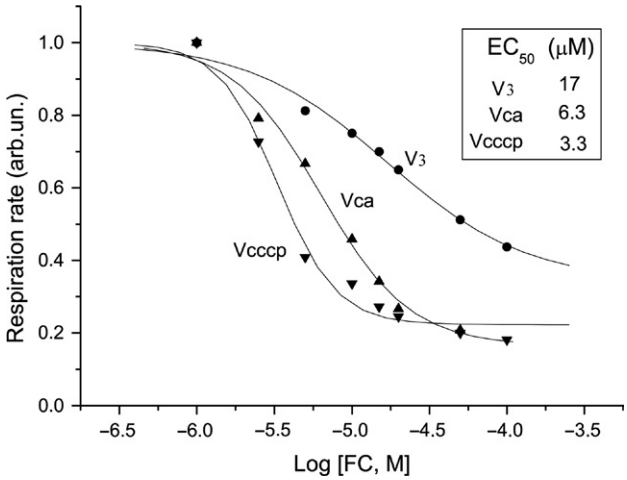

FIGURE 16.2 Effects of FC on respiration of rat liver MCh. Dependence of respiration rate activated by ADP (V_3), calcium transport (V_{Ca}), and protonophore CCCP (Vcccp) upon concentration of FC. Substrates: pyruvate + malate.

Physiological and Biochemical Effects of FA

Effects of FA and FC on Mitochondria and Other Intracellular Organelles

Functional disturbances of mitochondria (MCh) precede the appearance of structural anomalies (Buffa and Pasquali-Ronchetti, 1977) and consist of their decreased capacity to oxidize the substrates introduced. Within the mitochondrial matrix, FA induces changes that develop over several minutes, resulting in swelling and loss of electronic density. These changes are explained by the accumulation of citrate, rise of osmotic pressure, and decrease of energy production; change in the level of ATP is not caused by uncoupling of respiration and phosphorylation (Corsi and Granata, 1967). Mitochondrial volume changes are accompanied by their conformational reorganizations: they are displacement of granules, disintegration of cristae, and extension and rupture of their membranes. Axonal cylinders stretch in 3–4 h after small doses of the poison and 1–2 h after lethal doses. The cylinders are filled with MCh (most of which are swollen and degenerated), multilamellar lisosome-like bodies, vesicules, and neurofibrils. In the Golgi complex, a condensation of cisternae takes place (McDowell, 1972). Concurrently, a disruption of endoplasmic reticulum (ER), swelling of nucleus, and reduction of aggregated chromatin can be seen.

Having studied *in vitro* effects of FC on rat liver MCh, we revealed that maximal inhibition of respiration was registered when MCh were uncoupled (Figure 16.2). The level of alkalinization of the medium at the addition of adenosine diphosphate (ADP) was much lower in the presence of FC, thus evidencing an inhibition of ATP synthesis. The amplitude of alkalinization was also decreased, which could be caused by incomplete ATP synthesis, an additional transmembrane redistribution of protons, and/or change of the binding constant of ADP. FC induced a leak of Ca^{2+} from MCh, which was consistent with the observed inhibition of oxygen consumption in respiratory state 1. Addition of the substrates caused the reentry of Ca^{2+} into MCh. In the presence of FC, the MCh only partially took up the Ca^{2+} ions added to the medium, followed by their spontaneous efflux through an electroneutral $2H^+/Ca^{2+}$ exchanger with $K_{1/2} = 10\,\mu mol/L$ (Teplova et al., 1992).

The effects observed under exposure of MCh to SFA developed at much higher concentrations (from 4 mmol/L) than for FC and greatly depended on respiratory substrates. With pyruvate as substrate, the time period of oxidative phosphorylation (OP) and the level of nicotinamide adenine dinucleotide (NADH) oxidation increased linearly at increasing SFA concentration in the medium (Zinchenko et al., 2007). However, with utilization of succinate and especially glutamate, SFA had no effect on OP in concentrations as high as 8 mmol/L (Figure 16.3A) and even 16 mmol/L (not shown in the figure). Moreover, the effect of SFA with pyruvate as respiratory substrate can be prevented by incubation of MCh with cyclosporine A (CsA), a known inhibitor of the mitochondrial transition pore (Figure 16.3B). This means that under exposure to FA, development of mitoptosis and apoptosis is possible, but opening of the pore is reversible in nature, and preventing oxidation or

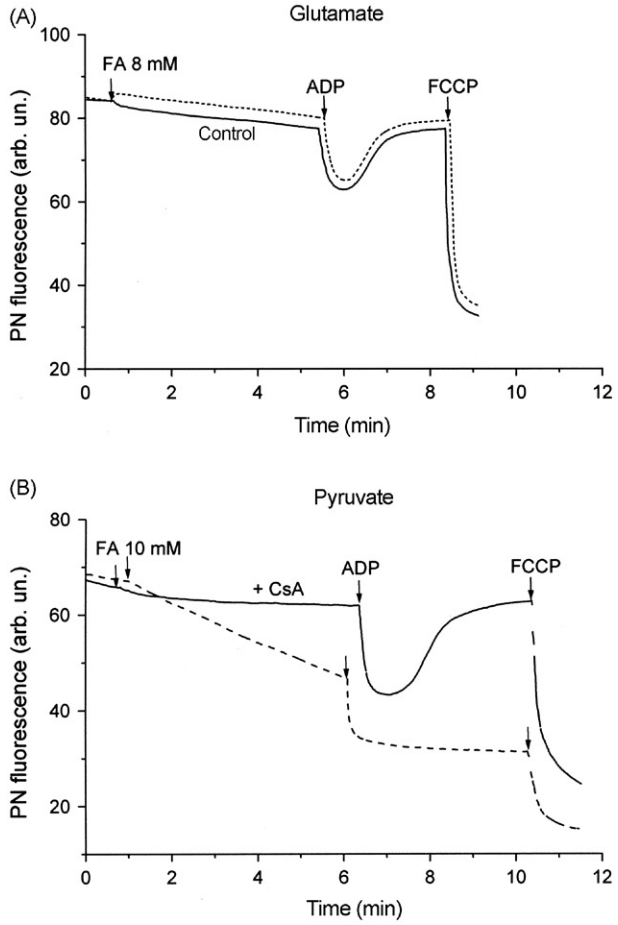

FIGURE 16.3 Effects of FA on the redox state of PNs of rat liver MCh. (A) Glutamate as respiratory substrate. (B) Prevention of PN oxidation, leakage, or both by CsA when pyruvate is used as a respiratory substrate. Additions: (A) SFA 8 mmol/L (dots) or sodium acetate 8 mmol/L (control line), ADP 120 µmol/L, FCCP 1 µmol/L; (B) SFA 10 mmol/L (dots) or SFA 10 mmol/L + CsA 1 µmol/L (control line).

leak of NADH from MCh can return them to the normal functional state.

Effects of FA on Isolated Cells

The effects of FA on the physiological and biochemical status of cells and tissues are closely dependent upon the level of their oxidative metabolism. Thus, FA does not inhibit phagocytosis because of the low level of trichloroacetic acid (TCA) cycle activity within macrophages (Cifarelli et al., 1979). We investigated a series of cell types, transformed lines, and those obtained from animals under exposure to FA or FC. The level of NADPH in Ehrlich ascite tumor (EAT) cells slowly decreased and the level of Ca^{2+} increased when the cells were incubated with SFA (Zinchenko et al., 2007). SFA obviously induced depletion of intracellular calcium stores and activation of influx of extracellular Ca^{2+} ions through the store-operated calcium (SOC) channels. Discovery of other calcium channels, such as TRPV5 and TRPV6 (Hoenderop et al., 2003; van de Graaf et al., 2006), which remain inactivated when cytosolic $[Ca^{2+}]_i$ is increased and become activated when $[Ca^{2+}]_i$ is decreased, stimulated the investigation of the level of calcium ions in ER with chlortetracycline. FC does not affect the velocity of calcium efflux from ER, so the signal transmission from P2Y receptor via G-protein is not inhibited in EAT cells exposed to FC (Zinchenko et al., 2007). However, FC induced a growth in both amplitude of Ca^{2+} leakage and velocity of its influx into ER. A rather long period (8–10 min) of Ca^{2+} influx into ER was observed, which indicated efflux of intracellular Ca^{2+} from cells by plasma membrane Ca-ATPase immediately after mobilization and leaving ER. This greatly reduces $[Ca^{2+}]_i$ for transport back to ER. It has been demonstrated (Zinchenko et al., 2001) that the velocity of return transport of Ca^{2+} into ER depends upon the activity of SOC channels in plasma membrane. Therefore, we suggest that FA (or FC) can induce the entry of calcium ions into cells through SOC channels.

The toxic effects of FC on endothelial cells have been shown to be similar to its effects on other energy-dependent tissues: namely, reduction of ATP level and oxygen consumption but accumulation of lactate and considerable decrease of protein synthesis (Rist et al., 1996). We have demonstrated with rat aorta endothelial cells in culture a gradual decrease of the mitochondrial membrane potential and elevation of $[Ca^{2+}]_i$ under exposure to SFA, similar to that observed with EAT cells (not shown in this chapter). Conversely, in cardiomyocytes, SFA induced a slow enhancement of the mitochondrial membrane potential with a rise of basal $[Ca^{2+}]_i$; propagation of calcium waves along the surface of sarcoplasmic reticulum or visible elevation and velocity of spreading of the preexisting waves was also observed (Zinchenko et al., 2007). Probably the increased level of $[Ca^{2+}]_i$ is the reason for its transport into MCh with a subsequent inhibition of the proton ATPase and rise of membrane potential. Mechanistically, this phenomenon can be explained by the existence of a Ca^{2+}-dependent protein inhibiting H^+-ATPase (Hubbard and McHugh, 1996).

We have also studied kinetic parameters of platelet aggregation in experiments with rats *in vitro* and *ex vivo* (Mindukshev et al., 2006). Aggregation of platelets was induced with ADP over the concentration range 10–100 nmol/L. The median effective concentrations (EC_{50}) of ADP for the cells exposed to SFA (10 and 5 mmol/L) were calculated to be 25 and 35 nmol/L, respectively, and these platelets can be characterized as hypersensitive to ADP. Studying the kinetic parameters of platelet aggregation under intoxication of rats with SFA, we encountered a problem of spontaneous aggregation of the platelet-rich plasma, which agreed with the data on primary transition of the platelets to the hypersensitive state. However, the cells that avoided aggregation demonstrated an extremely high level of desensitization. In some experiments, ADP could not

induce platelet aggregation at very high (nonphysiological) concentrations near 10 µmol/L. Thrombocytopenia found in cats after poisoning agrees with our observations (Collicchio-Zuanaze et al., 2010).

Under intoxication with SFA, a significantly reduced thymus, as well as a prominent reduction of the quantity of freshly obtained thymocytes and elevation of apoptosis, were revealed (Figure 16.4A). SFA also caused an acceleration of apoptosis of intact and dexamethazone-treated human lymphocytes *in vitro* (Figure 16.4B), although spontaneous apoptosis of human neutrophils was inhibited (not shown in this chapter). Moreover, SFA either inhibited (high doses) or had little effect on reactive oxygen species (ROS) production by peritoneal macrophages of mice in our experiments *in vitro* (not shown here). In this regard, it should be noted that dexamethasone, in contrast to its effect on lymphocytes, also inhibits the apoptosis of neutrophils induced by ROS (Ruiz et al., 2002). One can suggest that the inhibitory effect of FA on neutrophil apoptosis is realized through ROS, whereas the enhanced apoptosis and depression of the cells responsible for adaptive immunity is a nonspecific reaction under SFA intoxication, reflecting a general decline and redistribution of energy resources of the organism. Leukopenia was again found in cats after poisoning (Collicchio-Zuanaze et al., 2010).

Biochemical Parameters Under Intoxication with FA

Among the biochemical effects caused by FA are accumulation of citrate and disturbance of its transport from MCh; elevation of lactate and disturbances in carbohydrate metabolism; decrease or increase of free fatty acid (FFA) concentration; elevation of adenosine and ammonia; disbalance of bivalent cations and acid-base equilibrium; hypokalemia; changes in gamma-aminobutyric acid (GABA) balance in brain; hyporphosphatemia or hyperphosphatemia; and rise of various enzymes in blood plasma, including creatine kinase enzyme and creatine kinase cardiac isoenzyme (Buffa and Peters, 1950; Engel et al., 1954; Maytnert and Kaji, 1962; Stewart et al., 1970; Buffa et al., 1973; Liang, 1977; Taitelman et al., 1983a; Bosakowski and Levin, 1986; Szerb and Redondo, 1993; Tsuji et al., 2009; Collicchio-Zuanaze et al., 2010). Recently, we specified changes in triglycerides and FFAs after acute intoxication with SFA (Table 16.1). As expected, the total level of triglycerides and FFAs changed in opposite directions within 3 h after poisoning; the former reduced down to 60% and the latter elevated up to 75%. Reduced level of triglycerides persisted in 24 h, whereas total concentration of FFA dropped below the normal level, though not significantly. GC-MS analysis of 36 fatty acids after extractive alkylation revealed reliable changes in concentration for 7 of them, in agreement with total FFA changes.

However, among this variety of biochemical changes, citrate seems to be the only parameter whose qualitative (but not quantitative) trends are not controversial. In rat hearts under acute intoxication with FA, the concentration of citrate can exceed control values 8–15 times (Bosakowski and Levin, 1986). Elevation of citrate concentration is in direct proportion to the respiratory activity of a tissue: metabolically active tissues, such as the heart, kidneys, and spleen, maximally accumulate citrate. Though in the liver, which is also characterized by high respiratory levels and metabolic activity, a small

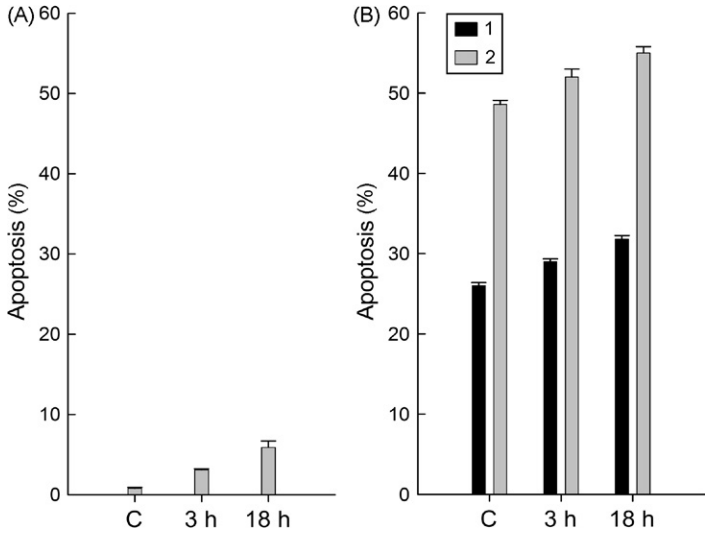

FIGURE 16.4 Effect of SFA on the development of apoptosis of rat thymocytes 3 and 18 h after administration of SFA at 1/2 LD_{50}. Registration of apoptosis with Hoechst-33258. (A) Apoptosis in freshly isolated thymocytes; (B) Apoptosis in thymocytes cultivating for 20 h after isolation in the absence (1, black) and presence (2, gray) of dexamethasone.

TABLE 16.1 Triglycerides and FFA Concentrations in Rat Blood Plasma in Normal State and After Introduction of SFA, 1/2 LD_{50}

		Free Fatty Acids							
	Triglycerides (mM)	**Total (μM)**	**Dodecanoate, C12:0 (μg/mL)**	**Palmitoleate, C16:1n7 (μg/mL)**	**Palmitate, C16:0 (μg/mL)**	**Linoleate, C18:2n6c (μg/mL)**	**Oleate, C18:1n9c (μg/mL)**	**Elaidate, C18:1n9t (μg/mL)**	***cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester, C22:6n3 (μg/mL)**
Control	1.62 ± 0.24	100.6 ± 13.6	0.49 ± 0.18	5.3 ± 1.1	33.3 ± 5.8	50.2 ± 14.0	13.5 ± 6.1	2.1 ± 0.6	2.0 ± 0.9
3 h	0.96 ± 0.11*	174.6 ± 9.46*	0.88 ± 0.24*	7.7 ± 2.4	49.3 ± 11.2*	76.4 ± 16.0*	20.9 ± 4.2	3.6 ± 1.1**	3.6 ± 1.0*
24 h	0.98 ± 0.13*	82.4 ± 16.8	0.66 ± 0.29	4.5 ± 1.8	28.0 ± 11.2	47.6 ± 23.9	8.6 ± 6.9	1.4 ± 0.6	1.4 ± 0.7

*$P < 0.05$. **$P < 0.01$.

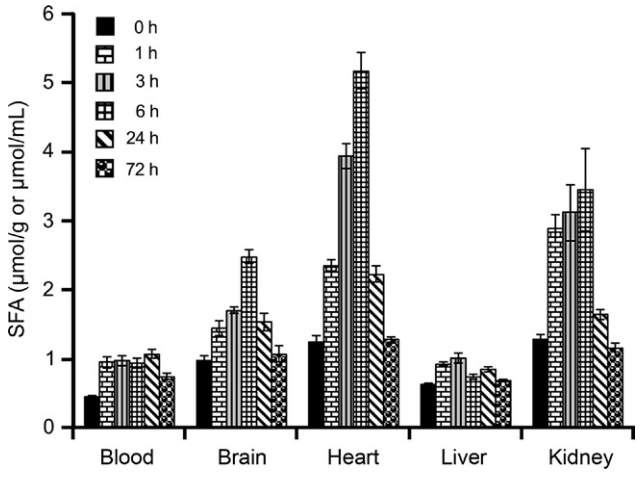

FIGURE 16.5 Concentration of citrate in blood and organs of rats after intoxication with SFA at 1/2 LD_{50}.

accumulation of citrate has been observed (Cole et al., 1955; Twigg et al., 1986). In our experiments with rats poisoned with SFA at a dose of 1/2 LD_{50}, the concentrations of citrate in blood plasma and organs increased within 1 h (Figure 16.5). The most prominent elevation of citrate was revealed 6 h after the poisoning in heart (5*x*), kidneys (3*x*), and brain (2.5*x*). There was doubling of the level of citrate in blood plasma after 1 h, though it was the only biochemical parameter of plasma that remained elevated for 3 days.

Transfer of citrate through the inner membrane of MCh is provided by a tricarboxylate transporter (m.w. 32.5 kD), which also catalyzes transport of *treo*-D_s-isocitrate, *cis*-aconitate, and other tricarboxylates (Kaplan et al., 1990). This is electroneutral exchange for either another tricarboxylate or dicarboxylate (e.g., malate or succinate), or for phosphoenolpyruvate. Formation of GSH-citryl thioester is irreversibly inhibited by (−)erythrofluorocitrate (IC_{50} = 25 pmol FC/mg protein), which makes a stable adduct with the synthase (Kun et al., 1977). However, the block of citrate transport is not absolute OR universal for all the organs and tissues. There are data on citrate transfer from MCh to cytosol with its subsequent utilization by cytoplasmic aconitase (c-aconitase), which is virtually unaffected under FA intoxication, and then by cytoplasmic NADP-dependent isocitrate dehydrogenase (cICDH) (Max and Purvis, 1965). Around 32% of citrate produced in MCh can be transported to cytosol (Buffa et al., 1972). These processes should be regarded as adaptive and positive; they lead to reduced oxygen consumption because the NADPH generated does not require further oxidation in the respiratory chain and can be utilized in other metabolic pathways.

Among the negative consequences of citrate accumulation is a change of electrolyte composition and acid-base imbalance in the organism. Moreover, elevation of citrate level in cells leads to disturbance of glucose metabolism due to inhibition of the key glycolytic enzyme phosphofructokinase (Bowman, 1964; Peters, 1972). Hyperglycemia during intoxication with FA can be very prominent, in spite of inactivation of gluconeogenesis (Godoy et al., 1968; Bobyleva-Guarriero et al., 1983). Nevertheless, we could not find significant changes in rat blood glucose throughout the periods of intoxication with FAA or SFA at a dose of 1/2 LD_{50}; at the same time, there was significant increase of glucose level in the liver, heart, and brain (not shown in this chapter). This may signify a utilization of glucose by other tissues (by skeletal muscles first of all). As a result, the local increase of glucose in organs is not reflected by the level of glucose in blood. Thus, glucose cannot serve as a reliable criterion of intoxication.

Some researchers considered the elevated glucose level to be a result of decreased insulin secretion by pancreatic β-cells due to their damage by FA (Cole et al., 1955; Karam and Grodsky, 1962). Along with hyperglycemia, there was hyperketonemia, observed characteristically for the diabetic state and caused by inhibition of the TCA cycle and depletion of oxaloacetate (Buffa et al., 1973; Taitelman et al., 1983a). Also consistent with diabetes is inhibition of hormone-induced lipolysis in adipose tissue (Taylor et al., 1977). Moreover, FA increased glucose conversion to fatty acids, and such a coincidence of antilipolytic and

lipogenetic effects of FA provides a basis for suggesting a relation between the effects of FA and insulin. However, injection of insulin does not alleviate FA intoxication in general and "FA diabetes" in particular (Reichelt, 1979). During FA intoxication, the initial hyperglycemia can even be reversed into hypoglycemia (Boquist et al., 1988), so this effect of FA was considered to be an insulin like phenomenon (Zieve et al., 1983). The principal distinction, however, should be the depletion of glycogen stores in different tissues under FA intoxication (Godoy et al., 1968; Boquist et al., 1988). After poisoning with FA, glycogen levels in animal tissues may decrease by 75% in 1 h and by 90% in 2 h (Buffa et al., 1973; Zhou et al., 1984). According to our data, during SFA intoxication (1/2 LD_{50}), glycogen levels are maximally decreased after 6 h in both liver (by 55%) and brain (by 40%), and the dynamics of the glycogen levels were similar in these organs. Such a decrease could result from the indirect action of adrenalin or sympathetic regulation (Buffa et al., 1973). In addition, inhibition of *de novo* glycogen synthesis has been reported (Zhou et al., 1984).

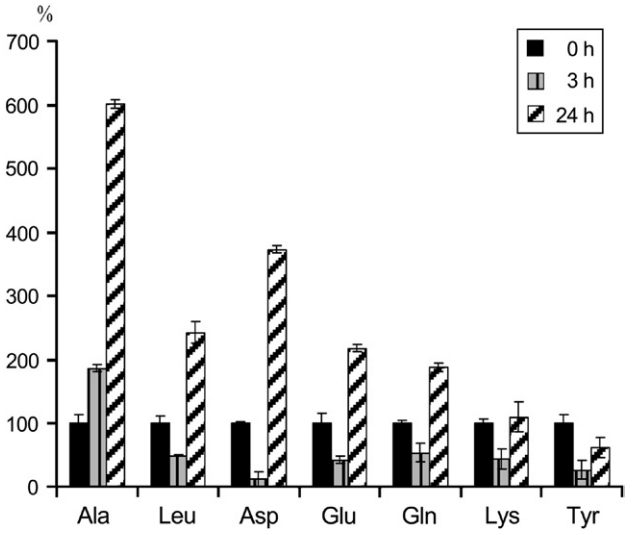

FIGURE 16.6 Changes of some amino acids in rat brain 3 and 24 h after administration of SFA at 1/2 LD_{50}.

Nevertheless, disturbances in hormonal regulation during the FA intoxication can also take place: reduction of calcium concentration in blood plasma could be caused by a poor reabsorption of calcium ions in kidneys due to a decrease of parathormone level; an excess of Ca^{2+} excretion of up to 0.173 mg/min (the control rate being 0.06 mg/mL) has been registered (Perez and Frindt, 1977). Decrease in calcium levels could be the reason of the so-called hypocalcemic tetanus (Roy et al., 1980), manifesting itself as typical convulsions, disturbances of blood clotting, and hypotension leading to vascular attacks. The level of decrease of calcium correlates with extension of the QT interval on electrocardiography (ECG), which is a consequence of broad spectrum cardiac arrhythmia (Buffa and Peters, 1950; Arena, 1970).

ATP level is usually reduced, and ADP and adenosine monophosphate levels can be elevated in the first hours of FA intoxication, with subsequent decreases (Bowman, 1964; Stewart et al., 1970). There are other reports of a constant level of ATP in some organs and tissues. For example, FA did not affect ATP and GTP, nor did it affect cyclic nucleotides levels in hepatocytes *in vitro* (Dohi and Murad, 1981). When dogs were intoxicated with sublethal doses of FA, there was no decrease in oxygen consumption and ATP level, which was explained by the utilization of glutamate and aspartate which can enter the TCA cycle distally of aconitase (Liang, 1977). The inversion of reactions at glutamate dehydrogenase (GDH) is a simple and effective compensative mechanism during blockade of the TCA cycle in kidney cells: instead of glutamine synthesis, glutamate is deaminated to form 2-oxoglutarate to support the flow of reducing equivalents in the TCA cycle and ATP synthesis, while the ammonia that is produced neutralizes local tissue acidosis (Yu et al., 1976). Such utilization of glutamate may account for the significant reduction of glutamate levels in rat organs, beginning from the first hour after intoxication with FA. The data obtained according to the GC method of Matsumura et al. (1996) have also shown decreases of glutamate, aspartate, and some other amino acids in rat brain (Figure 16.6), as well as decrease of glutamate and nearly complete absence of glutamine in blood plasma of rats and rabbits (not shown here) 3 h after poisoning with SFA. The levels of amino acids in the blood plasma of animals indicates the extent of protein breakdown in muscles on one hand, and the level of their utilization by other organs and tissues on the other. Under intoxication with FA, glutamate and its precursor glutamine are probably the main nutrients. Elevation of amino acid levels in blood plasma of rats within 3 h after poisoning signifies an elevation of protein breakdown. Furthermore, this indicates that other amino acids, because of their transport, catabolism, and other factors, are not nutrients of primary importance under energetic deficit conditions.

Elevation of lactate level in the blood of animals poisoned with FA has been reported (Engel et al., 1954; Taitelman et al., 1983a). In agreement with these results, we observed a prominent rise in lactate levels in blood just after convulsions (not shown here). In the rat heart and brain, lactate levels decreased under intoxication with SFA or FAA, regardless of convulsions. During SFA intoxication, the decrease in lactate level (and increase of glucose level) in rat heart takes place earlier and to a greater extent than for FAA intoxication: 38% decrease in 3 h for lactate, as compared with 25% in 6 h in the case of FAA; and 100% increase in 3 h for glucose, as compared with 67% in 6 h in the case of FAA. Also, the maximal

increase of citrate was registered at 24 h after poisoning with FAA, but at 6 h after poisoning with SFA. These and other biochemical data are consistent with clinical pictures of intoxication with equipotential doses of SFA and FAA: intoxication with SFA is generally more violent and takes a shorter period of time.

Effects of FA on the Cells of Nervous System: Interaction of Glia and Neurons

Acetate is metabolized in astrocytes nearly 18 times faster than in cortical synaptosomes, although the activity of acetyl-CoA synthase in synaptosomes is almost double that in astrocytes (5.0 and 2.9 nmol/min per mg of protein, respectively). The principal difference in the acetate metabolism rates is explained by differences in the kinetics of its transport, which is mediated by a monocarboxylate carrier (Hosoi et al., 2004); acetate uptake by astrocytes, unlike synaptosomes, rapidly increases and follows saturation kinetics (V_{max} = 498 nmol/mg protein/min, K_M = 9.3 mmol/L) (Waniewski and Martin, 1998). Having penetrated into astrocytes at one site, FA can diffuse into other cells through gap junctions (Ransom, 1995). Citrate accumulating in astrocytes is readily released from cells and effectively penetrates other astrocytes (Westergaard et al., 1994). The TCA cycle in nerve tissues is blocked by FA, but not completely—only by 35–55% (Patel and Koenig, 1968). This leads to decreased consumption of glucose and increased consumption of glutamine (if the latter is available); no reduction of ATP was observed (Hassel et al., 1994). The natural metabolic pathway is switched over to utilization of glutamine, glutamate, and 2-oxoglutarate in the TCA cycle. GDH of astroglia plays a big role in this switch, promoting the ATP-independent utilization of glutamate (Plaitakis and Zaganas, 2001). The absence of an aspartate/glutamate mitochondrial exchanger (the key component of the malate/aspartate cycle) in astrocytes also plays a role in supporting this (Xu et al., 2007). There is little GDH in neurons as compared to astrocytes, with activity of GDH depending not only upon proximity to glutamatergic fibers and terminals, but also upon activity of neighboring neurons regardless of their functional specialization; a deficiency of GDH activity in astroglia may be a cause of cytotoxic effects of glutamate and aspartate (Aoki et al., 1987).

The uptake of glutamate by astrocytes is an electrogenic process in which one molecule of glutamate is cotransported with three sodium ions (or 2 Na^+ and 1 H^+), being exchanged for 1 K^+ and 1 OH^- or 1 HCO_3^- (Bouvier et al., 1992). To reestablish the ionic balance, Na^+/K^+-ATPase would work with ATP provided by phosphoglycerate kinase binding to plasma membrane. This stimulates glycolysis and lactate production in astrocytes. Lactate is released from astrocytes and then is taken by neurons to be further oxidized. Pyruvate, which is also produced in astrocytes, can be utilized in the TCA cycle to form 2-oxoglutarate, or transaminated to form alanine; the latter can also enter neurons (Tsacopoulos and Magistretti, 1996; Tsacopoulos, 2002). However, the rate of alanine metabolism through alanine transaminase (ALT) in synaptosomes is much less than the rate of its uptake; moreover, neuronal ALT and aspartate aminotransferase work mainly to synthesize alanine and aspartate (Erecinska and Silver, 1990). Hence, we observed a stable elevation of alanine level in contrast to that of other amino acids in the brain of rats poisoned with SFA (Figure 16.6). The role of alanine as a source of glutamate increases during the restoration period after ischemia/hypoxia, when alanine concentration is elevated and glutamate concentration is reduced (Erecinska et al., 1994).

Inhibition of glutamate uptake by astroglial cells can be one of the causes of convulsions observed under intoxication with FA (Szerb and Issekutz, 1987). The toxic effect is governed mainly by citrate, which chelates calcium ions (Fonnum et al., 1997). Intrathecal injection of FC in mice caused convulsions in about 15 s, while in about 37 min by intracerebroventricular injection (Hornfeldt and Larson, 1990). Moreover, intrathecal injection of sodium citrate caused the same effect. This means that the main target of FC and citrate, and the area for generation of convulsions, should be the spinal cord. Convulsions could also be generated by other compounds having the common property of chelating calcium ions; these are ethylenediaminetetraacetic acid, ethylene glycol tetraacetic acid, glutamate, and lactate (Hornfeldt and Larson, 1990). Thus, along with elevation of citrate level, activation of anaerobic oxidation of glucose in neurons followed by accumulation of lactate in cerebrospinal fluid could also lead to coma and convulsions (Stewart et al., 1970). Chelation of zinc and other divalent cations by citrate enhances the signaling activity of *N*-methyl-D-aspartate (NMDA) receptors (Westergaard et al., 1995). In addition, disturbances of GABA metabolism were revealed as a result of the TCA cycle blockade: after injection of FA, there was initially an elevated level of GABA registered in different regions of the brain, followed by its reduction concurring with the start of clonicotonic convulsions (Maytnert and Kaji, 1962; Stewart et al., 1970).

The convulsive state is aggravated by increasing concentration of ammonia ions (Raable, 1981), an excess of which can lead to redistribution of K^+ and Cl^- ions, disturbances of neuronal depolarization and hyperpolarization, and impairment of postsynaptic inhibition. The neuron dysfunctions observed result in encephalopathy, ataxia, convulsions, and coma (Iles and Jack, 1980; Raable and Lin, 1983; Xiong and Stringer, 1999). Normally, ammonia specifically promotes GSH synthesis and export from astrocytes and increases its extracellular

degradation, which may improve the availability of precursors for GSH synthesis in neurons and their resistance to ammonia toxicity; FA abrogates this defense mechanism (Hilgier et al., 2010). Lymphocyte-mediated neuroprotection is also mediated by astrocytes, and it is no surprise that it is inhibited by FA (Shrestha et al., 2014). Moreover, FA impairs the enhancement of synaptic transmission and facilitation of long-term memory caused by nicotine, the effect being mediated by the gliotransmitter D-serine, an endogenous co-agonist of NMDA receptors (López-Hidalgo et al., 2012). On the other hand, FC affecting astroglia causes a drop in membrane potential and depolarization and a decrease of $[K^+]_i$ (Largo et al., 1997), which should lead to compensatory transport of bicarbonate ions into astrocytes and acidification of the extracellular medium. Together with the natural carbonate acidification of the medium close to chemoreceptors of the retrotrapezoid nucleus, this activates the diaphragmal nerve and increases the expired minute ventilation (Erlichman et al., 1998; Holleran et al., 2001): maximum ventilation is attained at 4% CO_2 against 8–10% in control hypercapnic trials. Control of extracellular pH in nervous tissue is coupled with functioning of the Na^+/HCO_3^- cotransporter, which exists in plasma membrane of astrocytes but is lacking in that of neurons (Deitmer, 1992; Romero and Boron, 1999). This transport has an electrogenic character because two (or even three) bicarbonate ions are transferred per sodium ion. Again, however, a continuous supply of glutamine to the glutamatergic nerve terminals is the necessary condition of respiratory rhythm generation; blockade of the TCA cycle in astroglial cells with FA can impair the respiratory activity (Hulsmann et al., 2000).

Physiology of Blood Vessels Under Intoxication with FA

FA does not affect circulation in resting organs, but significant increase of blood flow can be seen in working respiratory muscles (Johnson and Reid, 1988). Conversely, a reduction of blood was registered in the hepatic artery, and contractive activity of isolated portal veins was suppressed after introduction of FA into the medium (Liang, 1977). These data, along with data on the effects of FA on endothelial cells *in vitro*, suggested that the endothelium of blood vessels could be one of the primary targets for FA. If so, the endothelium-dependent relaxation of blood vessels would be affected. To test the hypothesis, we administered SFA to rats subcutaneously at a dose of 2–3 mg/kg (LD_{50}–LD_{84}), and investigated endothelium-dependent relaxation of rat aorta 3 and 24 h after the poisoning. Norepinephrine in saturating concentrations induced a rapid constriction of aorta followed by a smooth transition to plateau; in contrast, the vasoconstricting hormones angiotensin II, vasopressin, and 5-hydroxytryptamine induced a bell-shaped vasoconstricting response of aorta. To assess the functional state of endothelium, carbocholine was introduced at 10^{-5} mol/L. Acting on muscarinic receptors of endothelial cells, it induced the generation of nitric oxide and release of endothelium-derived hyperpolarizing factor (McCulloch et al., 1997). All the agonists applied had similar effects on the contraction of aortas obtained from control and poisoned animals (not shown in this chapter). The experiment clearly demonstrated that FA has no influence upon the contractile properties of isolated rat aorta at 3 and 24 h after poisoning. This endothelial function is not affected, at least directly, by intoxication with FA.

Body Temperature of Rats and Rabbits After Intoxication with FA

One of the main pathophysiological features of intoxication with FA is decrease of the body core temperature of endotherms, which indicates a disturbance of heat production, regulation, or both (Brockmann et al., 1955; Misustova et al., 1980; Taitelman et al., 1983b). It is interesting to note that the effects of FC are comparable with those of selective inhibitors of p38 MAP-kinases (activation of which precedes production of pyrogens) and antagonists of cytokines TNF, IL-1, and IL-6 (Milligan et al., 2001, 2003). In our experiments, following administration of lethal doses of SFA to male rats, a marked decrease of rectal temperature was registered beginning 1 h after the poisoning and gaining minimal levels in 6 or 24 h depending on doses. Then a gradual increase in temperature took place in the surviving rats, returning to normal in 2–7 days. For example, under intoxication with SFA at a dose of LD_{50}, a minimal rectal temperature of the surviving male rats (31.5°C, as compared to 38.5°C in control animals) was registered in 6 h, and 7 days after the poisoning, the temperature was 1°C below the control level. Under intoxication with FAA at equipotential doses, a minimal rectal temperature of the surviving male rats (32.6ºC, as compared to 38.9ºC in control animals) was registered in 2 days, and even 7 days after the poisoning, the temperature was 3°C below the control level. However, we observed a much smaller change of rectal temperature in rabbits after subcutaneous (s.c.) administration of SFA at a dose of LD_{50}: maximal decrease was only 1°C (38°C, as compared to 39.1°C) in 6 h. According to our observations, decrease of the temperature below 38°C in 3 h can serve as a reliable sign forecasting the lethal fate of the poisoned rabbits.

Electrophysiological Studies of FA Intoxication

Clinical analysis of ECG of rats poisoned with SFA or FAA (Kuznetsov et al., 2007) revealed a similar dynamic of the temporal parameters of ECG, with slowdown and delay of the repolarization processes being the most important (Table 16.2). A drop in amplitudes of the atrial and ventricular ECG complexes can be observed within 1 h after poisoning, followed by decrease of the systolic

TABLE 16.2 Parameters of ECG (Averaged Cardiocycle) of Adult Rats in Normal State and Different Terms After Introduction of SFA, 1/2 LD_{50}

	Parameters										
	Amplitude (mV)				Duration (s)						
Terms	**P**	**R**	**S**	**T**	**P**	**T**	**PQ**	**QRS**	**QT**	**RR**	**SI**
Background	0.297 ± 0.019	0.973 ± 0.131	0.723 ± 0.137	0.747 ± 0.071	0.018 ± 0.001	0.036 ± 0.001	0.050 ± 0.001	0.019 ± 0.001	0.057 ± 0.001	0.140 ± 0.003	40.7
1h	0.144 ± 0.016*	0.669 ± 0.083	0.235 ± 0.088**	0.469 ± 0.089**	0.018 ± 0.001	0.049 ± 0.002*	0.050 ± 0.001	0.022 ± 0.002	0.070 ± 0.002*	0.139 ± 0,005	50.4
3h	0.170 ± 0.017*	0.826 ± 0.066	0.161 ± 0.059***	0.518 ± 0.083	0.021 ± 0.001**	0.044 ± 0.002***	0.057 ± 0.004	0.022 ± 0.001**	0.067 ± 0.002*	0.182 ± 0.011***	36.8
1 day	0.124 ± 0.030*	1.153 ± 0.171	0.089 ± 0.050***	0.538 ± 0.143	0.019 ± 0.001	0.051 ± 0.002*	0.048 ± 0.002	0.024 ± 0.002**	0.075 ± 0.004*	0.237 ± 0.036**	31.7
3 days	0.195 ± 0.023***	1.309 ± 0.122	0.102 ± 0.052***	0.403 ± 0.102***	0.019 ± 0.004	0.046 ± 0.003***	0.052 ± 0.004	0.025 ± 0.001***	0.071 ± 0.003*	0.158 ± 0.005***	44.9
7 days	0.167 ± 0.012*	1.106 ± 0.113	0.450 ± 0.088	0.458 ± 0.041***	0.018 ± 0.001	0.062 ± 0.003*	0.052 ± 0.002	0.025 ± 0.001***	0.088 ± 0.003*	0.171 ± 0.007*	51.5

SI, systolic index, calculated after formula SI = (QRST*100)/RR.
*$P < 0.001$.**$P < 0.05$.***$P < 0.01$.

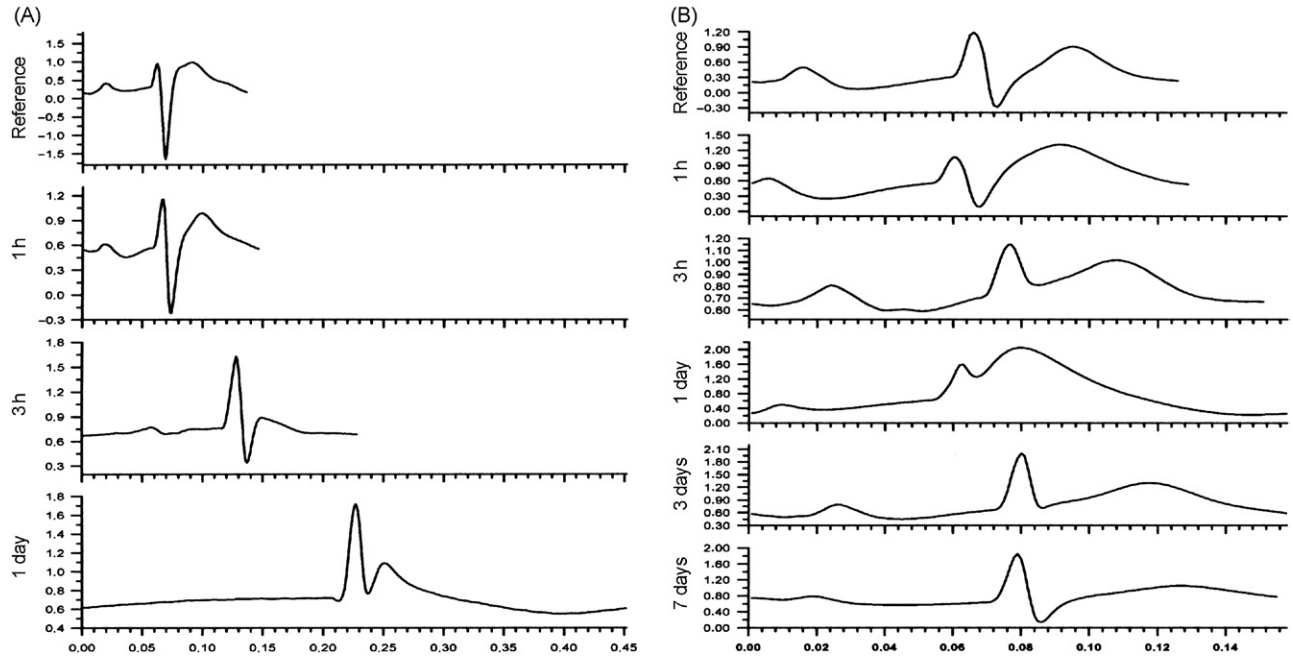

FIGURE 16.7 (A) ECG (averaged cardiocycle) of rat that died nearly 2 days after the introduction of SFA at LD_{50}. (B) ECG (averaged cardiocycle) of a rat that survived after introduction of SFA at LD_{50}. Along the *x*-axis: time (s); along the *y*-axis, amplitude (mV).

index in 24 h. This indicated impairment of the contractile capacity of the myocardium. Registration of ECG of rats that died in 2 days of intoxication with SFA revealed a sharp drop in heart rate (down to 120–180/min, the normal rate being 420–500 beats/min) 24 h after the poisoning, together with complete absence of the P wave, which reflects atrial depolarization (Figure 16.7A). Simultaneous reduction of both amplitude and duration of the T wave can be seen. An upward shift of the ST segment, though not accompanied by growth of the T wave amplitude, was registered in 70% of rats (Figure 16.7B). The cumulative evidence of the shape and amplitude changes of the ECG waves indicates a development of acute myocardial ischemia, though it was transient and maximally expressed 24 h after the poisoning. Reduction of the S wave amplitude could be caused by disturbances of excitation processes in basal ventricular regions and in some areas of the right ventricle. Taking into account an increase in duration and shape distortions of the ventricular complex, one cannot exclude an incomplete right bundle-branch block. A significant extension of the T wave during the course of examination indicates deceleration of the fast repolarization of the myocardium, though the process of slow repolarization (the QT interval in ECG, corresponding to the systole of ventricles) is accelerated within 3–24 h after the poisoning.

Respiratory rhythm gradually increased in rats after intoxication with SFA, and there were additional respiratory components in 50% of animals 24 h after administration of the poison (Figure 16.8) that may indicate disturbances of innervation of respiratory muscles. Spectral analysis of the respiratory curve demonstrated that there was an enhanced synchronization of the respiratory rhythm observed within 3 h after the poisoning. Simultaneously, the amplitude of respiration increased, followed by a gradual decrease that took place by the third day. Over the same period, a certain reduction of lability of respiratory rhythm was noted, accompanied by the appearance of two distinct peaks corresponding with frequencies of 90 and 120 cycles of respiration per minute. By the seventh day, the respiratory spectrogram was similar to the initial one, though the frequency of respiration was not completely restored. Comparison of spectrograms of respiration and ECG demonstrates disturbances of control mechanisms underlying generation of the second order waves (respiratory arrhythmia visible at the spectrogram as a peak in the high frequency region 0.8–2.5 Hz). One day after administration of SFA, there was a marked frequency maximum at the respiratory spectrum, in contrast to that of the ECG spectrogram.

Analysis of the heart rate variability (HRV) demonstrates that 1 h after poisoning an enhancement of parasympathetic influence took place, and this was accompanied by insignificant and paradoxical enhancement of heart and respiratory rates (Table 16.3). Then, against a background of enhancement of humoral (metabolic) and sympathetic influences and simultaneous decline of parasympathetic influence, a stable decrease

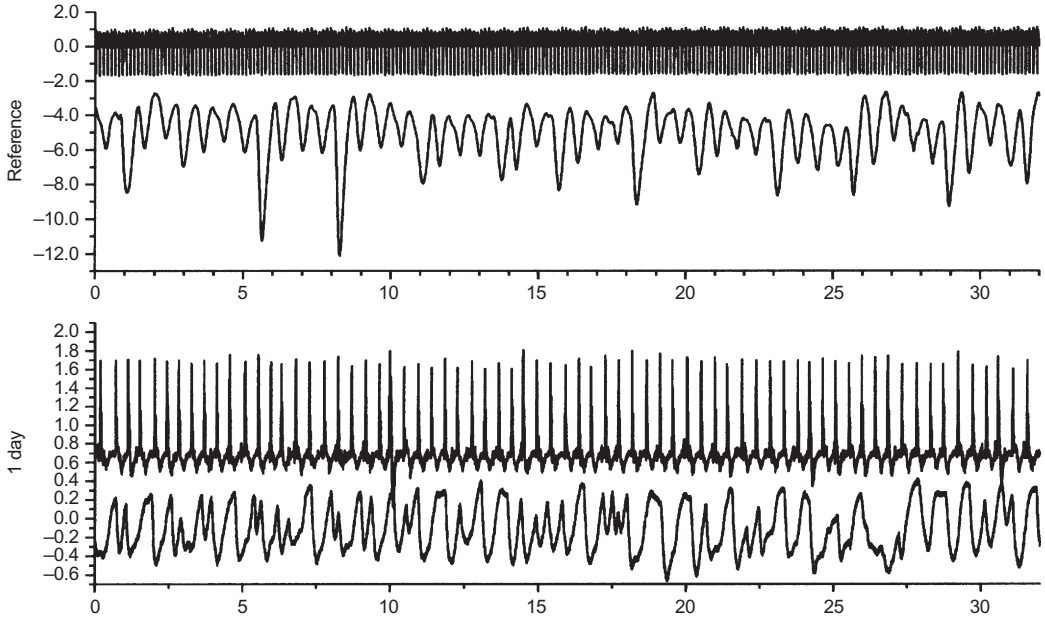

FIGURE 16.8 Records of ECG (upper) and respiratory rhythm (lower) from a narcotized rat before and 24 h after introduction of SFA.

of heart and respiratory rates took place, indicating a prominent divergence between vagosympathetic balance and resulting physiological parameters. Previously, it was shown in experiments with dogs that systemic, pulmonary, and coronary hemodynamic parameters during the first hours after introduction of FA were not mediated by the autonomic nervous system and adrenergic neuromediators (Liang, 1977). This is in partial agreement with our results obtained with rats, though this cannot be extrapolated to all the periods of intoxication and all animal species.

TOXICITY AND RISK ASSESSMENT

A characteristic feature of the clinical picture of intoxication with FA is a latent period of 0.5–6 h (Egekeze and Oehme, 1979). The duration of the latent period depends on animal species' metabolism and dose administered (Chenoweth, 1949; Goncharov et al., 2006). A broad variability of clinical manifestations of FA effects in different animal species is one of its characteristic features. There is a correlation between food specificity and toxic effect of FA; the cardiovascular system is mainly affected in herbivores, while the central nervous system (CNS) is mainly affected in carnivores. Accordingly, four groups were recognized in terms of clinical signs of intoxication (Chenoweth and Gilman, 1946). The first comprised herbivores (rabbits, goats, sheep, cattle, and horses), in which FA-induced ventricular fibrillation without notable CNS disorders (Marais, 1944; Chenoweth, 1949; Egekeze and Oehme, 1979). The second group comprised dogs and guinea pigs, in which the CNS was primarily affected. In dogs, a species highly sensitive to FA, symptoms of secondary intoxication appear after a latent period of 1–10 h (Chenoweth and Gilman, 1946; Egyed and Shupe, 1971). For animals in the third group (comprising rats and hamsters relatively tolerant of FA), the clinical pattern of intoxication is similar to that of the second group of animals, but slightly less pronounced. After a latent period lasting 1–2 h, tremor and elevated excitability were common symptoms. Death usually occurred within 4–6 h as a result of respiratory depression after exposure to high FA doses (Chenoweth and Gilman, 1946; Pattison, 1959). The surviving animals demonstrated depression, weakness, ataxia, and strongly pronounced bradycardia down to 30 heartbeats per minute. At sublethal doses of FA, full recovery can occur in 72 h after poisoning (Chenoweth and Gilman, 1946; Pattison, 1959). A mixed response to FA exposure was described in animals in the fourth group, which included cats, pigs, and rhesus monkeys; it included disturbances of both the CNS and cardiovascular system. On acute poisoning, adynamia, salivation, vomiting, frequent defecation, pupil dilatation, nystagmus, accelerated respiration, enhanced excitability, tremor, and clonicotonic convulsions were observed in these animals (Chenoweth and Gilman, 1946; Gammie, 1980). In cats, lesions were observed that were characteristic of degenerative and ischemic processes in the heart, kidneys, liver, brain, and lungs (Collicchio-Zuanaze et al., 2010).

Nevertheless, this classification was revised (Sherley, 2004). The division of animals into cardiac and neurological symptomatic groups is considered to be unnatural

TABLE 16.3 Analysis of HRV of Adult Rats in Time and Frequency Domains Under Intoxication with SFA at 1/2 LD_{50}

	Period of Examination											
	Control		1 h		3 h		24 h		3 days		7 days	
Parameters	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**
Heart rate (contr/min)	424.6 ± 6.1	–	441.6 ± 9.4	+4.0	340.1 ± 11.4***	−20.0	291.8 ± 18.5***	−31.3	370.6 ± 6.0***	−12.7	341.0 ± 6.0***	−19.7
Coefficient of arrhythmia (rel.un.)	0.049 ± 0.003	–	0.058 ± 0.005	+18.4	0.156 ± 0.042*	+218.4	0.245 ± 0.073*	+400	0.053 ± 0.003	+8.2	0.051 ± 0.006	+4.0
Value of VLF (ms^2)	0.041	8.8	0.053	7.6	0.087	11.8	0.060	9.7	0.043	10.1	0.044	10.9
Value of LF (ms^2)	0.075	16.1	0.107	15.2	0.128	17.3	0.114	18.4	0.073	17.1	0.079	19.6
Value of HF (ms^2)	0.350	75.1	0.542	77.2	0.523	70.9	0.445	71.9	0.311	72.8	0.280	69.5
Value of To (ms^2)	0.466	–	0.702	+50.6	0.738	+58.4	0.619	+32.8	0.427	−8.4	0.403	−13.5
LF/HF (rel.un.)	0.214	–	0.197	−7.9	0.245	+14.5	0.256	+19.6	0.235	+9.8	0.282	+31.8
HF/To (rel.un.)	0.751	–	0.772	+3.8	0.709	−5.6	0.719	−4.3	0.728	−3.1	0.695	−7.5
Respiration rate (min)	94.1 ± 3.0	–	101.9 ± 3.1	+8.3	94.4 ± 3.2	+0.3	98.8 ± 3.7	+5.0	111.9 ± 6.1*	+18.9	120.2 ± 8.1**	+27.7

Coefficient of arrhythmia = $(RR_{max} - RR_{min})/RR_{mean}$.Values of shifts (%) for *VLF*, *LF*, and *HF* indices are given against *To* index of corresponding period of examination. For other parameters the shift was calculated against the initial control value.
*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

because it ignores common neurological signs manifested in all the groups: these include tremor, ataxia, hypersensitivity, myotonic convulsions, weakness, and partial paralysis. The cardiac response in a pure form was not a common event and was described for a limited number of animals, though CNS involvement is obviously widespread.

As for humans, exposure to stock solution during formulation and dermal or respiratory exposure during application of bait, as well as accidental or intentional acute intoxication, are the main health concerns. Formulators and pest control workers are the largest occupational risk group (Norris, 2001; Beasley et al., 2009). Early monitoring indicated that exposures were highest in relation to cereal bait manufacturing and aerial carrot baiting procedures (Beasley et al., 2009).

The clinical picture of acute intoxication of humans is similar to that of rhesus monkeys; among the symptoms are nausea, vomiting, abdominal pains, salivation, irrational fear, weakness, tachypnea, cyanosis, and sometimes sweating and increased temperature (Brockmann et al., 1955; Pattison, 1959; Arena, 1970; Taitelman et al., 1983b). Psychomotor agitation and sometimes loss of spatiotemporal feeling can occur. In addition, tremor, nystagmus, involuntary dejection and urination, muscle spasms, hypertonus of the extremities, and even alalia, have been reported (Gajdusek and Lutheer, 1950; Robinson et al., 2002). The most characteristic signs of intoxication involve generalized recurrent convulsions alternating with deep depression. Sudden loss of consciousness and coma may occur. These symptoms were associated with metabolic acidosis and hypotension (Pattison, 1959; Chi et al., 1996, 1999), as well as cardiac rhythm disturbances, such as tachycardia, bradycardia, asystole, and sustained ventricular fibrillation (Gajdusek and Lutheer, 1950; Reigart et al., 1975; Trabes et al., 1983). Death usually occurs within 3 h to 5 days of heart block, arrhythmia, or respiratory failure (Reigart et al., 1975). Important diagnostic symptoms registered with ECG are arrhythmia, the QT and ST intervals, and the T wave (Pattison, 1959; Taitelman et al., 1983b, Chi et al., 1996). Kidneys are among the most sensitive organs: acute renal failure associated with uremia and increased level of creatinine in serum can be observed under acute FA poisoning (Chung, 1984; Chi et al., 1996). Pathomorphological abnormalities of humans poisoned with FA are also nonspecific and similar to those of animals. In the case of lethal outcome, petechial hemorrhages and excess blood filling of internal organs (Hayes, 1982), edema of lungs and brain, and sometimes mediastinal emphysema and acute inflammatory reaction with coagulating necrosis in esophagus were registered in humans (Brockmann et al., 1955). The morphological basis of cardiotoxic effects is acute myocardial dystrophy, a characteristic of which are diffuse lesions of cardiac muscle (Pattison, 1959; Taitelman et al., 1983b). Acute renal failure develops due to influence of FA on subcellular structures of kidneys. Metabolic acidosis aggravates the clinical course of renal failure. Diffuse degeneration of renal tubules was observed (Hayes, 1982). For cases that lack clinical and morphological specificity, biochemical data and primarily citrate and fluoride levels can be used for diagnostic purposes (Pattison, 1959; Schultz et al., 1982). Thus, under acute intoxication with FAA, citrate (108 µg/g in heart and 23.9 µg/g in kidney) and fluoride (6.3 mg/g dry weight of heart and kidney) were found in a human corpse; the dose of FAA was estimated to be near 23 mg/kg (Hayes, 1975). In addition, the indubitable diagnostic confirmation of the intoxication should be based on determination of the poison in tissues. Under acute SFA poisoning with lethal outcome, FA was found in urine (368 µg/mL), liver (58 µg/g), and brain (76 µg/g) (Harrison et al., 1952).

Among the aftereffects that develop following acute intoxication with FA or its derivatives are various neurological disturbances. These include impaired muscular tonus and reflex activity, transient spasmodic and meningeal syndromes, cerebellar dysfunction, such as ataxic gait, dysarthria, and intention tremor (Pridmore, 1978; Trabes et al., 1983; Kim and Jeon, 2009). Long after an acute poisoning (from 1.5 to 9 years), a tendency for epileptoid seizures, ataxia, extremity muscular hypertension, spastic tetraplegia, blindness of cortical origin, diffuse brain atrophy, and psychic disorders were observed. A case of chronic intoxication involving FA intoxication of a farm worker has been described (Parkin et al., 1977): the clinical signs included renal insufficiency and less pronounced injuries of other organs.

TREATMENT

Decades of studies on the toxicology of FA have led scientists to the conclusion that treatment of intoxications can be successful only if timely general and symptomatic therapy is applied, but not specific antidotes (Dorman, 1990; Norris, 2001). Much experimental work over an extensive period has been undertaken in an effort to find effective donors of acetate groups because of their property to inhibit conversion of FA to FC. Ethanol, monoacetin (glycerol monoacetate), acetamide, and cortisone acetate were tested for their ability to serve as antidotes (Chenoweth, 1949; Hutchens et al., 1949; Cole et al., 1955; Giller, 1956; Egyed, 1971; Egyed and Shlosberg, 1977). A therapeutic effect was revealed for the simultaneous introduction of ethanol and acetate (Hutchens et al., 1949; Tourtelotte and Coon, 1949). Negative effects of monoacetin and acetamide were the enhancement of hyperglycemia and metabolic acidosis, damage of capillaries and hemolysis of red blood

cells, and increase of citrate concentration in different organs (Engel et al., 1954; Egyed and Shlosberg, 1973). Administration of cortisone acetate inhibited FC synthesis and prevented the development of ketosis, though hyperglycemia increased (Cole et al., 1955).

Several antidotes were tested for their capacity to activate transport of the TCA cycle intermediates through mitochondrial membranes. For this purpose, fluoromalate was proposed, although positive results were negligible (Peters, 1972). Malate was also tested, but proved to be effective only in *in vitro* experiments (Buffa et al., 1972). GSH and a series of SH-containing compounds (cysteamine and *N*-acetylcysteine) were tested (Mead et al., 1985), also *in vitro.* However, they were incapable of replacing GSH in enzymatic defluorination of FA and have not found practical application. TCA cycle intermediates (succinate, malate, citrate, and glutamate) were tested, but they did not exhibit a protective effect (Hutchens et al., 1949). A positive result was observed in experiments with mice, which were administered calcium gluconate and succinate (Omara and Sisodia, 1990). This therapy was hardly more effective than ethanol, however. Some 16 years later, another research group tested the therapy with cats, which are known to be much more sensitive to FA. Again, differences in survival between treated and nontreated animals were insignificant ($P > 0.05$) (Collicchio-Zuanaze et al., 2006). Administration of calcium chloride to cats suffering acute intoxication with FA made it possible to postpone death up to 166 min; combination of calcium chloride with monoacetin gave a similar effect (Taitelman et al., 1983a). Nevertheless, calcium chloride reduced the QT interval and favored survival of humans after intoxication with FAA (Taitelman et al., 1983b).

Our strategy for developing therapeutic means of treating acute FA intoxication was based on a deep analysis of the biochemical literature, together with our own experimental data. Thus, a high sensitivity of aconitase to inhibition by superoxide anion and nitric oxide (Gardner et al., 1994; Andersson et al., 1998; Castro et al., 1998) means that ROS and NO could be competitive antagonists of FC to avert its effect on aconitase. Also, as considered earlier, glutamate could be utilized in the TCA cycle through GDH or transaminases during FA intoxication (Yu et al., 1976; Liang, 1977; Hassel et al., 1994). Moreover, the effects of FC could be prevented by prior introduction of isocitrate (bypass of inhibited aconitase) and fructose-1,6-bisphosphate (energy substrate for neurons; Lian and Stringer, 2004).

We have demonstrated that FA can adversely affect mitochondrial functions only if pyruvate was available as respiratory substrate, and that changes of redox state of pyridine nucleotides (PN) or their leakage from MCh could be critical factors that impair mitochondrial respiration and lead to cell death (Zinchenko et al., 2007). Opening of the mitochondrial pores is a reversible phenomenon: prevention of oxidation and leakage of NADPH from MCh can restore the normal functional state of MCh. For example, when succinate or glutamate was used as respiratory substrates, mitochondrial functions were not affected by FA (Figure 16.3A).

As for other alternative substrates, we suggest that the accumulating intracellular citrate could be among them. As discussed earlier, blockade of citrate transport from MCh under FA intoxication is not an obligatory event, and citrate can enter cytosol to be further utilized by cICDH (Max and Purvis, 1965; Buffa et al., 1972). The cICDH activity is almost equally distributed between cytosol and MCh of astroglia and microglia, whereas cICDH accounts for about 75% of activity in neurons and oligodendrocytes (Minich et al., 2003). We have not found data on the ratio of mitochondrial and cytoplasmic aconitases in the cells of the nervous system, but it is interesting to note that a similar ratio of mICDH and cICDH exists in hepatocytes (Rakhmanova and Popova, 2006), and that c-aconitase accounts for 65% of the aconitase in these cells (Konstantinova and Russanov, 1996). In rat heart, a similar ratio of m- and c-aconitases has been revealed: 35% and 65%, respectively (Medvedeva et al., 2002). Based on these data, one may suggest that an effective pathway for citrate utilization and NADPH synthesis exists in these (and other) cells in case of inhibition of m-aconitase. This alternative pathway could play a positive physiological role because NADPH might be used for anabolic reactions and heat generation, GSH reduction, and NO synthesis, and for regulation of blood vessel tone by ROS generation (Winkler et al., 1986; Bobyleva et al., 1993; Lee and Yu, 2002; Gupte and Wolin, 2006). As was pointed out earlier, studies focused on the pentose cycle as the main source of NADPH need to be reevaluated, taking into consideration the metabolic activity and substrate specificity of a tissue (Winkler et al., 1986). cICDH, along with malic enzyme and transhydrogenase, participate in NADPH regeneration to further reduce GSH in brain mitochondria (Vogel et al., 1999), but cICDH can provide a sevenfold increase in generation of NADPH compared to the malic enzyme (Winkler et al., 1986). The level of cytoplasmic NADPH can influence potassium channels and calcium balance (Wolin et al., 2005; Gupte and Wolin, 2006). In our *in vitro* studies, FA induced a slow elevation of $[Ca^{2+}]_i$ in different cells (Zinchenko et al., 2007). This could indicate activation of the SOC channels; the process is not affected by FA and does need ATP to be implemented, at least in glial cells (Lian and Stringer, 2004). We suppose that this mechanism is common for many types of cells, and this could explain a primary hypersensitivity of platelets exposed to FA (Mindukshev et al., 2006). In cardiomyocytes, elevated $[Ca^{2+}]_i$ can stimulate their functional activity observed in our experiments

in vitro and also supported *in vivo* by a primary increase of systolic index (Table 16.1). As for modulating effects of Ca^{2+} on the bioenergetics of MCh, it is pertinent to recall classic activation of the TCA cycle dehydrogenases, followed by increase of mitochondrial potential and NADH generation: 2-oxoglutarate dehydrogenase (OGDH) and mICDH can be activated by calcium ions through allosteric mechanisms, and pyruvate dehydrogenase is activated due to dephosphorylation by the Ca^{2+}-dependent phosphatase (McCormack et al., 1990; Hansford, 1994). The exact role of these dehydrogenases in the bioenergetic status of MCh affected by FA needs to be clarified, though one can suppose that OGDH could derive a special benefit from such an activation if it is provided with exogenic or endogenic 2-oxoglutarate.

In the above discussion, we have defined several directions for biochemical correction under acute intoxication with FA and suggested suitable preparations for therapeutic complexes: (i) competitive inhibition of FA and CoA interaction, (ii) competitive inhibition of FC and aconitase interaction, (iii) replenishment of the TCA cycle distally of aconitase, and (iv) utilization of accumulating citrate. In previous publications, we presented the first data on effectiveness of therapeutic complexes named METIS (Goncharov et al., 2006, 2009). These consisted of ethanol, methylene blue (MB), sodium glutamate (SG), and glycerol trinitrate (GT). Indices of therapeutic efficiency (ratio $LD_{50}^{treated}/LD_{50}^{non\text{-}treated}$) for different METIS complexes applied in different regimens are presented in Table 16.4. In addition to these data, a spectrum of physiological and biochemical data was also obtained. Animals treated with METIS complex experienced little change in body weight, temperature, and oxygen consumption. Dynamics of citrate in the brain, kidneys, and blood also improved, and the kinetic parameters of platelet aggregation were corrected. Comparative analysis of the FA level in the tissue homogenates, blood plasma, and urea of rats revealed that METIS complexes reduced the level of FA in the brain almost twofold, thus indicating inhibition of FA utilization (which first occurs in the cells of the nervous system).

TABLE 16.4 Assessment of Therapeutic Effectiveness of METIS Preparations Under Acute Intoxication of Rats with SFA

Therapy	Treatment Regimen After the Poisoning with SFA	Index of Therapeutic Efficiency Ratio LD_{50} Treated/LD_{50} Nontreated
Ethanol, $n = 42$	10 and 120 min	1.6
METIS-1, $n = 48$	10 and 120 min	2.5
METIS-2, $n = 92$	10, 60, and 120 min (ethanol and GT); 60 and 120 min (MB)	3.3
METIS-4, $n = 39$	10, 60, and 120 min, 24 and 48 h (ethanol, GT, and SG); 10 and 120 min, 24 and 48 h (MB)	4.3
Ethanol, $n = 43$	30 and 120 min	2.6
METIS-3, $n = 45$	30 and 120 min	3.2

n, number of animals used in experiment to calculate the index. *Experimental conditions and METIS components*. SFA was dissolved in distilled water and administered intragastrically at 0.2 mL per 100 g of rat body weight, after 7–8 h fasting. METIS-1 is a combination of two compounds: aqueous solution of MB administered subcutaneously (s.c.) at a dose of 5 mg/kg, and aqueous solution of SG administered intraperitoneally (i.p.) at a dose 250 mg/kg. METIS-2 is a combination of three compounds: MB (5 mg/kg, s.c.), ethanol and GT administered i.p. (corresponding doses for pure ethanol and GT were 1 g/kg and 10 mg/kg). METIS-4 is a combination of four compounds: MB (5 mg/kg, s.c.), and aqueous solution of ethanol (1 g/kg), GT (10 mg/kg), and SG (100 mg/kg) administered i.p. METIS-3 is a combination of three compounds: MB (5 mg/kg, s.c.), ethanol and SG administered i.p. (corresponding doses for pure ethanol and SG were 1 g/kg and 200 mg/kg).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The extreme toxicity of FA is determined by its similarity to acetate, which plays a central role in cell metabolism. FA enzymatically condenses with CoA-SH to produce fluoroacetyl-CoA, which replaces acetyl-CoA entering to the TCA cycle and produces FC. The latter reacts with aconitase and blocks TCA cycle. Energy production is reduced, as well as the concentration of metabolites generated distally to aconitase. Of these, 2-Oxoglutarate is the most important, being a precursor of glutamate, which is a neuromediator in the CNS and participates in neutralizing ammonia, either directly through glutamine synthase or indirectly through the urea cycle. Accumulation of citrate is one of the causes of metabolic acidosis. Chelating of Ca^{2+} is apparently one of the central events in pathogenesis of intoxication.

The first papers on the toxicology of FA were published in the 1940s. The long history of investigations was fruitful, with several important discoveries: biochemical mechanism of "lethal synthesis"; structure of aconitase; functional relations of glia and neurons; and switching of metabolic pathways. However, the main problem of toxicology (for any poison)—development of an effective therapy—was not solved. Analysis of the scientific literature has demonstrated that reciprocal relations of signaling and metabolic pathways under intoxication with FA are unclear. Inhibition of m-aconitase causes blockage of the TCA cycle, reduction of pyridine nucleotides, accumulation of citrate, disturbances of intracellular signaling, deenergization, and cell death. However, the dynamics and significance of these events are different depending of the type of cells and tissues, which is why it is very difficult to predict the primary reaction of different cells, let alone the whole organism.

Biochemical pathways underlie the basis of physiological rhythms; they should have a certain space-time structure and presuppose coordinated interactions of different cells. Thus, one of the causes of disturbances of normal respiration under FA intoxication could be disturbances in rhythmic activity of respiratory neurons; however, suppression of these neurons is a result of the inhibiting effects of FA on astrocytes, not neurons (Hulsmann et al., 2000). We have described the development of cardiac and respiratory tachyarrhythmia reflecting the reproduction of decasecond rhythms characteristic of immature or abnormal excitatory structures (Kuznetsov et al., 2007). Previously, it was suggested that such endogenic rhythmic activity could be determined by the level of pentose cycle activity (Kuznetsov, 2002). This cycle indeed plays an important role in neurons, protecting them from oxidative or traumatic stress (Ben-Yoseph et al., 1994; García-Nogales et al., 2003; Bartnik et al., 2005). However, it should be noted that although the activity of NADPH-generating enzymes of the pentose cycle in astrocytes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) is two to three times higher than in the brain as a whole, the activity of cICDH is also very high in these cells (Rust et al., 1991). This metabolic pathway is interesting primarily because under FA intoxication, citrate accumulates, and the pathway may be regarded as a form of biochemical adaptation that facilitates the use of the central metabolite. It was suggested that spatial and temporal division of m- and c-aconitases not only provide regulation of iron balance in cells, but also actually provide regulation of balance between catabolic and anabolic processes (Tong and Rouault, 2007).

If the cells have utilized citrate entering the cytosol, another problem that results should be utilization of generating NADPH. One of the possible mechanisms of PN oxidation, which is very important, is heat generation through shivering and nonshivering thermogenesis. A rise in activity of NADPH-generating enzymes and pathways, including c-aconitase and cICDH, is accompanied by enhanced thermogenesis (Bobyleva et al., 1993). It was shown that NADPH could be used together with (or even instead of) NADH as a reducing cofactor for cytoplasmic glycerophosphate dehydrogenase (Bobyleva et al., 1993; Fahien et al., 1999). But if the role of this pathway for transferring electrons from cytoplasm to MCh in skeletal muscles is clear, then the level of activity and functional state of glycerophosphate shuttle in brain cells are contradictive. For example, its activity in brain is explained by the need for glycerol-3-phosphate as a substrate for phospholipids synthesis in oligodendroglia (Adler and Klucznik, 1982; Nguyen et al., 2003). In neurons and astrocytes, the activity of glycerophosphate dehydrogenases is much lower than in oligodendrocytes (Rust et al., 1991; Nguyen et al., 2003). There are data, however, that indicate an important role of this shuttle in astrocytes, taking into consideration: (i) the absence of malate-aspartate shuttle in these cells (Waagepetersen et al., 2001; McKenna et al., 2006), and (ii) the elevated level of mRNA of cICDH in astrocytes after convulsions, under exposure to morphine, indometacine and some other preparations (Link et al., 2000).

It is noted that FA can prevent the development of tolerance to morphine (Song and Zhao, 2001). Another positive aspect of FA in nontoxic concentrations is its radioprotective power due to its capacity to reduce body temperature and oxygen consumption (Misustova et al., 1980). Moreover, we have estimated that SFA significantly inhibits the growth of Ehrlich tumor carcinoma. In experiments with autochthonous-induced by benzo[a]pyrene subcutaneous tumors, SFA was not active in monotherapy regimes, though exhibited enhancement of antitumor effect of cyclophosphamide significantly increased the number of mice with stabilized or decreased tumor volume, as well as the duration of this effect (Anikin et al., 2013). These findings provide a basis for additional studies of mechanism of the antitumor effects of SFA. Thus, we suggest that future progress in toxicological (and perhaps pharmacological) studies of FA will depend on comprehensive consideration of these and more recent data, along with reevaluation of old and forgotten data.

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CHAPTER

17

Strychnine

Jiri Patocka

INTRODUCTION

Strychnine is a poisonous alkaloid of indole type found in the genus *Strychnos*. Its basic compound forms colorless or white rhombic crystals. These have a bitter taste and melt at approximately 290°C. Strychnine was the first alkaloid to be identified in plants of the genus *Strychnos*, family Loganiaceae. *Strychnos*, created by Linnaeus in 1753, is a genus of trees and climbing shrubs of the gentian order. The genus contains 196 various species and is distributed throughout the warm regions of Asia (58 species), America (64 species), and Africa (75 species). Plants of the genus *Strychnos* have opposite leaves and bear cymes of white or yellow flowers that have a four-lobed or five-lobed calyx, a four-part or five-part corolla, five stamens, and a solitary pistil, and bear fruit in the form of a berry. The seeds and bark of many plants in this genus contain the powerful poison strychnine. Strychnine is obtained commercially from the seeds of the Saint Ignatius bean (*Strychnos ignatii*) and from the nux vomica tree (*Strychnos nux vomica*) (Volfova and Patocka, 2003). *S. nux vomica*, also known as poison nut, semen strychnos, and quaker buttons, is a deciduous tree native to India and southeast Asia. It is a medium tree that grows in open habitats. The seeds contain approximately 1.5% strychnine, and the dried blossoms contain 1.0% (Harry, 1968). However, the tree's bark also contains other poisonous compounds. Strychnine was first discovered by French chemists Joseph-Bienaime Caenoiu and Pierre-Joseph Pelletier in 1818 in the Saint Ignatius bean. In some *Strychnos* plants, a 9,10-dimethoxy-derivative of strychnine, alkaloid brucine, is also present (Li et al., 2006). Brucine is not as poisonous as strychnine.

BACKGROUND

Chemistry and Physico-Chemical Properties

Strychnine has a chemical formula of $C_{21}H_{22}N_2O_2$, and its structural formula is shown in Figure 17.1 (CAS No: 57-24-9 [base], 60-41-3 [sulfate]). It occurs as white crystals or powder that is odorless, with a melting point of 286°C, boiling point of 270°C at 5 mmHg, density of 1.36 g/cm^3, vapor density of 11.0 (air = 1), and vapor pressure 0 torr at 20°C (Mackison et al., 1981). Strychnine is a stable compound and incompatible only with strong oxidizing agents.

Water solubility of strychnine is negligible, only 160 mg/L at 25°C, and pH of saturated solution is 9.5. Octanol/water partition coefficient ($\log K_{ow}$) is 1.93 (Hansch et al., 1995). Strychnine is very slightly soluble in ether, slightly soluble in benzene, ethanol (6.7 g/cm^3), and acetone, and soluble in chloroform and pyridine (Budavari, 1996).

History

The toxic and medicinal effects of strychnine have been well-known since the times of ancient China and India. The inhabitants of these countries had ancestral knowledge of the species *nux vomica* and Saint Ignatius bean. The species *S. nux vomica* is a tree of native Indonesia that attains a height of 12 m. The tree has a crooked, short, thick trunk and the wood is close-grained and very durable. The fruit has an orange color and is approximately the size of a large apple with a hard rind and contains five seeds that are covered with a soft wool-like substance. The ripe seed looks like flattened

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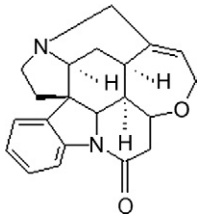

FIGURE 17.1 Structural formula of strychnine.

disks and are very hard. These seeds are the chief commercial source of strychnine and were first imported and marketed to Europe as a poison to kill rodents and small predators. *S. ignatii* is a woody climbing shrub of the Philippines. The fruit of the plant contains as many as 25 seeds embedded in the pulp. The seeds contain more strychnine than other commercial alkaloid plants. The properties of nux vomica and Saint Ignatius seeds are substantially those of the alkaloid strychnine.

Strychnine was discovered and identified as a main toxic principle of *Strychnos* in 1818, although *nux vomica*, the unpurified plant extract in which it is the active component, had been known and used for both medicinal and criminal purposes for some time. Historic records indicate that the strychnine alkaloid has been used to kill dogs, cats, and birds in Europe as far back as 1640. The structure of strychnine was first determined in 1946 by Sir Robert Robinson; in 1954, this alkaloid was synthesized in the laboratory by Robert W. Woodward (Woodward et al., 1954). This is one of the most famous syntheses in the history of organic chemistry. Both chemists won the Nobel prize, Robinson in 1947 and Woodward in 1965.

Therapeutic Purpose

Strychnine does have a history of use for therapeutic purposes, although in most cases this was entirely misguided and dangerous. It has a very bitter taste and so stimulates salivary and gastric secretion. This increases appetite and was used to counteract the loss of appetite associated with illness, giving the impression that strychnine had restorative properties. In fact, any constitutional improvement resulting from the increased appetite would probably be outweighed by the harmful effects of strychnine, and the underlying illness would be more or less unaffected. This is the same mechanism that gives tonic water its apparent "tonic activity," although in that case the bitter agent is quinine, which is much less dangerous.

Strychnine has an undeserved reputation as a useful therapeutic agent. There is no current justification for its presence in any medication. However, preliminary experimental reports suggest that judicious treatment with strychnine may modify the neurological deterioration in some infants with nonketonic hyperglycinemia, a rare metabolic disorder characterized by abnormally high concentrations of glycine in the brain and cerebrospinal fluid. Strychnine had been used as a tonic stimulant at one time for atonic constipation and as a stomachic and bitter. It produces no selective gastrointestinal effects and has no place in the therapy of any gastrointestinal disorder. At present, strychnine has no demonstrated therapeutic value in human medicine, despite a long history of unwarranted popularity. It was first used in medicine in 1540, but it did not gain wide usage until 200 years later (Gilman et al., 1985).

Although strychnine was previously used extensively in animals, it no longer has a rational place in the therapeutic armamentarium of the veterinarian. Its only interest is from a veterinary toxicology standpoint because animals are sometimes poisoned inadvertently or intentionally (Booth and McDonald, 1982; Gupta, 2012).

PHARMACOKINETICS AND TOXICOKINETICS

Absorptin, Distribution, Metabolism, and Excretion

Strychnine is rapidly absorbed from the gastrointestinal tract, mucous membranes, and parenteral sites of injection (Thienes and Haley, 1972), and also from the oral cavity (LaDu et al., 1971). A nonfatal case of strychnine poisoning through dermal exposure has been described (Greene and Meatherall, 2001). Strychnine is transported by plasma and erythrocytes, but protein binding is slight and distribution to the tissues rapidly occurs. Within a few minutes of ingestion strychnine can be detected in the urine, and excretion by that route accounts for approximately 15% of a sublethal (4 mg) dose over 6 h. Little difference was noted between oral and intramuscular administration of strychnine in a 4 mg dose. Blood levels in human poisoning were less than 0.5 μg/mL from 1 to 48 h after ingestion of a sublethal dose (700 mg), 2.7 μg/mL in a patient who survived the acute episode, and 40 μg/mL in a patient who died after massive ingestion (Haddad and Winchester, 1983). In persons killed by strychnine, the highest concentrations are found in the blood, liver, and kidney (Hayes and Laws, 1991).

The half-life of absorption is approximately 15 min and the half-life of metabolism is approximately 10 h. The apparent volume of distribution in one patient was 13 L/kg (Ellenhorn et al., 1997).

Strychnine is rapidly metabolized by the liver microsomal enzyme system and requires NADPH and O_2. Five metabolites formed *in vitro* by rabbit liver were isolated and identified as 2-hydroxystrychnine, 11,12-dehydrostrychnine, strychnine-21,22-epoxide, 21,22-dihydroxy-22-hydrostrychnine, and strychnine-*N*-oxide, which was

the major metabolite and accounted for approximately 15% of the metabolized strychnine. All other metabolites accounted for less than 1% (Mishima et al., 1985). Similar metabolites were identified in rat urine where the major metabolite was strychnine-21,22-epoxide (Oguri et al., 1989). Metabolic fate of strychnine in humans is unknown.

Toxicokinetics

Strychnine competes with the inhibitory neurotransmitter glycine, producing an excitatory state characterized clinically by hyperreflexia, severe muscle spasms, and convulsions. However, the toxicokinetics after overdose have not been well-described. In most severe cases of strychnine poisoning, the patient dies before reaching the hospital (Shadnia et al., 2004).

Palatnick et al. (1997) described a case of a 34-year-old man who presented to the emergency department 20 min after ingesting of 125 mL of 2% strychnine sulfate (2.25 g). He was alert and oriented and experiencing muscle spasms. His condition deteriorated, prompting sedation, muscle paralysis, and tracheal intubation. He was administered activated charcoal 100 g via nasogastric tube. He was admitted to intensive care, where he was managed with diazepam, pentobarbital, and pancuronium. Despite mild rhabdomyolysis, he recovered and was extubated on day 3. Although receiving prophylactic heparin therapy, a massive fatal pulmonary embolus ensued. Eighteen blood specimens for strychnine analysis were obtained from 20 min to 51 h after ingestion. Serum concentrations were determined with gas chromatography–mass spectroscopy. Disappearance followed a first-order process with a $t_{0.5}$ of 16 h. These results confirm the findings of a previous case report of 19 strychnine levels obtained between 4 and 19 h that described first-order kinetics with a $t_{½}$ of 10–16 h.

In a case report by Wood et al. (2002), a 42-year-old man ingested an unknown quantity of strychnine powder. Eight serum samples were taken over the first 5 days and analyzed subsequently for strychnine concentrations. The initial concentration at 1.5 h after ingestion was 4.73 mg/L, falling to 0.38 mg/L at 74 h postingestion. Serum concentrations followed a monoexponential elimination curve with a calculated elimination half-life of 12 h. The initial serum concentration of 4.73 mg/L is the highest reported concentration in a patient who has survived.

CLINICAL SYMPTOMATOLOGY

The symptomatology of human intoxication begins 15–30 min after ingestion of strychnine, usually without any warning, and the subject may experience violent convulsions. Convulsions lead to severe lactic acidosis that secondarily results in visceral (lung, heart, kidney, liver, and brain) collapse and death (Gordon and Richards, 1979). Prodromal symptoms are described such as apprehension, restlessness, heightened acuity of hearing, vision, and feeling, hyperreflexia, abrupt movement, and muscular stiffness of face and legs. Generalized convulsions last from 30 s to 2 min. At 10–20 min after exposure, the body's muscles begin to spasm, starting with the head and neck. At first convulsions are clonic, but a tetanic phase quickly intervenes. The body typically arches in hyperextension, the legs are adducted and extended, arms are flexed over the chest, and fists are tightly clenched. The jaw is rigidly clamped, face is fixed in grin, and eyes protrude in fixed stare (Philippe et al., 2004; Lages et al., 2013).

The convulsions progress, increasing in intensity and frequency until the backbone arches continually. Death comes from asphyxiation caused by paralysis of the neural pathways that control breathing or by exhaustion from the convulsions. The subject usually dies within 2–3 h after exposure. At the point of death, the body "freezes" immediately, even in the middle of a convulsion, resulting in instantaneous *rigor mortis*.

Initial symptoms of strychnine poisoning are tightness and twitching of the muscles agitation and hyperreflexia. Stiffness of the body, lockjaw, frothing of the mouth, cessation of respiration occur. Tetanus-like attacks appear every 10–15 min. During these attacks, the eyeballs protrude and the pupils enlarge. Severe cyanosis, which disappears after the attack subsides, also occurs. The attacks (each lasting approximately 3–4 min) appear to be spontaneous while other times they are the result of external stimuli, for example, noises, slight movements, or flashes of light. The patient never loses consciousness. When the poisoning is left untreated, each attack lasts longer than the previous and the interval between them grows shorter. Up to 10 attacks occur before death or recovery. This could happen from 10 min to 3 h and is a result of asphyxiation or inner tissue paralysis.

MECHANISM OF ACTION

Strychnine acts as a blocker or antagonist at the inhibitory or strychnine-sensitive glycine receptor, a ligand-gated chloride channel in the spinal cord and the brain (Song et al., 2006). The glycine receptor (GlyR) is the receptor for the amino acid neurotransmitter glycine (Rajendra et al., 1997). It is one of the most widely distributed inhibitory receptors in the central nervous system. Glycine receptors are found in most brain areas, including the hippocampus, amygdala, ventral tegmental area, and periaqueductal gray (Choi et al., 2013). Strychnine-sensitive glycine receptor is a member of

a family of ligand-gated ion channels. This ionotropic receptor can be activated by a range of simple amino acids, except glycine, β-alanine, and taurine, and can be selectively blocked by the high-affinity competitive antagonist strychnine. The receptor is arranged as five subunits surrounding a central pore, with each subunit composed of four α-helical transmembrane segments. There are presently four known isoforms of the α subunit (α_{1-4}) of GlyR that are essential to bind ligands and a single β-subunit. The adult form of the glycine receptor is the heteromeric $\alpha_1\beta$ receptor, which is believed to have a stoichiometry of three α_1 subunits and two β subunits or four α_1 subunits and one β subunit (Kuhse et al., 1993, 1995). The strychnine-binding subunit of the glycine receptor shows certain homology with nicotinic acetylcholine receptors (Grenningloh et al., 1987). The glycine receptor is highly enriched in microdomains of the postsynaptic neuronal surface apposed to glycinergic afferent endings. There is substantial evidence suggesting that the selective clustering of glycine receptor at these sites is mediated by the cytoplasmic protein gephyrin (Meier et al., 2000).

TOXICITY

Strychnine has been placed in toxicity category I, indicating a high degree of acute toxicity, for oral and ocular effects. Inhalation toxicity is also presumed to be high. Acute toxicity of strychnine to birds is very high. Mammalian studies indicate that strychnine is very highly toxic to small mammals on both an acute oral basis and a dietary basis. The extent of poisoning caused by strychnine depends on the amount and route of strychnine exposure at the time of the exposure; in humans, it depends on the person's condition of health. The signs of toxicity, including death, occur within 1 h.

Animal Toxicity

Reported toxic doses of strychnine at different routes of administration in some animals and humans are summarized in Table 17.1.

Strychnine toxicity in rats is dependent on sex. It is more toxic to females than to males when administered subcutaneously or intraperitoneally, and differences are attributable to higher rate of metabolism by male rat liver microsomes (Parke, 1968). Dogs and cats are more susceptible among the domestic animals, pigs are believed to be as susceptible as dogs, and horses are able to tolerate relatively larger amounts of strychnine (Humphreys, 1988). Birds affected by strychnine poisoning exhibit feathers fluffed or held tightly against the body, ataxia, wing droop, salivation, tremors, muscle tenseness, and convulsions. Death occurs as a result of respiratory arrest.

TABLE 17.1 Toxicity Values (LD_{50}) of Strychnine Found in Different Literature Sources

Organism	Route	LD_{50} (mg/kg)	Source
Bird—wild	Oral	16	Tucker and Haegele, 1971
Cat	Intravenous	0.33	RTECS, 1935[a]
Cat	Oral	0.5	Moraillon and Pinault, 1978
Dog	Intravenous	0.8	Longo et al., 1959
Dog	Subcutaneous	0.35	RTECS, 1935
Dog	Oral	0.5	Moraillon and Pinault, 1978
Duck	Oral	3.0	Tucker and Haegele, 1971
Human	Oral	100–120	Zenz et al., 1994
Human (adult)	Oral	30–100	Gossel and Bricker, 1994
Human (children)	Oral	15	Gossel and Bricker, 1994
Human	Oral	30–60	Lewis, 1996
Human	Oral	5–10	Ellenhorn et al., 1997
Human (adult)	Oral	50–100	Migliaccio et al., 1990
Human	Oral	100–120	Palatnick et al., 1997
Mouse	Intraperitoneal	0.98	Setnikar et al., 1960
Mouse	Intravenous	0.41	Haas, 1960
Mouse	Oral	2.0	Prasad et al., 1981
Mouse	Parenteral	1.06	Zapata-Ortiz et al., 1961
Mouse	Subcutaneous	0.47	Sandberg and Kristianson, 1970
Pigeon	Oral	21.0	Tucker and Haegele, 1971
Quail	Oral	23.0	Tucker and Haegele, 1971
Rabbit	Intravenously	0.4	Longo et al., 1959
Rabbit	Oral	0.6	RTECS, 1935
Rat	Oral	16.0	Spector, 1956
Rat	Oral	2.35	Ward and Crabtree, 1942

[a]*RTECS, Registry of Toxic Effects of Chemical Substances.*

The clinical signs of strychnine poisoning relate to its effects on the central nervous system. After the ingestion (swallowing) of strychnine, symptoms of poisoning usually appear within 15–60 min. The first clinical signs of poisoning include nervousness, restlessness, twitching of the muscles, and stiffness of the neck. As the poisoning progresses, the muscular twitching becomes more pronounced and convulsions suddenly appear in all the skeletal muscles. The limbs are extended and the neck is curved to opisthotonus. The pupils are widely dilated. As death approaches, the convulsions follow one another

with increased rapidity, severity, and duration. Death results from asphyxia because of prolonged paralysis of the respiratory muscles (Humphreys, 1988; Gupta, 2012).

Human Toxicity

People exposed to low or moderate doses of strychnine by any route will have the following signs or symptoms: agitation; apprehension or fear; ability to be easily startled; restlessness; painful muscle spasms possibly leading to fever and to kidney and liver injury; uncontrollable arching of the neck and back; rigid arms and legs; jaw tightness; muscle pain and soreness; difficulty breathing; dark urine; and initial consciousness and awareness of symptoms. The reported medium lethal doses of strychnine in humans range from 5 to 120 mg/kg.

There are some cases of human poisoning. A 46-year-old man presented 2 h after ingestion of approximately 250 mg strychnine with severe, violent, generalized convulsions triggered by external stimuli. During the convulsion-free periods there were no abnormal signs in the physical examination (Scheffold et al., 2004). A 28-year-old man was admitted 2 h after ingestion of 1–1.5 g of strychnine. He was severely agitated and in mild respiratory distress; blood pressure was 90/60 mmHg, pulse was 110/min, and peripheral pulses were weak. He had generalized hyperactive reflexes and had several generalized tonic–clonic convulsions in the emergency department. Treatment consisted of gastric lavage with water, oral administration of activated charcoal and sorbitol solution, continuous intravenous administration of midazolam, and then sodium thiopental, furosemide, sodium bicarbonate, and hemodialysis for acute renal failure. His clinical course included respiratory distress, agitation, generalized tonic–clonic convulsions, hyperactivity, oliguria, and acute tubular necrosis before recovery in 23 days. This patient ingested what would normally be a fatal amount of strychnine. He had signs and symptoms of severe toxicity and recovered, suggesting that with aggressive supportive care patients may have favorable outcomes (Shadnia et al., 2004). In another case report described by Wood et al. (2002), a 42-year-old man presented soon after ingestion of an unknown but warranted lethal quantity of strychnine powder. After respiratory arrest with intensive supportive management requiring admission to an intensive care unit, he survived.

People exposed to high doses of strychnine may have respiratory failure (inability to breathe), possibly leading to death or brain death, within the first 15–30 min of exposure. No postmortem lesions are observed with the exception of small pinpoint hemorrhages in the lungs resulting from death attributable to asphyxia. Rigor mortis occurs soon after death and persists for days.

Toxicity of strychnine in humans, expressed as LD_{Lo} (lethal dose low), is approximately 30 mg/kg. Strychnine is less toxic in humans than in most animals. If the person survives the toxic effects of strychnine poisoning, then long-term health effects are unlikely. However, long-term effects may result from damage caused by the poisoning, for example, brain damage from low oxygen or kidney failure. People severely affected by strychnine poisoning are not likely to survive.

At the present time, fatal strychnine poisoning is uncommon. It is no longer used as a therapeutic drug and its availability to the public is controlled by legislations in various jurisdictions. Nevertheless, it is still in use as a rodenticide and an adulterant in street drugs. Homicide by strychnine is extremely rare. Because the autopsy findings are subtle, strychnine poisoning could easily be overlooked and a homicide may go undetected. This communication highlights an unusual case of homicide by strychnine. It is important in deaths in which there are no gross autopsy findings, sudden death in particular, that routine toxicology be performed because strychnine is likely to be detected (Kodikara, 2012).

Diagnosis

A tentative diagnosis can be made based on clinical signs and history. However, a positive diagnosis can only be made by identifying strychnine in the stomach contents, viscera, or blood. The drug can be identified by chemical tests and microscopic identification of typical strychnine crystals.

RISK ASSESSMENT

Human Health Hazard

The human health assessment for strychnine is based on the acute toxicity. Strychnine has been placed in toxicity category I, indicating the greatest degree of acute toxicity, for oral and ocular effects. It has been reported that the probable lethal oral dose is 1.5–2 mg/kg (Gosselin et al., 1984). Inhalation toxicity is also presumed to be high. An oral dose of 1.5–2 mg/kg is equivalent to 70–93 mg/m^3 for 30 min for a 70 kg human.

Strychnine was first registered as a pesticide in the United States in 1947; however, this natural toxin had been used in many counties to control vertebrate animals for many years before 1947. Currently, strychnine is registered for use only below ground as a bait application to control pocket gophers. The end-use products are formulated as grain-based bait or a paste. Baiting can be performed manually or with the use of application equipment.

The European Union (EU) withdrawal of strychnine marked its end as a method of mole control. The EU directive 91/414/EEC is midway through an ambitious

program to review all pesticides used within Member States. This requires manufacturers to provide health and safety data to support the continued registration of their products. Strychnine was to be reviewed in the fourth part of this program, but manufacturers have failed to provide such data. Despite last ditch appeals by users, from September 1, 2006, it was no longer legal to use.

However, strychnine in the form of a homeopathic preparation is still used and in exceptional cases may also cause poisoning. Gicquel et al. (2012) presented the case of a bulimic woman who was admitted to the emergency unit for painful muscle spasms and hypertonic crisis with respiratory blocking, after application of homeopathic nux vomica mother tincture, which contains indole alkaloids including strychnine. Toxicological screening revealed the presence of strychnine in the blood sample. This was corroborated by the patient, who explained that she had swallowed a whole bottle of nux vomica mother tincture as an emetic during a bulimic episode. This corresponded to 212 mg strychnine ingested. The therapeutic patient management consisted of symptomatic medication by diazepam and paracetamol and monitoring biological parameters and vital functions including breathing functions. Determination of strychnine concentrations in blood samples contributed to the control of poisoning. A blood concentration of 3 mg/L was first evaluated at admission of the patient, followed by a progressive decrease to 0.5 mg/L on the second day and the favorable clinical outcome of the patient.

Safety Data

Strychnine oral reference dose (RfD) of 0.0003 mg/kg/day or 0.02 mg/day for a 70 kg person is derived from the Seidl and Zbinden (1982) short-term to subchronic study by applying an uncertainty factor of 10,000. This factor accounts for extrapolation from a less-than-chronic to a chronic exposure study, extrapolation from animals to humans, and differences in sensitivity among the human population. An additional factor of 10 is used because a LOAEL/FEL (2.5 mg/kg/day) was utilized in the estimation of the RfD instead of a NOAEL. The immediately dangerous to life and health dose for strychnine by NIOSH REL is 0.15 mg/m^3, and the current OSHA PEL is 0.15 mg/m^3.

The work of Seidl and Zbinden (1982) is the only oral short-term or subchronic study reported in which rats received daily doses of 0–10 mg/kg of strychnine by gavage for 28 days. Data recorded for the surviving animals included blood cell counts, electrocardiograms, eye examinations, urine chemistry, weight gain, tissue histology, organ weights, behavioral tests, and food and water consumption. Mortality was observed in 5 of 12 male rats receiving 10 mg/kg and in 1 of 12 in each of the 5 mg and 2.5 mg/kg groups. All deaths occurred 0.5–6 h after oral doses.

Additional studies (Gitzelmann et al., 1978) reported that a 6-month-old human patient received strychnine doses of 0.3–1.1 mg/kg/day over an 18-month period without any adverse effects. However, the patient may have had a higher strychnine tolerance as a result of nonketotic hyperglycinemia. Risk phases of strychnine are R26, R27, and R28.

TREATMENT

There is no specific antidote for strychnine, but recovery from strychnine exposure is possible with early hospital treatment. Treatment consists of removing the drug from the body (decontamination) and getting supportive medical care in a hospital setting. Supportive care includes intravenous fluids, medications for convulsions and spasms, and cooling measures for high temperature.

Treatment of strychnine poisoning involves an oral application of an activated charcoal infusion that serves to absorb any poison within the digestive tract that has not yet been absorbed into the blood. Unabsorbed strychnine can be removed from the stomach by gastric lavage of tannic acid (strong tea) or potassium permanganate solutions used as chemical antidotes. Seizures are controlled and anticonvulsants such as phenobarbital or diazepam are administered to control convulsions, along with muscle relaxants such as dantrolene to combat muscle rigidity. Because diazepam is the anticonvulsant of choice but is not effective in all cases, a combination with midazolam, fentanyl, and pancuronium is recommended in controlling the convulsions (Scheffold et al., 2004). Fatal outcome of strychnine poisoning demands aggressive management with early intubation, control of muscle tremors, and prevention of rhabdomyolysis and renal failure. If the patient survives past 24 h, then recovery is probable.

Small doses of strychnine were once used in medications as a stimulant, a laxative, and as a treatment for other stomach ailments. Strychnine has stimulant effects at low doses but, because of its high toxicity and tendency to cause convulsions, the use of strychnine in medicine was eventually abandoned once safer alternatives became available.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Strychnine is a highly poisonous natural substance that is used in some countries for the control of wild animals. Today, strychnine is used primarily as a pesticide, particularly to kill rats. Its use is restricted by law.

Because strychnine is highly toxic and can be rapidly absorbed through the mucous membranes of the mouth, stomach, and small intestines, theoretically it may be used as a military toxic or terroristic agent. There are three main ways that strychnine can enter the body: inhalation, ingestion, and broken skin.

Goal-directed misuse of strychnine against humans is unlikely. Its misuse against domestic animals is realistic and more likely.

Uncommonly, strychnine is found mixed with "street" drugs such as LSD, heroin, and cocaine. It is very probable that seizures observed occasionally after cocaine application may be caused by admixed strychnine (Haddad and Winchester, 1983; Wijesekera et al., 1988). After analysis of heroin samples seized in the Florence area between 1975 and the first half of 1981, no dangerous substances were found in the samples and strychnine, if present, was found in very low concentrations (Mari et al., 1982).

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CHAPTER

18

Superwarfarins

Michael J. Murphy and Andres M. Lugo

INTRODUCTION

Chemical warfare agents may be manufactured from a wide range of commercially manufactured household industrial products, such as bleach, antifreeze, fertilizers containing anhydrous ammonia, pesticides, or anticoagulant rodenticides, particularly superwarfarins (WHO, 2008). Some chemical warfare agents (e.g., nerve agents), when dispersed in air, are capable of harming or killing a large number of people. On the other hand, superwarfarins may be used to harm or terrorize people through the ingestion of contaminated food or water.

Superwarfarins, the second generation of anticoagulant rodenticides, are referred to as such in the modern medical literature (Pavlu et al., 2005; Sharma and Bentley, 2005; Dolin et al., 2006; POISINDEX, 2007). They are a group of commercially available, long-acting, anticoagulant rodenticides that are structurally similar to warfarin but are many times more potent. Many of them have the capacity to cause severe bleeding problems that may last for 2–8 months in humans (Ellenhorn et al., 1997; Goldfrank et al., 2002).

This group of long-acting anticoagulants may be used as chemical warfare agents because of their high potency and duration of action. The capability of terrorists to use these commercially available poisons depends upon the availability of large amounts of high-concentration product (whether obtained legally or illegally), the target population and its vulnerability, and the method of effective delivery and dissemination (EPA, 2005; WHO, 2008). Although these anticoagulants may be absorbed through the skin and lungs, the main route of exposure is ingesting food or water containing the product (Jones et al., 1984; Katona and Wason, 1989; Swigar et al., 1990; Wallace et al., 1990; Exner et al., 1992; Rauch et al., 1994; Gallo, 1998; Corke, 1997). Superwarfarin poisoning may result in a large number of casualties if these substances are ingested (Palmer et al., 1999; Baker et al., 2002; EPA, 2005; POISINDEX, 2007; HSDB, 2008).

Superwarfarin rodenticides are used to kill urban and agricultural rodent pests. They are readily available to the general public and pest controllers and are easy to obtain and conceal, so they may pose a risk of being used as chemical warfare agents (EPA, 2003, 2005; WHO, 2003, 2008). These rodenticides are available as meal bait packs, pellets, mini-pellets, blocks, mini-blocks, wax blocks, liquid bait formulations, tracking powder, and concentrate formulations (POISINDEX, 2007; WHO, 2008; Wilton, 1991).

BACKGROUND

Anticoagulants were discovered in the early twentieth century after livestock had eaten moldy sweet clover contaminated with bis-hydroxycoumarin and died of hemorrhagic disease. Newer long-acting warfarin derivatives such as brodifacoum, bromadiolone, diphenadione, and chlorophacinone can produce profound and prolonged anticoagulation and bleeding after a latency period that generally lasts 24–72 h (Gill and Redfern, 1980; FDA, 1985; Chong et al., 1986; Greeff et al., 1987; Smolinske et al., 1989; Swigar et al., 1990; Wallace et al., 1990; Routh et al., 1991; Wilton, 1991; Exner et al., 1992; Rauch et al., 1994; IPCS, 1995a–e; Hui et al., 1996; Tecimer and Yam, 1997; Chua and Friedenberg, 1998; Gallo, 1998; EPA, 2003, 2005) (Table 18.1).

In the 1940s, a small British pharmaceutical company suggested that dicoumarol might have rodenticidal properties. Trials carried out by Armour and Barnett (1950) confirmed the idea and started the era of anticoagulant rodenticides. Warfarin was the first anticoagulant rodenticide; it was introduced into the market shortly after World War II and became widely used in many countries. Other anticoagulant compounds with potency similar to that of warfarin were also synthesized. These early anticoagulant rodenticides have often been called

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TABLE 18.1 Common Commercial Products Containing Superwarfarins

Name	Molecular Formula	Commercial Names
Brodifacoum CAS: 56073-10-0	C_{31}-H_{23}-Br-O_3	D-Con Mouse-Prufe I & II®, Havoc®, Klerat®, Ratak Plus®, Talon G®, Void® Finale, Folgorat, Matikus, Mouser, Rodend, Volak, Volid
Difenacoum CAS: 56073-07-5	C_{31}-H_{24}-O_3	Compo®, Diphenacoum®, Frunax DS®, Matrak®, Neosorexa®, Rastop®, Ratak®, Ratrick®, Silo®
Bromadiolone CAS: 28772-56-7	C_{30}-H_{23}-Br-O_4	Apobas®; Bromard®; Bromone®, Bromatrol®; Bromorat®; Contrac®; Deadline®; Hurex®; Lanirat®; Maki®; Morfaron®; Musal®; Maki®, Ramortal®; Ratimon®; Rodine-c®; Slaymore®; Super-caid®; Toidon®
Diphacinone CAS: 82-66-6	C_{23}-H_{16}-O_3	Diphacine®, Ditrac®, Gold Crest®, Kill-Ko®, P.C.Q.®, Promar®, Ramik®, Rat Killer®, Rodent Cake®, and Tomcat®
Chlorophacinone CAS: 3691-35-8	C_{23}-H_{15}-Cl-O_3	Caid®, Liphadione®, Microsul®, Ramucide®, Ratomet®, Raviac®, Rozol®, Topidox®
Difethialone[a] CAS: 104653-34-1	C_{31}-H_{23}-Br-O_2-S	None to report
Pindone[a] CAS: 83-26-1	C_{14}-H_{14}-O_3	Pestanal®, Pindone®, Pival®, Pivalyn®, Pivalyl Valone®, Tri-ban®
Coumatetralyl[a] CAS: 5836-29-3	C_{19}-H_{16}-O_3	Racumin®
Coumafuryl[a] CAS: 117-52-2	C_{17}-H_{14}-O_5	Fumarin®, Tomarin®
Valone CAS: 83-28-3	C_{14}-H_{14}-O_3	None to report
Flocoumafen[a] CAS: 90035-08-8	C_{33}-H_{25}-F_3-O_4	None to report

Available forms include: meal bait packs, pellets, mini-pellets, blocks, mini-blocks, wax blocks, liquid bait formulations, and tracking powder.
[a]*No longer produced or used in the United States.*

TABLE 18.2 The Oral LD_{50} Values (mg/kg body wt) of Some Anticoagulant Rodenticides

Animals	Bromadiolone	Brodifacoum	Difenacoum
Rat (acute)	0.65	0.27	1.8
Rat (chronic)	(0.06–0.14) × 5	(0.05–0.08)	0.15 × 5
Mouse	0.99	0.4	0.8
Rabbit	1.0	0.2	2.0
Pig	3.0	10.0	80.0
Dog	10.0	3.5	50.0
Cat	25.0	25.0	100.0
Chicken	5.0	10.0–20.0	50.0
Guinea pig	2.8	–	–
Opossum	–	0.17	–
Sheep	–	10.0	100.0

first-generation anticoagulant rodenticides. These compounds generally have moderate toxicity, with acute LD_{50} values ranging from 10 to 50 mg/kg body weight (Table 18.2).

The first-generation compounds often needed continuous bait exposure for rodent control. Many rodent species developed a resistance to warfarin (Jackson et al., 1975), presumably due to continued exposure and widespread use. Consequently, new chemical structures were synthesized and used as anticoagulant rodenticides. These newer compounds are generally more toxic than warfarin, with acute LD_{50}s of 0.2–3.9 mg/kg body weight. For example, a bait concentration of only 50 ppm of brodifacoum is adequate to control the population in a single feeding for most rodents and noncommensal species (Matolesy et al., 1988). These newer compounds were called *second-generation anticoagulant rodenticides* and are often now referred to as *superwarfarins* in the contemporary medical literature (e.g., Chong et al., 1986; Greeff et al., 1987; Swigar et al., 1990; Wallace et al., 1990; Routh et al., 1991; Wilton, 1991; Exner et al., 1992; Rauch et al., 1994; Hui et al., 1996; Tecimer and Yam, 1997; Gallo, 1998; Chua and Friedenberg, 1998; Pavlu et al., 2005; Sharma and Bentley, 2005; Dolin et al., 2006).

During the past 30 years, there have been more than 600 articles published in medical literature relating to the clinical assessment, laboratory testing, and treatment of patients exposed to superwarfarins. A great number of these articles are about children under 6 years old who accidentally ingested small amounts of these products and, in most cases, did not experience adverse effects (Brands et al., 1995; Ingels et al., 2002; AAP, 2003; Osterhoudt and Henretig, 2003). Also, there are a few cases describing severe bleeding or bleeding-related complications from patients who intentionally ingested large amounts of the substance, as well as a few fatal ingestion cases (Wallace et al., 1990; Casner, 1998; Walker and Beach, 2002; AAPCC, 2006). Most of the health hazards are associated with accidental ingestion of superwarfarins, and the risk of dermal and inhalation exposure is minimal (Bruno et al., 2000; POISINDEX, 2007). One-third of these publications concern domestic

animals and a few wild animals (Newton et al., 1990; Stone et al., 1999), with a small number of reported deaths. All animal exposures are due to accidental direct and indirect ingestion of these rodenticides. The great majority of animal exposures include dogs, which may need gastric decontamination and, in some cases, referral to a veterinarian for further clinical evaluation and treatment (DuVall et al., 1989; Hornfeldt and Phearman, 1996; McConnico et al., 1997; Robben et al., 1997; Borst and Counotte, 2002). In most cases, gastric decontamination will be recommended and treatment with vitamin K_1 may be needed. There are few data about the incidence or mortality rates from animal exposures to rodenticides.

Warfarin and dicoumarol found application as both oral anticoagulants and as rodenticides. Sweet clover requires the action of molds to form dicoumarol; giant fennel does not. Giant fennel (*Ferula communis*) grows in Mediterranean countries. It has a naturally occurring anticoagulant effect. An association between the plant and anticoagulation was first reported in the 1950s (Costa, 1950a,b; Carta, 1951). It was further investigated in Italy (Corticelli and Deiana, 1957; Corticelli et al., 1957; Mazzetti and Cappelletti, 1957; Cannava, 1958), and then in Israel (Shlosberg and Egyed, 1983). The anticoagulant activity of the plant in Morocco has also been reviewed (Lamnaouer, 1999).

Warfarin and its congeners are still used as therapeutic agents. Oral anticoagulants available therapeutically in Europe include warfarin, phenprocoumaron, and nicoumalone—also called acenocoumarol (Shetty et al., 1993). Oral anticoagulants are used therapeutically to reduce thromboembolic events. Warfarin examples include a reduction in catheter-related thrombosis (Guidry et al., 1991; Magagnoli et al., 2006); early venous thrombosis after operations (Calnan and Allenby, 1975; Pan et al., 2005), including hip surgery; atrial fibrillation (Middlekauff et al., 1995; Reiffel, 2000); and myocardial infarction (Asperger and Jursic, 1970). A number of adverse events have been recognized, most of which are related to drug interactions (Dayton and Perel, 1971).

An association between vitamin K_1 and coagulopathies was made in the mid-1930s (Dam, 1935; Fieser et al., 1939). Soon thereafter, Karl Paul Link reported the discovery of dicoumarol in moldy hay (Last, 2002). Naturally occurring coumarin in sweet-clover hay is converted by fungi to dicoumarol. Dicoumarol was found to be the causative agent of the disease, so the elements needed for the disease were coumarin-containing plant material plus mold growth. Subsequently, a range of molecules were synthesized, and one of these, warfarin, became the most popular (Duxbury and Poller, 2001). Warfarin takes its name in part from the Wisconsin Alumni Research Foundation and the rest from from coumarin.

AAPCC Data on Superwarfarins

Every year, tens of thousands of accidental ingestions of long-acting anticoagulant rodenticides are reported worldwide in medical literature. These include the annual report from the American Association of Poison Control Centers (AAPCC). During the past 23 years, the AAPCC reported 209,047 exposures to long-acting anticoagulant rodenticides (LAARs), which included 24 deaths due to LAAR ingestion. All fatalities were in adults who committed intentional suicide.

CLASSIFICATION OF SUPERWARFARINS

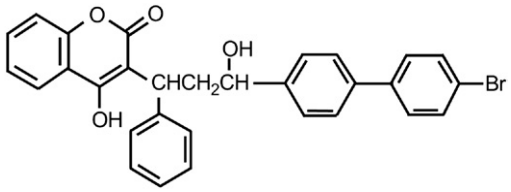
Anticoagulant rodenticides are also categorized by chemical structure. The chemical structure of the currently marketed products fits in one of two chemical classes: 4-hydroxycoumarins and indanediones.

4-Hydroxycoumarins

This group of compounds have a 4-hydroxycoumarin ring with different side-chain substituents at the 3-position. Commonly used superwarfarin anticoagulant rodenticides in this group are bromadiolone, brodifacoum, coumatetralyl, coumafuryl, and difenacoum. Brodifacoum, difenacoum, and bromadiolone are three of the most commonly used rodenticides around the world. Brodifacoum is the most frequently used rodenticide in the United States. These rodenticides share most of their physical and chemical characteristics, as well as their toxicokinetics, toxicodynamics, and mechanism of toxicity, and the medical toxicological management is the same for all superwarfarins.

Bromadiolone

Chemical formula: $C_{30}H_{23}BrO_4$

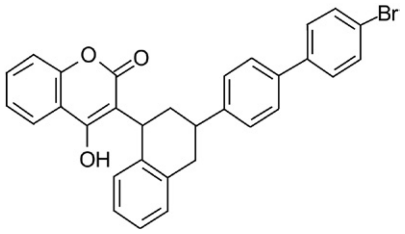


Bromadiolone [3-(3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenyl propyl)-4-hydroxycoumarin] was synthesized and marketed by the French company Lipha SA during the mid-1970s. It is used widely for control of commensal and field rodents in many countries. Technical-grade bromadiolone is 97% pure.

It is a yellowish powder and stable up to 200°C (Chalermchaikit et al., 1993). It is very soluble in dimethylformamide (730 g/L), but less soluble in ethyl acetate (25 g/L), and ethanol (8.2 g/L), and sparingly soluble in water (0.019 g/L). Bromadiolone is considered more palatable to rodents than most other anticoagulants. Its concentration in baits is usually 50 ppm (Chalermchaikit et al., 1993). Although bromadiolone is considered a second-generation anticoagulant rodenticide, some resistance problems have been reported with *Rattus norvegicus* and *Mus musculus* in the United Kingdom and Denmark (Rowe et al., 1981; Lund, 1984; IPCS, 1995d).

Brodifacoum

Chemical formula: $C_{31}H_{23}BrO_3$



Brodifacoum [3-(3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro naphth-1-yl)-4-hydroxycoumarin] is one of the newer and more potent second-generation anticoagulant rodenticides. It was first introduced in 1977 by Sorex Ltd. of London, and then developed by the Imperial Chemicals Incorporated (ICI) Plant Protection Division (Chalermchaikit et al., 1993).

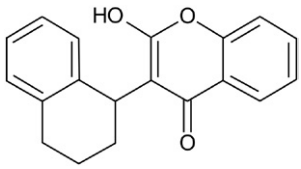
Pure brodifacoum is an off-white to fawn-colored powder with a solubility of 6–20 g/L in acetone, 3 g/L in chloroform, 0.6–6 g/L in benzene, and less than 10 mg/L water. It is very stable in the environment with no loss after 30 days of exposure to direct sunlight (Chalermchaikit et al., 1993).

Brodifacoum has been marketed in several countries for the control of a wide range of rodent pest species. It is available as a 0.005% pellet for rat and mouse control, a smaller 0.001% pellet for field rodent control, and as 29 g wax blocks for sewer rat control. It is the only anticoagulant rodenticide found to produce 100% mortality in most rodent species after only a 24 h dose (Chalermchaikit et al., 1993). Brodifacoum was effective against warfarin-resistant rats and mice in 1984, but the possibility of resistance has been raised (Lund, 1984).

There is variation in the susceptibility of species to brodifacoum. Dogs are susceptible and are commonly exposed to potentially toxic quantities of brodifacoum (Chalermchaikit et al., 1993).

Coumatetralyl

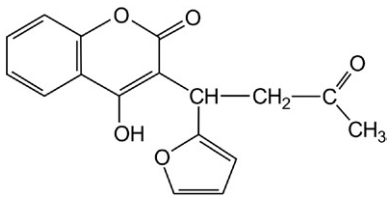
Chemical formula: $C_{19}H_{16}O_3$



Coumatetralyl [3-(alpha-tetralyl)-4-hydroxycoumarin] was introduced by Bayer AG with the trademark name of Racumin. It has been used for commensal rodent control in many countries. It is formulated as a dry bait (0.0375%), a liquid bait of its sodium salt, and a 0.75% tracking dust (Chalermchaikit et al., 1993). Pure coumatetralyl is a colorless powder that is stable at temperatures below 150°C. Its solubility is 20–50 g/L in propan-2-ol, 50–100 g/L in methylene dichloride, and 4 mg/L in water. The acute and chronic LD_{50}s of *R. norvegicus* are 16.5 and 0.3 mg/kg for five consecutive doses, respectively. Chickens are somewhat resistant to coumatetralyl, with a chronic LD_{50} of 50 mg/kg for eight consecutive doses. Signs did not appear in fish until the concentration of coumatetralyl reached 1,000 mg/L in water (Chalermchaikit et al., 1993). In spite of its low toxicity, it is reported to be a little more effective than warfarin against *R. norvegicus*, apparently due to a higher palatability. Coumatetralyl was introduced after the detection of warfarin-resistant rat populations and showed considerable success for a number of years, but resistant pests have been reported in the United Kingdom and Denmark (Rowe and Redfern, 1968; Lund, 1984).

Coumafuryl

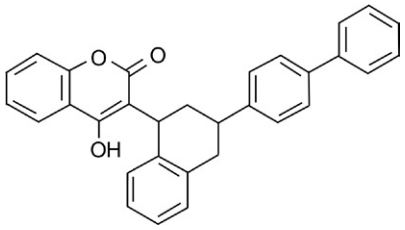
Chemical formula: $C_{17}H_{14}O_5$



Coumafuryl [3-(alpha-acetonylfurfuryl)-4-hydroxycoumarin] is a German anticoagulant, introduced in 1952, and is used at 0.025–0.05% in baits. Its toxicity is considered equal to warfarin for *R. norvegicus*, but slightly less efficient against *M. musculus*. The chronic LD_{50} in *R. norvegicus* is 1.4 mg/kg for five repeated doses. Cats and dogs seem to be almost as susceptible as rats, with dogs being killed by 2 mg/kg for five repeated doses and cats by 10 mg/kg for four repeated doses (Chalermchaikit et al., 1993).

Difenacoum

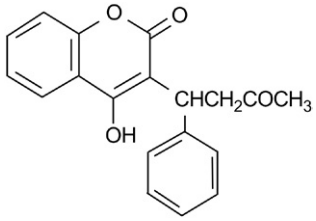
Chemical formula: $C_{31}H_{24}O_3$



Difenacoum [3-(3-*p*-diphenyl-1,2,3,4-hydronaphth-1-yl)-4-hydroxycoumarin] was synthesized in the United Kingdom and marketed in 1975 by Sorex Ltd under the trademark Neosorexa, and by ICI Plant Protection Division under the trademark Ratak as a 0.005% pelleted bait, and as a wax block. Pure difenacoum is an off-white powder with a solubility of greater than 50 g/L in acetone, 600 mg/L in benzene, and less than 10 mg/L in water. It is more toxic than warfarin but less palatable (IPCS, 1995c). Difenacoum is still effective against many populations of warfarin-resistant rats (Desideri et al., 1979), but resistance may be developing in the United Kingdom (Greaves et al., 1982).

Warfarin

Chemical formula: $C_{19}H_{16}O_4$



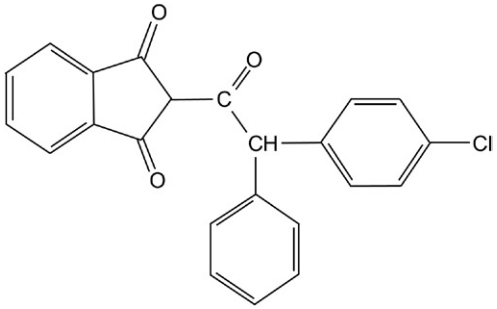
Warfarin [3-(a-acetonylbenzyl)-4-hydroxycoumarin] was the first anticoagulant rodenticide. It was introduced shortly after World War II after being developed by the Wisconsin Alumni Research Foundation. Warfarin is still used widely, especially for the control of *R. norvegicus* in areas where resistance has not yet developed. In its racemic form, warfarin is colorless and crystalline, insoluble in water but readily soluble in acetone, dioxane, and moderately soluble in alcohols. Warfarin is formulated as dry bait (0.005–0.05%), as well as a liquid bait, based on the sodium salt, and a tracking dust (0.5–1.0%). It is generally applied as the S-isomer, which has a toxicity ten times greater than the R-isomer. The acute and chronic LD_{50}s for *R. norvegicus* are around 10–12 and 0.75 mg/kg for five repeated doses, respectively (Colvin and Wang, 1974). Warfarin is sometimes combined with an antibacterial agent, sulfaquinoxaline, in order to reduce the bacterial production of vitamin K in the rat intestine, but the effectiveness of this combination has not been proven. Warfarin is considered one of the safest anticoagulants, as far as domestic and other nontarget animals are concerned. Serious resistance problems have been reported in Europe. It has recently been evaluated against sewer rats in London (Channon et al., 2000).

Indanediones

This group of compounds has a 1,3 indanedione structure with different side-chain substituents at the 2-position. The most common superwarfarins in this group are chlorophacinone and diphacinone.

Chlorophacinone

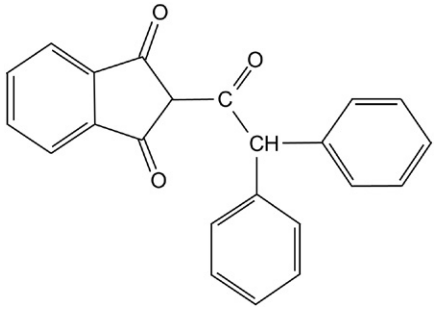
Molecular formula: $C_{23}H_{15}ClO_3$



Chlorophacinone [2-(alpha-4-chlorophenyl-a-phenylacetyl)-1,3-indandione] was introduced in the mid-1960s by Lipha SA at concentrations of 0.05% in baits and 0.2% in tracking dust. Pure chlorophacinone is a yellow crystalline solid that is very soluble in acetone, ethanol, ethyl acetate, but is only somewhat soluble in water. It is quite stable and resistant to weathering. Chlorophacinone does not induce bait-shyness and is compatible with cereals, fruits, roots, and other potential bait substances. Its acute LD_{50} in *R. norvegicus* is about 20.5 mg/kg, which is less toxic than warfarin, but it has a stronger initial effect on rats and mice. For control of house mice populations, a prolonged feeding period is needed. Chlorophacinone may not be effective against warfarin-resistant rodents (Chalermchaikit et al., 1993).

Diphacinone

Molecular formula: $C_{23}H_{16}O_3$



Diphacinone (2-diphenylacetyl-1,3-indandione) is an old anticoagulant rodenticide introduced by Vesicol Chemical Corp. and the Upjohn Co. It has been produced and used primarily in the United States as a 0.005% dry or liquid bait. Pure diphacinone is a yellow powder that is very soluble in chloroform (204 g/kg), toluene (73 g/kg), xylene (50 g/kg), and acetone (29 g/kg), but only somewhat soluble in water (0.30 g/L). It will decompose in water due to sunlight. The acute LD_{50}s in *R. norvegicus* are 22.7 mg/kg in females and 43.3 mg/kg in males. It is more toxic than warfarin to rats, mice, and dogs, but its palatability is somewhat lower. Diphacinone may not be effective against some warfarin-resistant rodents (Chalermchaikit et al., 1993). The anticoagulant rodenticides are marketed to have efficacy against a number of target pest species.

TOXICOKINETICS

Absorption, Metabolism, and Excretion in Laboratory Animals and Humans

Superwarfarins are primarily well absorbed from the gastrointestinal tract. Almost 90% is absorbed, with peak plasma concentrations often occurring within 12 h of ingestion. Binding to plasma proteins may prolong distribution and half-life. Toxicity after dermal or respiratory exposure is rare (Boermans et al., 1991; Berry et al., 2000), but not unreported (Spiller et al., 2003).

The metabolism and elimination of the trans-isomer was more rapid than those of the *cis*-isomer. The elimination from the liver is biphasic, with an initial rapid phase of 3 days and a slower phase with a half-life of 120–130 days. The liver is the major organ for accumulation and storage, which has been found mainly as the unchanged parent compounds. The major route of elimination in different species after oral administration is via the feces. The urine is a very minor route of elimination (Watt et al., 2005).

MECHANISM OF ACTION

The mechanism of action of all anticoagulant rodenticides is similar to that of warfarin, specifically inhibition of vitamin K_1 epoxide reductase (Park et al., 1979; Leck and Park, 1981; Breckenridge et al., 1985). In the coagulation cascade, the clotting factors II, VII, IX, and X must bind calcium ions to be active in clot formation. The Ca^{2+}-binding ability requires converting glutamyl residues on these clotting factors to carboxyl glutamyl residues by the process of carboxylation. This carboxylation uses vitamin K_1 hydroquinone as a cofactor. This vitamin K–dependent carboxylase reaction converts vitamin K_1 hydroquinone to its epoxide form, vitamin K_1 2,3-epoxide. In the normal cycle, vitamin K_1 2,3-epoxide is reduced to the original vitamin K_1 (phylloquinone) by epoxide reductase, and thus it gets recycled. The anticoagulant rodenticides produce their effect by interfering with vitamin K_1 epoxide reductase, resulting in the depletion of vitamin K_1 and subsequently impairing the synthesis of normal clotting factors II, VII, IX, and X (Craciun et al., 1997, 1998). Clinical coagulopathy soon follows the depletion of vitamin K_1 in the liver. In the dog, these clotting factors have plasma half-lives of 41, 6.2, 13.9, and 16.5 h, respectively. The coagulation system continues to function well until about 3–5 days after ingestion, when the natural decay of clotting factors occurs. It has been suggested that they bind more strongly to the liver than warfarin, resulting in more persistent effects (Jackson and Suttie, 1977; Suttie, 1986; Murphy and Gerken, 1989; Barnett et al., 1992; Babcock et al., 1993; James et al., 1998). The interrelationship of vitamin K, prothrombin, and gamma-carboxyglutamic acid is reviewed in Stenflo (1978). The interaction of warfarin and vitamin K is reviewed in Suttie (1990).

TOXICITY

Clinical Effects: Signs and Symptoms

Clinical signs and symptoms of acute intoxication by superwarfarins range from a mild tendency to bleed (in less severe poisoning cases) to severe coagulopathy. Mild bleeding tendencies often show themselves clinically as nose or gum bleeding, hemoptysis, ecchymosis, bloody or melenotic stools, hematuria, abdominal or flank pain, enhanced bruising, and ventral hematomas. Severe bleeding may lead to shock and death. Internal and external bleeding are the most frequent clinical signs, followed by tachycardia and hypotension, and then multiple organ failure due to substantial blood loss. The onset of the signs of poisoning may not be evident until a few days after absorption (Vogel et al., 1988; Nighoghossian et al., 1990; Swigar et al., 1990; Weitzel et al., 1990; Wilton, 1991; Ross et al., 1992; Corke, 1997; Casner, 1998; Chua and Friedenberg, 1998; Baker et al., 2002; Tsutaoka et al., 2003).

Animal Toxicology

Clinical signs are usually delayed until 24–36 h after ingestion. The most common signs include vomiting, diarrhea, dyspnea, weakness, depression, anorexia, hematuria, and melena. Other signs, such as pale mucous membranes, bleeding from nose and gums, and generalized bruising, may be noticeable as well. Internal bleeding also causes generalized pain; fever;

lameness from bleeding into a joint; bleeding into the thorax or abdomen, brain, or pericardium; or sudden death (Braithwaite, 1982; Berny et al., 1995; Munday and Thompson, 2003).

Pediatric Exposures

The great majority of human exposures are children under the age of 6 due to accidental or unintentional ingestion. They usually do not require any medical intervention or routine follow-up laboratory studies and can be adequately managed by poison control centers with home observation and parent education (Mullins et al., 2000; Ingels et al., 2002; Kanabar and Volans, 2002; Shepherd et al., 2002). Children with acute, unintentional superwarfarin ingestions may often be managed without gastric decontamination or prophylactic vitamin K. Laboratory testing for coagulopathy should be reserved for cases involving clinically evident bleeding abnormalities (Ingels et al., 2002).

A small number of reported cases of children have presented with mild to moderate hematological effects, requiring minimal to nonmedical intervention (Smolinske et al., 1989; Watts et al., 1990; Babcock et al., 1993; Travis et al., 1993; Golej et al., 2001; Osterhoudt and Henretig, 2003).

Intentional suicidal ingestion of large amounts of product conveys a greater risk for severe toxicity and increased mortality and should be referred to a healthcare facility for examination and treatment if needed (Ingels et al., 2002).

Adult Exposures

The great majority of adult exposures to rodenticides are due to deliberate acute and chronic surreptitious ingestion. Bleeding disorders may persist for 6 weeks to many months. Serious poisoning has been reported in adults with massive overdoses. These long-acting anticoagulants have produced rapid and persistent bleeding due to hypoprothrombinemia (Chong et al., 1986; Hoffman et al., 1988; Katona and Wason, 1989; Wallace et al., 1990; Routh et al., 1991; Barnett et al., 1992; Exner et al., 1992; Mack, 1994; Morgan et al., 1996; McCarthy et al., 1997; Gallo, 1998; Berry et al., 2000). There is a risk of spontaneous abortion with long-acting anticoagulants (Lipton and Klass, 1984; Zurawski and Kelly, 1997; Nelson et al., 2006).

The severity of the intoxication depends on the amount of rodenticide ingested, preexisting comorbidity, and coingestion of other toxic substances (Seidelmann et al., 1995; Palmer et al., 1999; Stanziale et al., 1997; Tecimer and Yam, 1997; Walker and Beach, 2002). Fatalities are usually due to intentional suicidal ingestion of large amounts. Bleeding disorders and organ failure have been described in adults due to deliberate acute and chronic surreptitious ingestion. A total of 24 deaths have been reported by the AAPCC in the past 20 years (Table 18.3; AAPCC-TESS annual reports from 1983–2006).

Household Pets and Farm Animal Exposures

Household pets and farm animals may be accidentally exposed to rodenticides. The possible effects on nontarget organisms can be considered in two types: direct poisoning and secondary poisoning. Secondary poisoning is generally considered to occur after an animal has consumed anticoagulant rodenticide–poisoned rodents. The most common type of exposure is direct poisoning by eating a cereal-based bait containing the rodenticide. The most commonly affected household pets are dogs, followed by cats, hamsters, rabbits, and pet birds (Redfern and Gill, 1980; Park and Leck, 1982; Boermans et al., 1991; Woody et al., 1992; Hornfeldt and Phearman, 1996; Peterson and Streeter 1996; McConnico et al., 1997; Robben et al., 1997, 1998; Munday and Thompson, 2003; Radi and Thompson, 2004) (Table 18.4).

Cats may be more resistant to the toxic effects of brodifacoum and difenacoum than dogs. Cases of abortion and hemorrhage in sheep and goats after misuse of brodifacoum have been reported (Jones, 1996; Watt et al., 2005).

Nontarget Wildlife Exposures

Nontarget wildlife may be exposed to rodenticides as well. The possible effects on nontarget organisms can also be considered in two types: direct poisoning and secondary poisoning. The potential for secondary poisoning is more likely in carnivorous wildlife (Mathur and Prakash, 1980; Mahmoud and Redfern, 1981; Greaves et al., 1982; DuVall et al., 1989; Newton et al., 1990; James et al., 1998; Borst and Counotte, 2002; Eason et al., 2002). The most commonly affected species are birds such as great horned owls, barn owls, eastern screech owls, golden eagles, red-tailed hawks, Cooper's hawks, and crows. Bird species varied in their susceptibility. Other wild animals also may be exposed, such as polecats, wildcats, and foxes. Brodifacoum was highly toxic for fish when tested as a technical material.

Laboratory/Monitoring and General Recommendations

Superwarfarins lower the blood concentrations of the vitamin K–dependent clotting factors II, VII, IX and X; this results in prolongation of prothrombin time (PT) and partial thromboplastin time (PTT). PT and PTT should be repeated at least twice daily until normal PT and PTT levels are established. Also, the blood clotting time and the bleeding time should be measured. Blood is often demonstrable in the excreta. Secondary hypochromic or microcytic anemia may be marked (Goldfrank et al., 2002; Nelson et al., 2006).

A PT 24–48 h after exposure in asymptomatic children with accidental ingestions of large or unknown amounts

TABLE 18.3 Number of Exposures to Long-Acting Anticoagulant Rodenticides and Deaths Reported by the American Association of Poison Control Centers—Toxic Exposure Surveillance System (AAPCC-TESS) Annual Reports: 24-Year Data

Year	No. of Participating Centers	Population Served (Millions)	No. of Human Exposures Reported (All Substances)	No. of Exposures/ Thousand Population	No. of Exposures to Long-Acting Anticoagulant Rodenticides (LAAR)	No. of Deaths Due to Anticoagulants (Alone and Combined with Other Substances)	Reason: Intentional Suicidal (IS)	% of Deaths from Exposure to (LAAR)
1983	16	43.1	251,012	5.8	1,724	0	No deaths	0.00%
1984	47	99.8	730,224	7.3	3,703	2	IS	0.05%
1985	56	113.6	900,513	7.9	5.098	2	IS	0.04%
1986	57	132.2	1,098,894	8.3	7,723	1	IS	0.01%
1987	63	137.5	1,166,940	8.5	4,382	0	No deaths	0.00%
1988	64	155.7	1,368,748	8.8	5,133	1	IS	0.02%
1989	70	182.4	1,581,540	8.7	6,116	0	No deaths	0.00%
1990	72	191.7	1,713,462	8.9	8,215	0	No deaths	0.00%
1991	73	200.7	1,837,939	9.2	10,826	1	IS	0.009%
1992	68	196.7	1,864,188	9.5	10,864	0	No deaths	0.00%
1993	64	181.3	1,751,476	9.7	10,692	1	IS	0.009%
1994	65	215.9	1,926,438	8.9	12,868	1	IS	0.008%
1995	67	218.5	2,023,089	9.3	13,423	1	IS	0.007%
1996	67	232.3	2,155,952	9.3	13,345	1	IS	0.007%
1997	66	250.1	2,192,088	8.8	13,405	3	IS	0.02%
1998	65	257.5	2,241,082	8.7	16,019	0	No deaths	0.00%
1999	64	260.9	2,201,156	8.4	15,982	2	IS	0.01%
2000	63	270.6	2,168,248	8.0	16,006	0	No deaths	0.00%
2001	64	281.3	2,267,979	8.1	16,423	2	IS	0.01%
2002	64	291.6	2,380,028	8.2	17,100	3	IS	0.02%
2003	64	294.7	2,395,582	8.1	16,891	2	IS	0.001%
2004	62	293.7	2,438,644	8.3	16,054	0	IS	0.00%
2005	61	296.4	2,424,180	8.2	14,740	1	IS	0.006%
2006	61	299.4	2,403,539	8.0	13,118	0	No deaths	0.00%
Total			43,482,940		222,165	24	IS	0.01%

should be obtained. In adults with deliberate ingestions and children with clinical evidence of bleeding, an initial PT and PTT should be obtained and then repeated at 24 and 48 h after ingestion (Manoguerra and Cobaugh, 2005).

Analytical Methods

A number of analytical methods have been reported for detecting anticoagulant rodenticides in various matrices. Early fluorimetric methods were used to detect warfarin in serum (O'Reilly et al., 1962; Corn and Berberich, 1967; Lewis et al., 1970; Welling et al., 1970; Keiser and Martin, 1974; Vesell and Shivley, 1974; Fasco et al., 1977; Hanna et al., 1978; Lee et al., 1981) and GLC for warfarin (Mildha et al., 1974).

Warfarin-specific methods were generally inadequate for anticoagulant rodenticides, so a number of other methods were developed. These include thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), mass spectroscopy (MS), and antibody-mediated tests. Coumarin anticoagulant rodenticides were initially detected using TLC (Lau-Cam

TABLE 18.4 Dosing of Ipecac

Adult[a]	15–30 mL
Adolescent[a]	15–30 mL
Child 1–12 years old	15 mL
Child 6–12 months	Dose: 5–10 mL (Position child in left lateral decubitus position to reduce risk of aspiration)
Child under 6 months of age	NOT recommended for pre-hospital use

[a]*Katona and Wason (1989).*

and Chu-Fong, 1972; Mallet et al., 1973). A high-performance TLC method with an estimated detection limit of 200 ppb and 87% recovery from liver has been reported (Berny et al., 1995).

Early HPLC methods focused on an individual chemical. For example, methods to detect chlorophacinone in formulations (Grant and Pike, 1979; Vigh et al., 1981), brodifacoum in serum (Murphy and Gerken, 1989), brodifacoum (Koubek et al., 1979; Keiboom and Rammel, 1981; Hoogenboom and Rammell, 1983; Ray et al., 1989), bromadiolone (Hunter, 1983; Subbiah et al., 2005), chlorophacionone (Hunter, 1985), difethiolone (Goldade et al., 1998), and difenacoum (Mundy and Machin, 1977) in tissue have been reported.

Then a method was developed to look for all the anticoagulant rodenticides on the market at the time. It succeeded in extracting and detecting eight anticoagulant rodenticides in serum and liver using fluorescence and ultraviolet (UV) detection. Samples were extracted with acetonitrile then cleaned up on solid phase columns. Four hydroxycoumarins were detected by fluorescence with excitation at 318 nm and emission at 390 nm. The indandiones were detected at 285 nm. An extraction recovery of 75% from serum and 69% from liver was reported. Hydroxycoumarins may be detected down to about 1 ng/mL of serum and 1 ng/g of liver, and indandiones down to 10 ng/mL of serum and 10 ng/g of liver (Felice and Murphy, 1989; Felice et al., 1991; Chalermchaikit et al., 1993). Another HPLC method for detecting brodifacoum in serum and liver using difenacoum as the internal standard has been reported (O'Bryan and Constable, 1991). There is also a method for the simultaneous detection of five superwarfarin rodenticides in human serum (Kuijpers et al., 1995).

Other serum methods have been reported; for example, detection limits of 3–12 ng/mL for fluorescence and 20–75 ng/mL for UV detection (Mura et al., 1992; Kuijpers et al., 1995; McCarthy et al., 1997; Feng et al., 1999). Tissue methods include a solid phase cartridge extraction from liver, with recoveries ranging from 52% for difenacoum to 78% for warfarin. The limit of detection is 10 ppb for warfarin and difenacoum and 110 ppb for chlorophacinone (Addison, 1982; Jones, 1996; Fauconnet et al., 1997).

HPLC methods have also been published to distinguish *cis*- and *trans*-isomers of difenacoum with detection limits of 5 ng/mL (Kelly et al., 1993). An early, interesting approach was to use a post-column pH shift to enhance fluorescence detection (Hunter, 1983, 1985). Several earlier HPLC methods have also been reported (AOAC, 1976a,b; Mundy and Machin, 1982; Hunter, 1983): diphacinone (Bullard et al., 1975, 1976), fluorescence for bromadiolone (Deepa and Mishra, 2005), brodifacoum (Fu et al., 2006), brodifacoum in tissues (Hoogenboom and Rammell, 1983), difenacoum (Hadler and Shadbolt, 1975), determination of Rozol in parafinized formulations (Kawano and Chang, 1980), and bromadiolone in tissues (Nahas, 1986).

A recent method uses diode-array detection (DAD) (Yang et al., 2001). An interesting new method uses HPLC to detect anticoagulant rodenticides in soft drinks (Dimuccio et al., 1991). An ion pair liquid chromatography method has been reported to detect chlorophacionone and diphacinone in oats (Primus et al., 1998).

Contemporary confirmatory methods use MS. Most recently, liquid chromatography-electrospray ionization-mass spectroscopy (LC-EIS-MS) has been reported for the analysis of 10 anticoagulant rodenticides with a quantity limit of about 5 µg/L (Grobosch et al., 2006). Other recent methods use LC-MS-MS for unknown drugs, including warfarin (Marquet et al., 2003), and LC-ESI-MS and HPLC UV to detect anticoagulant rodenticides as low as 20 ng on a column (Mesmer and Flurer, 2000). One of the earlier MS methods used a direct-probe technique to detect indandione residues in food animals (Braselton et al., 1992).

A cell culture/enzyme-linked immunosorbent assay (ELISA) has been developed to detect anticoagulant rodenticides in treated grain (Lawley et al., 2006). A prior immunoassay was used to detect diphacinone and chlorophacionone (Mount et al., 1988). Enantiomers of warfarin, coumachlor, and coumafuryl can be separated chromatographically (Armstrong et al., 1993).

Serum concentration of dogs with anticoagulant rodenticide poisoning ranged from less than 10–851 ng/L for brodifacoum, difethialone, and difenacoum (Robben et al., 1998).

Animal samples are routinely analyzed in veterinary diagnostic laboratories. For example, the Texas Veterinary Medical Diagnostic Laboratory, in College Station, TX (http://tvmdl.tamu.edu/), performs such analyses. A list of other laboratories performing anticoagulant rodenticide analyses on animal samples can be obtained from the American Association of Veterinary Laboratory Diagnosticians (www.aavld.org). Human samples are generally analyzed at either the National Medical Services Laboratory in Willow Grove, PA, or the Medtox Scientific Laboratories in St Paul, MN (Table 18.5).

TABLE 18.5 Dosing of Activated Charcoal

Charcoal dose Recommended to Dilute 240 mL of Water per 30 g Charcoal (FDA, 1985)	
Adults and adolescents	50–100 g
Children aged 1–12 years	25–50 g
Infants up to 1 year old	1 g/kg of body weight

Chyka and Seger (1997).

GENERAL TREATMENT RECOMMENDATIONS

Referral to Healthcare Facilities

In case of a suspected terrorist act, misuse, intentional criminal, or any deliberate intentional suicidal ingestion, or when the amount ingested is either large or indeterminate, the patient should be referred to a healthcare facility for clinical and laboratory assessment, as well as treatment if necessary (Manoguerra and Cobaugh, 2005; POISINDEX, 2007).

Home Observation Criteria

Accidental ingestion of a small amount (less than a few pellets) can be adequately managed at home by either poison control centers or a healthcare professional with home observation and parent education. Usually, these types of exposures do not require any medical intervention or routine follow-up laboratory studies. Gastric decontamination has no effect on the clinical outcome after "taste" (one fifth of a spoonful) amounts are ingested by children (Mullins et al., 2000; Kanabar and Volans, 2002; Shepherd et al., 2002).

If the amount ingested by a child is a "moderate amount" (defined as more than a handful, or a mouthful), or is questionably high, then it is recommended that the parent contact a physician or call a local poison control center for instructions on how to induce emesis with syrup of ipecac (Tenenbein et al., 1987; AAP, 2003).

There is no risk of poisoning for animals ingesting taste amounts, but if the amount ingested is unknown, or cannot be estimated, then a local poison control center or veterinarian should be contacted for instructions on how to induce emesis and to obtain a 24- to 48-h blood test (PT) to determine the need for treatment with vitamin K_1 (Murphy and Gerken, 1989; Munday and Thompson, 2003).

Treatment at Healthcare Facilities

A PT should be obtained 48 h after exposure in asymptomatic children with accidental ingestion of a suspected large amount of rodenticide (Babcock et al., 1993; Berry et al., 2000). Adults with intentional ingestion and children with clinical evidence of bleeding should obtain an initial PT and PTT, repeated at 24 and 48 h after ingestion (Greeff et al., 1987; Hoffman et al., 1988; Goldfrank et al., 2002). If any significant prolongation or evidence of bleeding is observed, PT should be repeated every 6–12 h. Determination of factors II, VII, IX, and X may be abnormal in patients with a normal PT and PTT, which may provide evidence of earlier significant ingestion (Brands et al., 1995; Corke, 1997; Pavlu et al., 2005; Spahr et al., 2007). Hemoglobin and hematocrit should be monitored in patients with clinical evidence of bleeding or significant coagulopathy. Determination of the ABO blood type may be necessary in cases of toxic ingestions and bleeding. Patients may require red blood cell transfusions or the administration of fresh frozen plasma (Ellenhorn et al., 1997; Bruno et al., 2000; Laposata et al., 2007; Olmos and Lopez, 2007).

Emesis

Currently, there is controversy regarding the use of syrup of ipecac; the American Academy of Pediatrics reversed its policy position about using syrup of ipecac to help with poisoning emergencies in children (AAP, 2003). Simultaneously, the AAPCC is still indicating that syrup of ipecac does have a place in therapy, and "concluded that individual practitioners and poison control centers are best able to determine the particular patient population, geographic and other variables that might influence the decision to recommend having ipecac on hand" (Manoguerra and Cobaugh, 2005). We recommend that the first action for a caregiver of a child who may have ingested one of these rodenticides is to consult with their local poison control center (AAP, 2003).

Emesis with syrup of ipecac has been recommended for children with a history of accidental ingestion of small amounts (defined as more than a "grain or two,") if it can be administered within 1 h from the time of ingestion (Katona and Wason, 1989).

Emesis is contraindicated in patients with a prolonged PT or a bleeding disorder due to the risk of bleeding following ipecac-induced increased intracranial pressure (POISINDEX, 2007). Taste amounts (a few pellets or a bite of one block of bait) do not require emesis. If the amount is more than two mouthfuls or one block of bait, or if an unknown amount is ingested, then emesis is most effective if initiated within 30 min to 1 h from the time of ingestion. The decision to induce emesis or not is often controversial; it must be carefully considered. It could be most appropriate in pre-hospital settings and is not recommended once the patient is in the emergency room (Chyka and Seger, 1997; Krenzelok et al., 1997).

Contraindications: Patients with a bleeding disorder, particularly those under treatment with anticoagulants

or with histories of chronic, long-acting anticoagulant ingestion, are at risk from gastrointestinal and central nervous system (CNS) bleeding from ipecac-induced emesis. The administration of activated charcoal is preferred when large amounts have been ingested or chronic ingestion has occurred. Also, it is contraindicated if there is a risk for choking or aspiration, CNS excitation or depression, coma, seizures, or signs of oral, pharyngeal, or esophageal irritation (Chyka and Seger, 1997; Golej et al., 2001; Goldfrank et al., 2002).

Before or after ipecac is administered, patients should be encouraged to drink water. Adults are given approximately 8 oz (240 mL), and children 4–8 oz (120–240 mL) (Goldfrank et al., 2002; POISINDEX, 2007).

Activated Charcoal

For patients with a potentially toxic ingestion who are awake and able to protect their airway, activated charcoal diluted in water may be administered before going to the hospital. It is more effective when administered within 1 h after ingestion. It is recommended to dilute the mixture with 240 mL of water per 30 g charcoal (Chyka and Seger, 1997).

In patients who are at risk from the abrupt onset of seizures or depression, activated charcoal should be administered by medical or paramedical personnel capable of airway management to prevent aspiration in the event of spontaneous emesis (POISINDEX, 2007).

Use of a cathartic with activated charcoal is not routinely recommended, as there is no evidence that cathartics reduce drug absorption, and cathartics can cause adverse effects such as nausea, vomiting, abdominal cramps, electrolyte imbalances, and occasionally hypotension. Complications include emesis and aspiration (Chyka and Seger, 1997; Golej et al., 2001).

Gastric Lavage

Gastric lavage is recommended within 1–2 h after ingestion. For chronic ingestion, it is not recommended, as it may induce bleeding in adults with significant coagulopathy. In addition, it is not necessary in children after accidental ingestion (Brands et al., 1995).

Laboratory Monitoring

PT and PTT values should be obtained 24 and 48 h post-ingestion in asymptomatic children with nonintentional ingestion of a "large amount." Adults with intentional ingestions and children with clinical confirmation of bleeding should obtain initial PT and PTT readings, repeated at 24 and 48 h post-ingestion, followed by blood type verification (Barnett et al., 1992; Ellenhorn et al., 1997; Robben et al., 1998). If any significant prolongation or evidence of bleeding is observed, PT should be repeated every 6–12 h. Determination of factors II, VII, IX, and X may be abnormal in patients with a normal PT and PTT, which may provide proof of earlier significant ingestion. Serial hemoglobin and hematocrit in patients should be followed with clinical evidence of bleeding or significant coagulopathy (Babcock et al., 1993; Robben et al., 1998). Hematocrit should be monitored closely at least every 4 h until the patient is stable (Brands et al., 1995).

Hematest should be performed in stools and vomit for occult blood, and PT and PTT monitored routinely. PT and PTT readings obtained within 48 h post-ingestion may not be predictive of subsequent coagulopathy (Greeff et al., 1987). A 24 h and 48 h PT and PTT, therefore, are recommended every 6–12 h to assess efficacy of therapy. If prolongation is observed, then PT or international normalized ratio (INR) measurements should be repeated (Hoffman et al., 1988; Smolinske et al., 1989).

Antidote: Vitamin K_1 (Phytonadione: AquaMephyton®, Mephyton®) is the specific antidote and should be administered to any patient with a prolonged PT (Braithwaite, 1982; Bruno et al., 2000; Tsutaoka et al., 2003). Blood and fresh or frozen plasma are recommended if the anticoagulation is severe.

Administration of vitamin K_1 is recommended if anticoagulation is excessive. A small intravenous dose of 1–5 mg, titrated to return PT to the therapeutic range, has been recommended. In anemic patients, the hematocrit should be monitored about every 4 h until it is stable. Stools and vomit may also be tested using Hematest (Hornfeldt and Phearman, 1996).

Oral vitamin K_1 may be administered in small doses after the patient has been stabilized. Recommended doses are 15–25 mg p.o. in adults, 5–10 mg in children (Greeff et al., 1987), and 2.5–5 mg/kg body weight in animals. A large daily maintenance dose of vitamin K_1 may be required for prolonged therapy in severe overdoses, particularly in patients in whom vitamin K_1 absorption is variable (Lipton and Klass, 1984; Hoffman et al., 1988; Ross et al., 1992; Murphy, 2012).

Intravenous phytonadione may be instituted in severe cases where rapid correction is needed. The adult dose is a minimum of 10 mg diluted in saline or glucose, injected intravenously (i.v.) at a rate not exceeding 5% of the total dose per minute. Doses should be repeated at intervals of 6–8 h. Initial i.v. doses of 25, 100, 150, 160, and 400 mg have been required in patients actively bleeding (Hoffman et al., 1988; Vogel et al., 1988). Anaphylaxis may occur if vitamin K_1 is injected too rapidly.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Today, there is a greater risk than ever that extremist or terrorist groups may use industrial or household chemical substances to harm, kill, or terrorize our society

because these substances are very easy to obtain and conceal. Also, numerous highly toxic chemicals can be stolen and released or detonated from storage tanks or from transportation and manufacturing facilities. These commercial and industrial chemicals stored in large quantities are considered agents of opportunity because they are readily and commercially available. A large number of industrial and household chemicals, including the superwarfarins, have the potential to be used as chemical warfare or terrorist agents. However, the Federal Insecticide, Fungicide, and Rodenticide Act classifies these rodenticides in the "low toxicity group." Nevertheless, they may be used to harm and terrorize people through the ingestion of contaminated food or water. Superwarfarins are available to consumers as meal bait packs, pellets, mini-pellets, blocks, mini-blocks, wax blocks, liquid bait formulations, and tracking powder in diluted to concentrated formulations. The ingestion of small amounts may not cause any bleeding problems. Ingestion of greater amounts provides increased risk of severe bleeding in 36–48 h. The coagulopathy may last several weeks to months despite vitamin K_1 treatment. Inhibition of synthesis of vitamin K_1–dependent clotting factors may also occur following repeated ingestion of small amounts, making these agents insidious.

The use of chemical weapons by terrorists remains a major threat. Until recently, UN resolutions required complete disclosure of chemical warfare agents and their destruction. More action is needed to prevent radical groups from accessing or accumulating these agents. Participation of the entire community, particularly the civilian population, is needed. Participation may increase due to educational programs that boost awareness, improved surveillance, and reporting to the authorities of any suspicious activity, as well as the purchasing and stockpiling of large quantities of these commercial products.

What should be done at this time? Be wary if people from a community that attended the same event, meal, party, or restaurant show similar signs of bleeding disorders. Ingestion of superwarfarins may go unnoticed when they are mixed with food, and signs or symptoms are delayed 36–48 h. Consequently, the victims may not associate the ingestion with the coagulopathy.

Identification of superwarfarins is easy today. Analytical methods for the detection of these products in serum and tissues are readily available. Routine laboratory tests for coagulopathy may help support the need for such analytical chemistry testing. It may be useful to create stockpiles of vitamin K_1, particularly in places where large stocks of superwarfarin rodenticides are used, such as livestock areas.

Although viral infections manifest with different signs and symptoms, it is possible that members from a community who are victims of superwarfarins may begin to panic, thinking that they were victims of a virus such as Ebola, but the signs and symptoms of superwarfarin ingestion differ from viral infection. Superwarfarin intoxication may have no signs or symptoms other than the appearance of bleeding in the stools, urine, mucous membranes, or the thoracic or abdominal cavities. The Ebola virus causes sudden hemorrhagic fever, weakness, muscle pain, headache, and sore throat, followed by vomiting, diarrhea, rash, limited kidney and liver functions, and both internal and external bleeding.

Consequently, good differential diagnostic and laboratory work-up algorithms are needed for both humans and animals. Teams of subject matter experts for both humans and animals may be useful in this regard. Also, community education programs should be developed to inform residents about superwarfarin rodenticides and other household commercial chemicals that could be used as chemical warfare or terror agents. Fact sheets may be one method of disseminating such information to the public. Identifying those at risk, as well as deciding when they should be informed, may be a useful part of this education campaign. Such a program may also improve security in industrial plants and limit access from outsiders to all industrial and storage facilities where flammable or highly toxic chemicals are stored, particularly livestock facilities.

Finally, surveillance of food sources, particularly those derived from livestock, should be assessed to determine whether these agents are available to consumers in low concentrations, which may lead to long-term accumulation and eventual coagulopathies.

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CHAPTER

19

PCBs, Dioxins and Furans: Human Exposure and Health Effects

Bommanna G. Loganathan and Shigeki Masunaga

INTRODUCTION

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated dibenzo-*p*-dioxins (PBDDs), and polybrominated dibenzofurans (PBDFs) are members of the group of halogenated aromatic hydrocarbons. This group of chemicals has been identified by the national and international agencies as priority environmental pollutants posing significant effects on aquatic and terrestrial animals, including humans (Loganathan and Kannan, 1994; Jacobson, 1994; International Programme on Chemical Safety, 1998; Van den Berg et al., 1998; Kodavanti et al., 2008). PCBs do not occur in nature except for forest fires and were synthesized by humans. PCBs were produced in large quantities (millions of pounds) for a variety of industrial uses during 1940 to the late 1970s, especially by developed nations. However, PCDDs and PBDDs (dioxins) and PCDFs and PBDFs (furans) were never produced commercially but are formed in small quantities as by-products of combustion of various industrial as well as natural processes. Because of their persistent, bioaccumulative, and toxic properties, residues of these compounds are found in every component of the global ecosystem (Lipnick et al., 2001; Kodavanti et al., 2008). Some PCBs (coplanar PCBs), dioxins, and furans are three structurally and toxicologically related families of compounds that are classified as the most toxic synthetic chemical substances to a variety of animal species, including humans (Tucker et al., 1983; Loganathan et al., 1995; ATSDR, 1998). Production of PCBs was banned during the 1970s; however, already produced PCBs are still causing environmental and health problems (Loganathan et al., 2008; Sajwan et al., 2008; Loganathan, 2012). Fortunately, these compounds were never used as chemical warfare agents, weapons of mass destruction, or agents of threat or terror by contamination of air, water, or food/feed. However, inadvertent poisonings by these chemicals have caused significant environmental and health problems (Yusho Support Center, 2007; Loganathan, 2012; Kodavanti and Loganathan, 2012). In this chapter, historical background, chemical characteristics, analysis, pathways of human exposure to these compounds, and toxic effects associated with the exposures are presented.

HISTORICAL BACKGROUND

PCBs were first synthesized in the early 1880s (Schmidt and Schultz, 1881) and commercial production began in 1929. Biphenyls were reacted with Cl_2 in the presence of ferric chloride catalyst, where some of the hydrogen atoms are replaced by chlorine atoms. PCBs were produced as complex mixtures potentially containing 209 congeners formed by chlorinating biphenyl with from 1 to 10 chlorines (Figure 19.1). The amount of chlorination of biphenyls corresponded to the duration of chlorination process. For example, Aroclor 1221, 1242, 1248, 1254, 1260, and 1268 are commercial preparations that were formerly produced by the Monsanto Chemical Company in the United States (St. Louis, MO) that contain 21%, 42%, 48%, 54%, 60%, and 68% chlorine by weight, respectively, as indicated by the last two digits in the numerical designation (Giesy and Kannan, 1998). The PCB mixture formulations were different depending on the country of origin and were produced in Germany (Clofen), France (Phenoclor and Pyralene), Japan (Kanechlor), Italy (Fenclor), Russia (Sovol), and Czechoslavakia (Delor). PCB mixtures were produced

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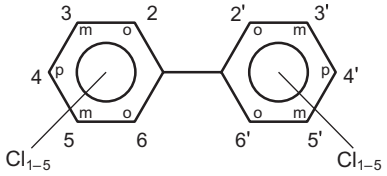
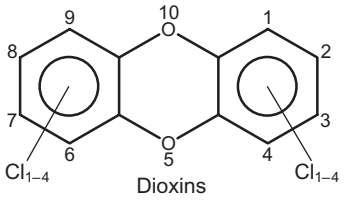
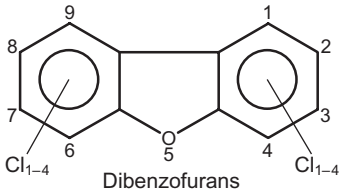
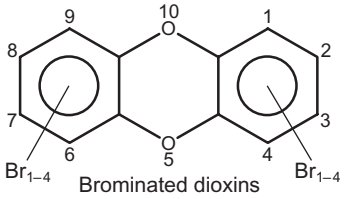
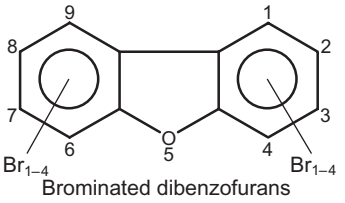
Compound	Molecular Wt	Chemical structure
PCBs	188–498	
PCDDs	218–460	
PCDFs	202–444	
PBDDs	263–816	
PBDFs	247–800	

FIGURE 19.1 Generalized structures of PCBs, dioxins, and furans.

for a variety of uses such as fluids in electrical transformers, capacitors, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, and as additives in plastics, paints, copying paper, printing inks, adhesives, and sealants (Loganathan et al., 1989; Safe, 1990).

Dioxins and furans are not produced deliberately but are produced unintentionally as by-products of combustions of organic matter in the presence of chlorine. Dioxins and furans consist of 135 possible chlorinated (or brominated) dibenzofuran and 75 chlorinated (or brominated) dibenzo-*p*-dioxins with from one to eight chlorine (bromine) substituents (Figure 19.2). PCDDs/DFs are found as by-products during the manufacture of some industrial chemicals such as PCBs, polychlorinated naphthalenes, chlorinated phenols, chlorinated phenoxyacids, polychlorinated diphenyl ethers, polyvinyl chlorides, and chlorinated phenoxy-2-phenols (Hutzinger et al., 1985; Hryhorczuk et al., 1986; ATSDR, 2001; Masunaga et al., 2001a). Similarly, PBDDs/DFs are found as by-products in brominated organic chemicals, such as brominated flame retardants (polybrominated diphenyl ethers (PBDEs), decabromobiphenyl, 1,2-bis(tribromophenoxy) ethane, tetrabromobisphenol A (TBBPA)) (International Programme on Chemical Safety, 1998). These compounds are also formed during incineration of industrial and municipal waste, forest fires, fireplaces, and combustion engines (Loganathan et al., 1997; Feil and Larsen, 2001). Because of anthropogenic as well as natural processes, PCBs, dioxins, and furans are widely dispersed in the global environment and their presence was reported in air, water, soil, sediment, aquatic organisms, and terrestrial organisms, including human tissues (Safe, 1990;

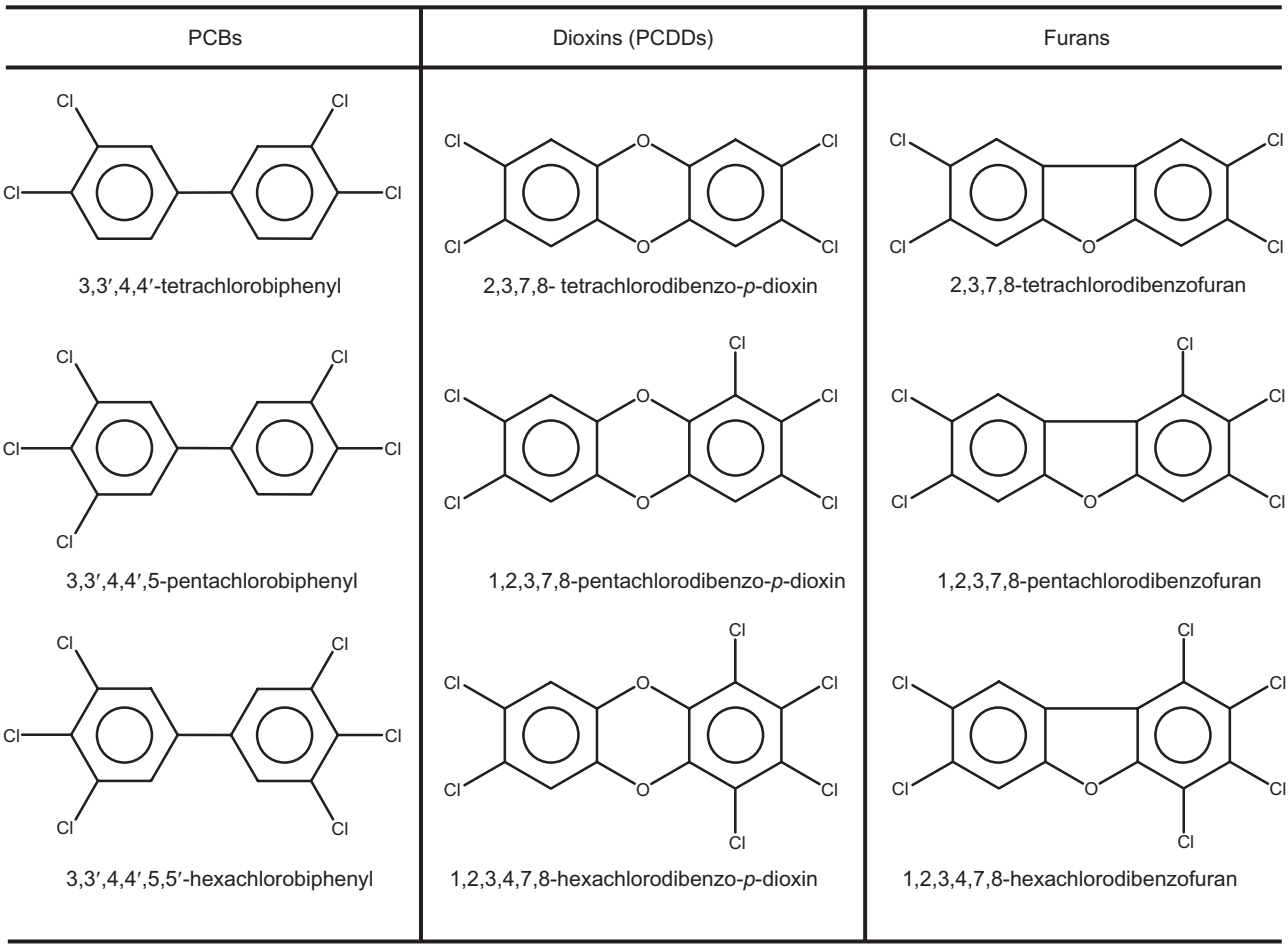


FIGURE 19.2 Structures of highly toxic PCBs, dioxins, and furans.

Loganathan and Kannan, 1994; Giesy and Kannan, 1998; Masunaga et al., 2001b; Ogura et al., 2001).

HUMAN EXPOSURE TO PCBS, PCDDS, AND PCDFS

Direct human exposure to PCBs/PCDF occurred because of inadvertent poisoning due to consumption of PCB contaminated food, resulting in Yusho and Yucheng poisoning (oil disease) in Japan and Taiwan during 1968 and 1979, respectively. The Yusho Support Center Report (2007) states that "39 years have passed since the outbreak of YUSHO, the PCB/dioxin tragedy—the most unprecedented incident in the history of mankind whereby people ingested toxic chemicals unknowingly, directly through food." The outbreak of a strange disease "Yusho" (Kanemi Oil Poisoning) occurred in the western part of Japan in 1968. The major symptoms and signs of the disease consisted of acne-like eruptions, pigmentation of the skin, nails, and conjunctivas, increased discharge from eyes, and numbness in the limbs (Yao et al., 2002; Yusho Support Center Report, 2007). The epidemic was identified later (1969) to be an unprecedented mass food poisoning caused by the ingestion of commercial brand rice oil that had been contaminated by PCB and their related compounds. The number of people who reported to have ingested the rice oil was approximately 14,000 and 1,867 persons were designated as Yusho victims. A similar outbreak of "oil disease" occurred in Taiwan in 1979. Toxicological studies revealed that PCDF congeners, including 2,3,4,7,8-pentachlorodibenzofuran, played an important role in the manifestation of these diseases. Research conducted with Yusho victims revealed harmful effects of the exposure continued two generations (Yusho Support Center Report, 2007).

Direct human exposure to dioxin occurred in southern Vietnam and also in Seveso, Italy. It was estimated that southern Vietnam has been contaminated by 160–600 kg of dioxin as a result of 80 million liters of defoliant herbicides (Agent Orange, a 50:50 mixture of 2,4,5-T and 2,4-D) being sprayed by the US military over a large area of forests and crops of southern Vietnam from 1962 to 1971 (Westing, 1984; Schecter et al., 2006;

FIGURE 19.3 Dioxin poisoning. Ukrainian former Prime Minister and presidential candidate Viktor Yushchenko, with his face disfigured by illness due to dioxin poisoning. *Source: Photo: www.mindfully.org; November 19, 2004 (accessed on July 13, 2008).*

Le Hong Thorn et al., 2007). The defoliant was contaminated with a very toxic form of dioxin (TCDD) known to have caused adverse effects on human health. Dioxin may cause harmful effects on the whole body and can affect separately the functioning of the systems such as the nervous system, immune responses, carcinogenicity, hepatotoxicity, and metabolic and enzyme toxicity (Le Hong Thorn et al., 2007). Another example of direct human poisoning of dioxin was food poisoning of Viktor Yushchenko (Figure 19.3), Ukrainian Presidential candidate, in 2004. The dioxin poisoning caused a mysterious illness that resulted in his face becoming pockmarked and ashen (www.mindfully.org). Manahan (1992) classified dioxin (2,3,7,8-TCDD) as super toxic in comparison with other known toxic substances (Table 19.1).

TABLE 19.1 Toxicity Level of Various Chemical Compounds (Prepared from Manahan, 1992)

Toxicity Level	Compounds	LD_{50} Estimated from Laboratory Animals[a] (mg/kg)
Slightly toxic	Ethyl alcohol	10,000
	Sodium chloride	5,000
Moderately toxic	Malathion (organophosphorus pesticide)	1,000
	Chlordane (termite exterminator)	500
Very toxic	Heptachlor (pesticide)	100
Extremely toxic	Parathion (pesticide)	10
	Dioxin (2,3,7,8-TCDD)	5 (hamster)
	Tetraethyl pyrophosphate (pesticide, raticide)	1
Super toxic	Tetrodotoxin (toxin of blowfish)	0.1
	Dioxin (2,3,7,8-TCDD)	0.0006 (guinea pig)
	Botulin (toxin of botulinum)	0.00001

[a]*LD_{50} are rough values estimated from oral-dose experiment on laboratory animals (usually rats). Unit: mg/kg body weight.*

PHYSICO-CHEMICAL PROPERTIES AND GLOBAL DISTRIBUTION

The unusual industrial versatility of PCBs was directly related to physical and chemical properties that include resistance to acids and bases, compatibility with organic materials, resistance to oxidation and reduction, excellent insulating properties, nonflammability, and thermal stability (Hutzinger et al., 1985). Physical and chemical stability of PCBs are vital to the industrial applications and the same properties have been responsible for global environment contamination. In addition, multi-media releases and volatility lead to long-range environmental transport, both via water and atmosphere, resulting in widespread environmental contamination of humans and wildlife at sites distant from their use (Lipnick and Muir, 2001; Loganathan et al., 2008). In PCBs, dioxins, and furans, the properties vary widely and depend on the number and position of chlorine (or bromine) atoms attached to the molecule. In general, vapor pressure, water solubility, and biodegradability decrease with increasing number of chlorine atoms. Lipophilicity adsorption capacity shows a reverse trend (Loganathan and Kannan, 1994). Because of these unique properties, PCBs, dioxins, and furans have been detected in air and water (rivers, lake ecosystems) (Pearson et al., 1997; Loganathan et al., 1998a,b, 2001). Apart from this, these compounds were recorded in fish, birds, and marine mammals of several other ecosystems such as the Atlantic, Baltic, and Pacific Oceans and Swedish environments (Kawano et al., 1988; Loganathan et al., 1999). They have been identified in processed fish and other food products (Kannan et al., 1997; Patandin et al., 1999). Further, the residues of these contaminants were found in human adipose tissue, blood, and milk, and also in numerous other matrices (Loganathan et al., 1993, 1998a,b, 1999; Petreas et al., 2001; Czaja et al., 2001). The comparison of PCDD/DFs and PBDD/DFs in terms of toxic equivalents (TEQs) calculated based on WHO-TEF (World Health Organization's toxic equivalent factors) in pooled human milk samples of 17 countries showed that PBDD/DF levels are significantly lower than the PCDD/DF (mean PBDD/DF:PCDD/DF-TEQ ratio was 0.13 and its range was 0.06–0.25), indicating that exposure

of PCDDs/DFs is much greater than PBDD/DFs and more important to human health (Kotz et al., 2005). Because of the large production and indiscriminate use of PCBs in industries, these contaminants extended their boundaries of distribution over the global environment, and this was evidenced by their detection even in pristine environmental media and biota such as the Arctic and Antarctic atmosphere, hydrosphere, and biosphere (Muir et al., 1988; Corsolini et al., 2002; Kumar et al., 2002). The discovery of the widespread environmental occurrences, the increased general environmental concern, and the apparent link to carcinogenesis and other health disorders prompted public outcry that resulted in prohibition of PCBs as well as chlorinated pesticides in several developed nations during the early 1970s. The following section deals with the chemical analysis of PCBs, dioxins, and furans in environmental and biological samples.

ANALYTICAL METHODS

PCBs and PCDDs/DFs consist of a total of 419 individual congeners and PBDDs/DFs have 210 congeners. In addition, dioxins and furans with mixed chlorine and bromine substitution can occur. These congeners have quite a variety of toxicity and some of these congeners, especially planar dioxins, furans, and dioxin-like PCBs (non-*ortho* chlorine substituted coplanar PCBs), are extremely toxic even at very low concentrations (Table 19.1). Therefore, determination of some toxic congeners to a very low concentration (parts per trillion) has become important.

Congener-specific determination was required for those congeners that have toxic equivalency factors (TEFs), namely 2,3,7,8-chlorine substituted dioxins and dioxin-like PCBs (Table 19.2). Thus, they are analyzed using high-resolution gas chromatography–high-resolution mass spectrometry (HRGC-HRMS). A schematic flow chart of a representative analysis procedure for environmental and biological samples is shown in Figure 19.3 based on standard analytical methods such as US EPA Methods 1613, 1668, 1668a, and JIS K0312 (US EPA, 1994, 1997, 1999; JISC, 2008).

Biological samples are either freeze-dried or dewatered (homogenized) with anhydrous sodium sulfate salt and spiked with internal standards (clean-up spike) and then extracted using Soxhlet apparatus. Obtained extracts are concentrated and their solvents are changed to an appropriate solvent such as hexane, followed by a series of clean-up procedures to remove lipids and other interfering chemicals. Then, the eluates are concentrated again and spiked with internal standards. The prepared samples were injected into HRGC-HRMS and monitored by multiple ion monitoring mode. Concentrations are calculated by isotope dilution method. Stable isotope-labeled target compounds are used as internal standards and spiked into samples. Calibration with internal standards and determination by isotope dilution are necessary to obtain reliable data under very low concentrations and after repeated pretreatment and clean-up procedures.

Solid samples such as soil and sediment are air-dried and extracted using the Soxhlet/Dean-Stark apparatus. Aqueous samples are separated into solids and filtrates by filters. Solids are Soxhlet extracted similar to biological samples and filtrates are liquid–liquid extracted. Then, these extracts are cleaned and injected into HRGC-HRMS, similar to biological samples.

In cases when congener-specific information is not necessary, other simpler methods can be used. EPA Methods 608 and 8082a use GC/ECD to determine concentrations of PCBs in terms of Aroclor (Federal Register, 1984; US EPA, 2007). EPA Method 680 (US EPA, 1985) uses GC/MS (low-resolution mass spectrometry) to determine the homolog concentration of PCBs (Figure 19.4).

MECHANISM OF ACTION AND TOXICITY

The toxicity of PCBs, PCDDs/DFs, and PBDDs/DFs is complicated by the presence of a large number of congeners, each with its own toxicity. Commercial PCB mixtures elicit a broad spectrum of toxic responses that are dependent on several factors, including chlorine content, purity, dose, species, age, sex, and duration of exposure. Immunotoxicity, carcinogenicity, and developmental toxicity as well as biochemical effects of commercial PCB mixtures have been studied extensively in various laboratory animals, fish, and wildlife (Giesy and Kannan, 1998). Several studies have confirmed the common receptor-mediated mechanism of action of toxic halogenated aromatics and have resulted in the development of a structure–activity relationship for this class of chemicals (Safe, 1990). The most toxic halogenated aromatics is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is assigned the maximum toxicity factor of 1; the relative toxicities of individual halogenated aromatics have been determined relative to TCDD (i.e., toxic equivalents). The 17 congeners of PCDD/DFs and the 12 congeners of dioxin-like PCBs are assigned TEFs lower than TCDD (Table 19.2). In June 1997, the World Health Organization established the levels of toxicity factors (WHO-TEFs) to be applied to evaluating the risks for humans and animals (Van den Berg et al., 2006). The WHO consultation set the tolerable daily intake (TDI) between 1 and 4 pg TEQ/kg body weight, emphasizing that the aim was to lower the TDI to a level less than 1 pg TEQ/kg body weight (Guerzoni and Raccanelli, 2004).

TABLE 19.2 Toxic Equivalency Factors (TEFs) for Dioxins, Furans and Dioxin-like PCBs.

	TEF				
	I-TEF	Van den Berg et al. (1998)			Van den Berg et al. (2006)
Congener	Human	Human and Mammals	Fish	Bird	Human and Mammals
PCDDs					
2,3,7,8-TCDD	1	1	1	1	1
1,2,3,7,8-PeCDD	0.5	1	1	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1	0.5	0.05	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1	0.01	0.01	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.1	0.01	0.001	<0.001	0.01
OctaCDD	0.001	0.0001	–	–	0.0003
PCDFs					
2,3,7,8-TCDF	0.1	0.1	0.05	1	0.1
1,2,3,7,8-PeCDF	0.05	0.05	0.05	0.1	0.03
2,3,4,7,8-PeCDF	0.5	0.5	0.5	1	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01	0.01	0.01
OctaCDF	0.001	0.0001	0.0001	0.0001	0.0003
NON-ORTHO-PCBs					
3,4,4′,5-TeCB (#81)		0.0001	0.0005	0.1	0.0003
3,3′,4,4′-TeCB (#77)		0.0001	0.0001	0.05	0.0001
3,3′,4,4′,5-PeCB (#126)		0.1	0.005	0.1	0.1
3,3′,4,4′,5,5′-HxCB (#169)		0.01	0.00005	0.001	0.03
MONO-ORTHO-PCBs					
2,3,3′,4,4′-PeCB (#105)		0.0001	<0.000005	0.0001	0.00003
2,3,4,4′,5-PeCB (#114)		0.0005	<0.000005	0.0001	0.00003
2,3′,4,4′,5-PeCB (#118)		0.0001	<0.000005	0.00001	0.00003
2′,3,4,4′,5-PeCB (#123)		0.0001	<0.000005	0.00001	0.00003
2,3,3′,4,4′,5-HxCB (#156)		0.0005	<0.000005	0.0001	0.00003
2,3,3′,4,4′,5′-HxCB (#157)		0.0005	<0.000005	0.0001	0.00003
2,3′,4,4′,5,5′-HxCB (#167)		0.00001	<0.000005	0.00001	0.00003
2,3,3′,4,4′,5,5′-HpCB (#189)		0.0001	<0.000005	0.00001	0.00003

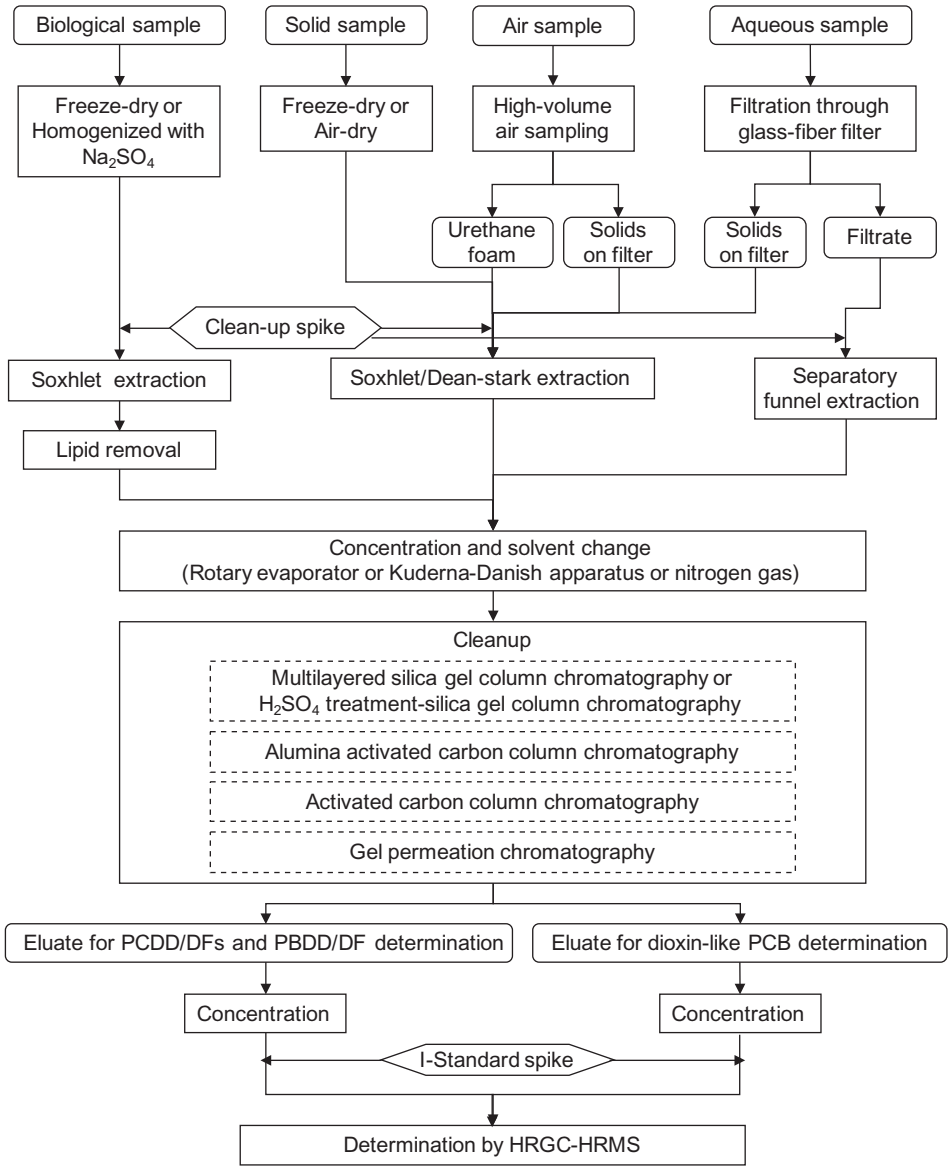


FIGURE 19.4 Analytical procedure of dioxins, furans, and dioxin-like PCBs.

The most toxic PCB congeners are those that have chlorine substitution in most of the non-*ortho* positions, such as 3, 4, and 5 in each ring. These coplanar PCB congeners (Figure 19.2) are structurally similar to highly toxic 2,3,7,8-TCDD and exhibit similar toxic responses (Ah receptor–mediated toxicity) (Figure 19.5). There are no TEFs for 2,3,7,8-substituted PBDDs/DFs that have international agreement; however, use of the same TEF values for the corresponding PBDD/DF congeners as described for the chlorinated analogues appears to be justified (International Programme on Chemical Safety, 1998). 2,3,7,8-TCDD and structurally related halogenated aromatic compounds induce a variety of microsomal enzymes primarily in the liver. 2,3,7,8-TCDD evokes dose-related induction of cytochrome-P-450–associated aryl hydrocarbon hydroxylase (AHH) activity. The most widely studied of these responses are induction of AHH and EROD (markers of CYP1A activity) in mammalian cell cultures and in laboratory rodents (Goldstein and Safe, 1989).

Ah receptor–mediated toxicity resulted in a wide range of biological responses, including alterations in metabolic pathways, body weight loss, thymic atrophy, impaired immune responses, hepatotoxicity, chloracne and related skin lesions, developmental and reproductive effects, and neoplasia.

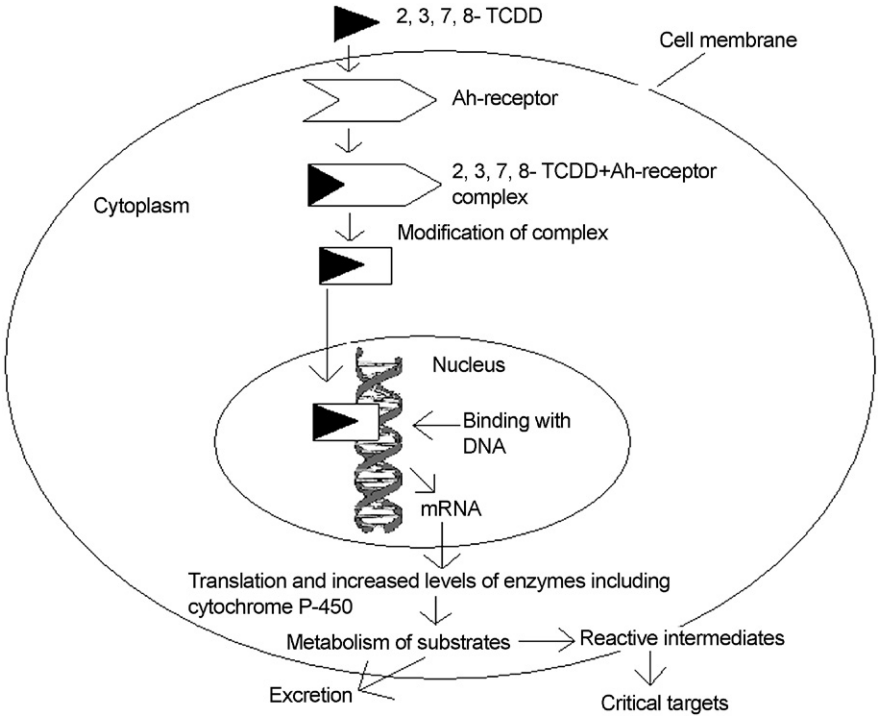


FIGURE 19.5 Possible mechanism of toxic action of 2,3,7,8-TCDD.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

PCBs, dioxins, and furans are persistent organic pollutants that have negative effects on the environment and health of humans, including skin toxicity, immunotoxicity, neurotoxicity, negative effects on reproduction, teratogenicity, endocrine disruption, and a predisposition to cancer. A major pathway of exposure to these chemicals is through consumption of food contaminated by PCBs and dioxins. The Committee of Experts on Food of the European Commission proposed a dose called "tolerable weekly intake" given by the total of dioxins and PCBs of 14 pg TEQ/kg of body weight, which is an average of 2 pg TEQ/day/kg of body weight (Guerzoni and Raccanelli, 2004). By reducing the environmental contamination, we can diminish the food chain accumulation and, ultimately, we can reduce the intake levels of PCBs and dioxins and their toxic effects.

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CHAPTER

20

Polycyclic Aromatic Hydrocarbons: Implications for Developmental, Molecular, and Behavioral Neurotoxicity

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INTRODUCTION

Airborne polycyclic aromatic hydrocarbons (PAHs) arise from incomplete combustion of carbon-based fuels. They are common in the environment, arising from multiple sources including industrial emissions from coal-fired power plants, motor vehicle emissions (especially diesel exhaust), tobacco smoke, and dietary sources such as charbroiled foods (Banks et al., 2014). Exposure to PAHs is rapidly being recognized as a global problem, particularly in major urban centers in the United States and developing nations such as China, where urban environmental protections tend to be weaker and where biomass is used as a source of indoor fuel (Zhang and Tao, 2009). In these situations, these contaminants accumulate to toxic levels in the body within a short period of time and enter the environment rapidly (ATSDR, 1995; WHO, 2010). Chronic exposure to even low concentrations of PAHs cause long-lasting damage, such as cancer, infertility, and neurotoxicity to humans and wildlife (Banks et al., 2014).

The goal of this chapter is to encourage research activity into both novel intervention strategies and therapeutic approaches to mitigate the neurotoxicity associated with *in utero* exposure to PAHs as combustion-derived agents of urban industrialization. The focus of this chapter is to highlight current epidemiological data pertaining to the known neurobehavioral outcomes from PAH exposures, as well as supporting data in animals. An attempt is made to suggest potential operative pathways contributing to PAH exposure–induced neurodevelopmental deficits.

A critical review of the post–September 11, 2001, literature has resulted in a specific focus on studies that have revealed critical neural signaling/activity pathways and the identification of primary targets of PAH toxicity, as well as toxicity modifiers. It is hoped that this chapter stimulates multidisciplinary efforts to look at the conduct of temporal integrative analyses of developmental processes affected by exposure to PAHs.

BACKGROUND

Epidemiological Evidence for the Negative Effects of PAHs on Pregnant Women

A number of cohort studies have been undertaken to assess pregnancy outcomes and early life impacts associated with the September 11, 2001, attack and other airborne PAH exposures. To study the acute exposure of pregnant women either working in the World Trade Center (WTC) or residing in the communities of lower Manhattan on September 11, 2001, a Mount Sinai Hospital cohort was assembled (Berkowitz et al., 2003). Of the 187 recruited pregnant women, 12 (about 6%) were inside the WTC towers at the time of attack, and an additional 121 (65%) were within eight blocks (Wolff et al., 2005). A comparison group (n = 2,367) consisted of all private patients not known to have been near the

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WTC who delivered at Mount Sinai Hospital during the same time period.

Another cohort of 300 women who lived, worked, or delivered their infants in lower Manhattan in the weeks and months after September 11 were recruited to examine chronic exposure after the attacks (Lederman et al., 2004). These women were followed by the Columbia Center for Children's Environmental Health. The New York City Department of Health and Mental Hygiene and the Agency for Toxic Substances and Disease Registry (ATSDR) established the World Trade Center Health Registry, a voluntary registry to prospectively monitor physical and mental health in the aftermath of the attacks among those with a high probability of direct exposure, including pregnant women (Lipkind et al., 2010).

In recognition of the impact of environmental toxicants encountered *in utero* and the disparities in adverse birth outcomes among inner-city, minority populations, the Columbia Center for Children's Environmental Health established a prospective cohort to follow pregnant women and their children longitudinally (Perera et al., 2003). Nonsmoking Dominican and African-American women residing in three NYC neighborhoods were monitored for exposure to environmental tobacco smoke (ETS), airborne PAHs, and pesticides. A parallel prospective cohort was assembled in Krakow, Poland, with the same eligibility criteria, study design, and air-monitoring methods (Jedrychowski et al., 2009). In Tongliang, China, the effects from PAH exposure secondary to coal combustion was studied prior to and after the shutdown of a coal-fired power plant (Perera et al., 2005a; Tang et al., 2006).

Conclusions from Prospective Epidemiology Cohort Studies

Table 20.1 outlines the main findings from follow-up studies of the cohorts described earlier in this chapter. Analysis of the 187 exposed women in the Mount Sinai WTC cohort revealed nearly a twofold increase in the odds of intrauterine growth retardation in their babies (IUGR; adjusted odds ratio (AOR) 1.90; 95% CI, 1.05–3.46) (Berkowitz et al., 2003). Biologically plausible causes of IUGR in these babies include exposures to fine particulate matter (PM) and PAHs. Previous studies have found associations between particulate air pollution and IUGR (Dejmek et al., 1999; Bobak et al., 2001). Other investigations have linked air pollution to preterm births (Ritz et al., 2000). Additionally, high levels of PAH-DNA adducts in umbilical cord leukocytes are associated with reduced birth size (Perera et al., 1998).

Among the cohort of 300 nonsmoking women who were pregnant on September 11, 2001, and lived within a 2-mile radius of the WTC during the following month, a number of decrements were found in their babies compared to the babies of other pregnant women studied. Infants born to exposed women showed a decrease of 122 g in birth weight and 0.74 cm in birth length after adjusting for gestational duration, sociodemographic, and biomedical risk factors (Lederman et al., 2004). Infants born to women who were in their first trimester at the time of the attacks had significantly shorter gestation (−3.6 days) and smaller head circumference (−0.48 cm) compared to infants born to mothers exposed during their second or third trimester. The 446 births recorded in the World Trade Center Registry also showed a variation in mean gestational age by trimester on September 11, in addition to differences in birth weight (Lipkind et al., 2010). Analysis of births from previous years, however, revealed that the effect of trimester observed may have been due to a seasonal effect unrelated to exposure. It is important to note that the odds of low birth weight and preterm delivery were higher in registry-linked births to mothers that probably had posttraumatic stress disorder (PTSD; Lipkind et al., 2010).

Effects of Maternal Stress

In addition to PAH exposure during pregnancy, maternal stress associated with a terrorist attack or disaster has been shown to affect birth outcomes and later neurocognitive development. Among the 187 women in the Mount Sinai cohort who were pregnant and living or working within close proximity to the WTC on September 11, 52 women completed at least one psychological assessment prior to the delivery of their child. In this analysis, Engel et al. (2005) found that posttraumatic stress symptomatology (PTSS) was associated with decrements in infant head circumference at birth. These decrements could later influence neurocognitive development, although follow-up on this cohort has not yet been reported.

Analysis of live singleton births to women enrolled in the World Trade Center Health Registry between September 11, 2001, and October 31, 2002, also found an apparent effect of maternal stress. When comparing women enrolled in the registry (who were exposed) to those not in the registry and who lived in a NYC census tract more than 5 miles from the WTC site, adjusted analyses found no differences in birth weight, gestational age of delivery, the odds of low birth weight, or the odds of preterm delivery. However, when comparing women with a high PTSD score to those with a lower score, increased odds of preterm delivery (AOR 2.48, 95% CI 1.05–5.84) and low birth weight (AOR 2.49, 95% CI 1.02–6.08) were detected (Lipkind et al., 2010).

In analysis of birth certificate data obtained from the Office of Vital Statistics in New York State and NYC, excluding lower Manhattan, Eskenazi et al. (2007) found

TABLE 20.1 Summary of Findings from Studies Examining Cognitive and Developmental Outcomes Resulting from *In Utero* Exposure to PAHs

Cohort and Authors	Age	Development Measure	Findings
Tongaliang, coal-fired power plant pre- and post-shutdown (Perera et al., 2008)	2 years of age	GDS	Significant association between elevated cord blood adducts and decreased motor development quotient and average development quotient in 2002 cohort (pre-shutdown), but not 2005 (post-shutdown)
Tongaliang, coal-fired power plant pre- and post-shutdown (Tang et al., 2013)	Summary of birth—2 years	GDS	Children born after the power plant shutdown had greater head circumference ($P = 0.001$), reduced levels of cord blood adducts ($P < 0.001$) and were exposed to lower levels of ambient PAHs compared to the cohort born prior to shutdown ($P = 0.01$)
Tongaliang, coal-fired power plant pre- and post-shutdown (Tang et al., 2014)	Summary of birth—2 years	GDS	An inverse association was found between BDNF and PAH-DNA adducts in cord blood. Developmental quotient scores were positively associated with BDNF levels
A subset of the CCCEH cohort pregnant on 9/11 or became pregnant in following month ($n = 300$) (Perera et al., 2007)	3 years	BSID-II	A significant interaction between cord blood DNA adducts and *in utero* exposure to ETS on MDI score ($P = 0.02$), but cord blood adducts or ETS alone were not significant predictors of cognitive development
The 182 of the WTC cohort versus Mt. Sinai births (Mt. Sinai Pregnancy Outcome Study) (Berkowitz et al., 2003)	Birth outcomes		No differences in mean gestational age, mean birth wt, frequency of preterm birth Twofold increase in IUGR among WTC exposed women No association found between probable PTSD and RR of preterm birth, LBW, or IUGR
The **52** of the 182 of the WTC cohort versus Mt. Sinai births (Mt. Sinai Pregnancy Outcome Study) (Engel et al., 2005)	Birth outcomes		PTSS and depression were associated with longer gestational duration PTSS was associated with decrements in head circumference
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2003)	Birth outcomes		Among African Americans, high PAH exposure was associated with lower birth weight ($P = 0.003$) and smaller head circumference ($P = 0.01$)
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2006)	3 years	BSID-II	Prenatal exposure to PAHs significantly associated with lower MDI at age 3, but not at 1 or 2 years. Prenatal PAH exposure was also unrelated to PDI
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2009)	5 years	WPPSI-R	High PAH levels, above the median of 2.26 ng/m^3, were associated with full-scale IQ (4.31 lower, $P = 0.009$) and verbal IQ (4.67 lower, $P = 0.002$) reductions compared to less exposed children
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2012)	6–7 years	CBCL	High PAH exposure, defined as greater than the median exposure from personal air monitoring, or detectable and higher maternal and cord adducts, was associated with symptoms of Anxious/Depressed and Attention Problems ($P \leq 0.05$)
Krakow, Poland cohort (2001–2006 enrollment) (Edwards et al., 2010)	5 years	RCPM	Children with higher than the median (17.96 ng/m^3) prenatal exposure to airborne PAHs had decreased RCPM scores at age 5. These reduced scores correspond to an estimated average decrease of 3.8 IQ points

GDS, Gesell Development Schedules; CBCL, Child Behavior Checklist; WPPSI-R, Wechsler Preschool and Primary Scale of Intelligence-Revised; BSID-II, Bayley-II Scales of Child Development; ETS, Environmental Tobacco Smoke; MDI, Mental Development Index; PDI, Physical Development Index; RCPM, Raven Coloured Progressive Matrices.Included are studies related to the events of September, 11, 2001, as well as international studies of PAH exposures.

an increased chance of very low birth weight (<1,500 g) around the start of 2002 (i.e., among infants that would have been in their first or second trimester of gestation at the time of the September 11 attacks). This same finding was noted in births 33–36 weeks after the disaster (i.e., those conceived around or shortly after September 11). The authors suggest that these effects could be the result of the increased stress associated with a terrorist attack (Eskenazi et al., 2007). A study of maternal stress resulting from the 1998 ice storm in Quebec, Canada, found that more severe prenatal maternal stress was associated with decrements in Bayley Scales of Infant Development (BSID)–Mental Development Index (MDI) scores and parent-reported language abilities at 2 years of age (Laplante et al., 2004).

An interaction between exposure to PAHs and maternal stress has been suggested. Among children who experienced high prenatal exposure to PAH, maternal demoralization during pregnancy has a greater negative effect on children's neurobehavioral development. In the longitudinal study of 248 children from the Krakow cohort, maternal demoralization had an effect on syndromes of depression (anxious and withdrawn), rule-breaking and aggressive behaviors, and composite internalizing and externalizing scores at age 9 (Perera et al., 2003). These effects were seen only in those with high prenatal exposure. Both prenatal PAH exposure (Tang et al., 2014) and prenatal stress (Neeley et al., 2011) have been found to decrease hippocampal mature brain-derived neurotrophic factor (mBDNF) expression and signaling cascades for long-term potentiation (LTP), providing a potential mechanism for the observed interaction.

PAH-DNA Adducts

PAH-DNA adducts provide a reflection of individual variation in exposure and toxicokinetics and have been associated with risk of cancer and reproductive and developmental effects (Tang et al., 2006; Perera et al., 2007). In the cohorts followed by researchers from the Columbia Center for Children's Environmental Health, mean DNA adduct concentrations in both maternal and fetal cord blood, as well as the proportion of samples with detectable adducts, increased across the populations studied in a manner consistent with the trend in estimated ambient exposure to PAHs ($P < 0.001$, northern Manhattan < WTC < Krakow < Tongliang). Data from these four populations indicate that the developing fetus may have a 10-fold greater susceptibility to DNA damage than mothers (Perera et al., 2005a).

Higher adduct levels have been associated with a number of adverse birth and neurocognitive developmental outcomes. In a cohort of 150 mother-infant dyads in Tongliang County, China, that received exposure from a seasonally operated, coal-fired power plant, those with higher than median PAH-DNA cord blood adducts weighed less than their low-exposure counterparts at 18, 24, and 30 months of age (Tang et al., 2006). Exposure for a longer duration was associated with shorter length at birth and height at 18, 24, and 30 months. In children who were *in utero* during power plant operation, there was a 91% increase in the odds of being developmentally delayed in the motor area for each 0.1-unit increase in cord blood adducts (0.1 adducts/10^8 nucleotides). The mean cord adduct levels in this cohort were 0.32 adducts/10^8 nucleotides (Perera et al., 2008). After the shutdown of the power plant, the head circumference of the children was greater than that of those born prior to the shutdown (33.766 cm versus 34.130 cm; $P < 0.05$), and consistent with the reduced levels of ambient PAHs and reduced levels of cord blood adducts (Tang et al., 2013).

In comparisons of these pre- and post-shutdown cohorts, it was also found that the mean level of mBDNF was significantly higher in the 2005 post-shutdown cohort (1266.56 versus 752.87 pg/mL; $P < 0.05$) (Tang et al., 2014). The brain-derived neurotrophic factor (BDNF) levels were inversely correlated with PAH-DNA adducts in cord blood in both cohorts ($P < 0.01$) and positively associated with average, motor area, and social area development quotients. Based on their analyses, Tang et al. (2014) deemed BDNF to be a potential mediator between PAH-DNA adducts measured in cord blood and later cognitive outcomes. This is consistent with findings that prenatal exposure to ETS, of which PAHs are a component, was linked to down-regulation of BDNF through increased methylation of the BDNF exon (Toledo-Rodriguez et al., 2010).

Among women in the WTC cohort who were also exposed to ETS during pregnancy, increased PAH-DNA adducts were associated with reduced birth weight (276 g; 8% for a doubling of adducts) and head circumference (1.3 cm; 3% for a doubling of adducts) (Perera et al., 2005b). At age 3, children born to these mothers had an average reduction in MDI of 6% for a doubling of adducts (Perera et al., 2007). Lower MDI at age 3 was also found in the cohort of African-American and Dominican mothers and children (Perera et al., 2006).

Assessment of gene and environment interactions has found that both maternal and newborn haplotypes of CYP1A1 and CYP1B1 appear to be important effect modifiers of MDI at 12, 24, and 36 months of age (Wang et al., 2008, 2010). Subsequent analysis has identified a potential relationship between maternal haplotypes of XRCC1 and GSTM3, in addition to a maternal haplotype of CYP1B1, maternal PAH exposure, and newborn cord blood adducts in African Americans (Iyer et al., 2014). This study also found interactions with maternal PAH exposure and newborn cord blood adducts with newborn CYP1A2 and XRCC1 in African Americans,

maternal XRCC1 among Dominican women, and newborn NQO1 in Dominican infants (Iyer et al., 2014).

Refinement of Our Susceptibility-Exposure Paradigm to Access the Effects of *In Utero* Exposure to PAH Aerosols on Neurodevelopmental Processes

The determination of the embryonic "critical period of development" for the brain structures involved in learning and memory processes in mice is based on the original work by Rodier (1976). This study identified the embryonic time frames for peak neurogenesis and neuroepithelial proliferation for cerebral cortex, hippocampus, septum, amygdala, corpus striatum, thalamus, hypothalamus, cerebellum, and olfactory bulb as the period from embryonic day E14 through E17. Rodier documented almost 40 years ago that the specific time of the central nervous system (CNS) insult is an important factor in subsequent effects on both anatomy and behavior. Therefore, this early work established what we refer to as the embryonic "critical period of development." The report suggested that the behavioral effects of toxicants such as benzo(a)pyrene (B(a)P) are similar in both rats and mice. This study was one of the first to demonstrate that mice could be used successfully as subjects in a variety of behavioral evaluation experiments.

Novel object discrimination testing has been used for over two decades because it is perfectly suited to test the effects of pharmacological and genetic interventions on learning and memory processes (Dere et al., 2007). When comparisons are made among experimental model systems of learning and memory, the object discrimination test is more closely related with the conditions under which human recognition memory is measured. This is primarily due to a shortened training period coupled with the fact that novel object discrimination does not induce high levels of arousal and stress in animals (Ennaceur and Delacour, 1988). The component of memory likely affected by PAH is the medial prefrontal cortex (mPFC) and is referred to as *relative recency memory* (Fuster, 2001). In support of this argument concerning relative recency memory, it is known that lesions to the mPFC impair relative recency discrimination in humans, nonhuman primates, and rodents across a wide range of stimulus modalities (Fuster, 2001). This is true despite the fact that in some instances, recognition of novel and familiar stimuli is preserved.

Conversely, studies have reported that after bilateral mPFC lesions, an impairment or deficit is observed with respect to the ability to differentiate between previously presented and recently presented familiar objects (Mitchell and Laiacona, 1998). Results from our laboratory testing *in utero* of B(a)P aerosol–exposed $Cpr^{lox/lox}$ offspring in object discrimination paradigms were consistent with results from the aforementioned bilateral mPFC lesion studies. They support the suggestion that the mPFC is involved (at least in part) in making judgments regarding the sequence and order of object presentations. Additionally, the involvement of cortical glutamate receptors in performance of the novel object discrimination task in rodents is well established, as deficits and impairments have been reported following the application of AMPA (CNQX) or NMDA (AP-5) antagonists. By characterizing the effects of *in utero* exposure to PAH on $Cpr^{lox/lox}$ and brain-*Cpr*-null offspring, we further validate novel object discrimination phenotying as a measure of prefrontal and limbic circuit integrity by demonstrating that this behavior is negatively affected subsequent to *in utero* B(a)P exposure.

Refinement of our Susceptibility-Exposure Paradigm to Access the Effects of *In Utero* Exposure to PAH Aerosols on Behavioral Phenotypes

Cytochrome-P_{450} is an important modifier of mental development at an early age. Recently, significant interactions were reported among a maternal haplotype in the cytochrome P_{450}1B1 (CYP1B1) gene (ACCGGC), PAH exposure, and reductions in the MDI in a cohort of children (Wang et al., 2010). These studies have important implications for our society; they are valuable from a translational standpoint because they can inform the design of molecular neurotoxicology studies using experimental model systems.

Recently, Xia et al. (2011) evaluated the effects of subchronic exposure to B(a)P (intraperitoneal injections of 6.25 mg/kg B(a)P/day for 14 weeks) versus diluent on both neurotransmitter receptor gene expression and performance in a Morris water maze. Mircoarray results revealed that 1,016 genes were differentially expressed in B(a)P-treated specimens versus diluent controls. The Database for Annotation, Visualization, and Integrated Discovery was used to analyze those genes differentially expressed in the gene ontology and Kyoto Encyclopedia of Genes and Genomes pathways. Their analysis showed that the most significantly affected category was behavior, and the fourth-highest was learning and memory. They ranked 22 genes involved in learning and memory and 25 genes associated with neuroactive ligand–receptor interactions. Both lists included up-regulation of the ionotropic glutamate receptor *N*-methyl D-asparate 2A (GRIN2A). A conclusion was that "neuroactive ligand–receptor interactions" were among the most negatively affected by B(a)P exposure at ($P < 7.7$ E−6). In the Morris water maze test, B(a)P-treated rats had spatial learning deficits and had a decreased number of platform crossings and time spent in the target area, suggesting that B(a)P caused a deleterious effect on long-term

memory. Several other studies have also suggested that neurotransmitter and neurotransmitter receptor gene expression play important roles in modulating neurobehavioral effects, especially within the context of learning and memory.

These results confirmed the notion that the PAH component can have a direct negative impact on the developmental expression of (i) key glutamatergic regulators of NMDA-mediated processes (receptor tyrosine kinase-MET) and (ii) behavioral deficit phenotypes.

PAH EXPERIMENTAL MODEL SYSTEMS

Toxicological Observations from Modeling B(a)P Aerosols

In our experimental model system, *Cpr*$^{lox/lox}$ and brain-*Cpr*-null timed-pregnant dams were exposed to a B(a)P aerosol on E14–E17 exhibiting a trimodal distribution with a 93% cumulative mass less than 5.85 μm, 91% cumulative mass less than 10 μm, 57.6% cumulative mass less than 2.5 μm, and 43% less than 1 μm (Figure 20.1 and Table 20.2). The characterization of the aerosol atmospheres generated in these studies was comparable to those generated previously for rat studies (Hood et al., 2000). The B(a)P aerosol used in our earlier rat studies comprised a mass median aerodynamic diameter with a geometric standard deviation of 0.9 ± 0.09 μm, compared to 1.0 ± 0.07 in the present *Cpr* mouse experiments. Analysis of live birth indices revealed no significant differences in the number of mouse pups born per litter between control *Cpr*$^{lox/lox}$ dams and B(a)P-exposed *Cpr*$^{lox/lox}$ dams. The analysis of the live birth index for control *Cpr*$^{lox/lox}$ dams was 6.12 ± 0.42, compared to 5.9 ± 0.9 for B(a)P-exposed *Cpr*$^{lox/lox}$ dams, and 5.7 ± 0.31, compared to 5.99 ± 0.41 for the control and B(a)P-exposed brain-*Cpr*-null dams, respectively. Statistical analysis (P = 0.241) indicated no significant difference in this index between these groups. This finding is consistent with our previous reports using rat and mouse models (Hood et al., 2000, 2006; Wormley et al., 2004a,b; Brown et al., 2007; Sheng et al., 2010). During the prenatal exposure period, as well as the subsequent preweaning period, there were no identifiable B(a)P-related effects on conventional reproductive indices of toxicity, and there were no convulsions, tremors, or abnormal movements noted in any of the control or B(a)P-exposed *Cpr* litters.

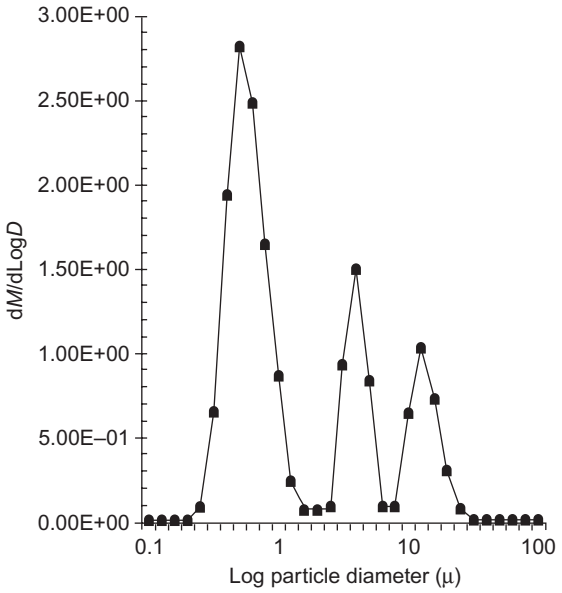

FIGURE 20.1 Differential particle-size distribution representative of B(a)P:carbon black aerosol delivered to timed pregnant *Cpr*$^{lox/lox}$ and brain-*Cpr*-null dams (100 μg/m^3). During a typical exposure, aerosol was collected on substrates every 30 min during a 4 h exposure period from E14 to E17. Subsequent to the exposure period each day, substrate post-weights were uploaded into a custom impactor data reduction program to generate average particle-size distributions as previously described (Wu et al., 2003).

In Situ Generation of "Oxidative Metabolites" in Neocortical Tissue from *In Utero* Exposure to B(a)P Aerosol

The quantitation of B(a)P metabolites from *Cpr*$^{lox/lox}$ and brain-*Cpr*-null offspring is shown in Figure 20.2A and B. In neocortical tissue from *in utero* B(a)P aerosol–exposed *Cpr*$^{lox/lox}$ offspring, we found 4.5-, 7.8-, 9-, and 10-diols, 3.6- and 6.12-diones, and 3-OH and 9-OH B(a)P metabolites, indicating the presence of an active phase 1 biotransformation pathway (Figure 20.2A). In contrast, in brain-*Cpr*-null offspring, all metabolites were essentially below the level of detection (note the change in the *y*-axis values in Figure 20.2B). The important point in Figure 20.2A is the identification of the developmental period over which there is formation/accumulation of the reactive 3-OH and 9-OH metabolites. These metabolites can further oxidize to form B(a)P quinones that can undergo redox cycling and generate reactive oxygen species (Li et al., 2012).

F_2-isoprostane measures assessed the *in vivo* prefrontal cortex (PFC) oxidative milieu occurring primarily in neuronal membranes. Figure 20.3 shows the quantitation of F_2-isoprostanes derived from neocortical homogenates from control and B(a)P-exposed *Cpr*$^{lox/lox}$ offspring (Figure 20.3A), and control and B(a)P-exposed brain-*Cpr*-null offspring (Figure 20.3B). (Note: There are no control *brain-Cpr-null* mouse isoprostane data for P3 to P15 due to the lack of available tissue.) The data document a sustained high neocortical tissue burden of F_2-isoprostanes in B(a)P-exposed *Cpr*$^{lox/lox}$, including the period from P7–P14, the extent of which was not observed in null mice (Figure 20.3B).

TABLE 20.2 Modeling of B(a)P: Carbon Black Aerosols for *Cpr* Studies

Q_{tot} (slpm)	Q_{vap} (slpm)	Q_{hum} (slpm)	Q_{RBG} (lpm)	T_{mixch} (°F)	T_{vap} (°F)	$\rho(T_{vap})$ (μg/m³)	T_{sat} (°F)	TCp (°F)	$T_{premix1}$ (°F)	$T_{particle2}$ (°F)	T_{mix} (°F)	Mixture $\rho(T_{mix})$ (μg/m³)	Mixture CB(a)P at T_{mix} (μg/m³)
10	2	7	1	132	234	3	192	276	270	79	132	5,900	100
10	**2**	**7**	**1**	**114**	**234**	**3**	**192**	**288**	**281**	**80**	**135**	**7,500**	**100**
10	2	7	1	104	234	3	192	295	288	80	137	8,800	100
10	2	7	1	115	234	3	192	297	290	80	138	9,100	100

TC_p and T_{premix} were generally high enough for top side of orifice plate > T_{sat}, rows 1–3 of each group of four rows. For each group, values greater than the second row are given in the fourth row for the purposes of evaluating overheating of the humidified B(a)P stream. Bold values in row 2 indicate the experimental conditions under which the studies were conducted. Assume T_{Wright} = 70°F; m = 0.565; b = 37.432 (for details, see Li et al., 2012).

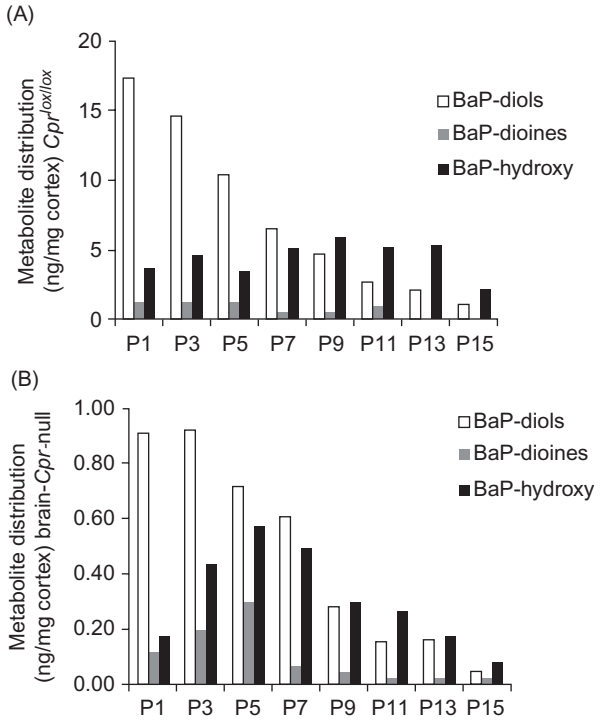

FIGURE 20.2 Metabolite distribution of cortical B(a)P metabolites during the critical postnatal period when synapses are forming for the first time in B(a)P-exposed *Cpr* offspring. Timed-pregnant $Cpr^{lox/lox}$ or brain-*Cpr*-null dams received either carbon black only or 100 μg/m³ B(a)P via nose-only inhalation on E14–E17 for 4 h/day. Offspring were sacrificed on P1, 3, 5, 7, 9, 11, 13, and P15. Shown are the distribution of metabolites detected in B(a)P-exposed (A) $Cpr^{lox/lox}$ offspring or (B) brain-*Cpr*-null offspring.

Temporal Modulation of NMDA-Mediated Developmental Processes as a Result of *In Utero* Exposure to B(a)P Aerosol

Evidence supporting the hypothesis that an early insult to an NMDA-mediated signaling system leads to phenotypical changes that manifest later in life as elevated glutamate concentrations in mPFC is provided in Figure 20.4A. Figure 20.4A shows a time course plot indicating basal levels of glutamate in C57BL background controls, $Cpr^{lox/lox}$, B(a)P-exposed $Cpr^{lox/lox}$, and B(a)P-exposed brain-*Cpr*-null offspring obtained by microdialysis. Studies were carried out in a control group of mice performing the same task and B(a)P aerosol–exposed *Cpr* offspring over a 120 min sampling period. Quantitation of the results in Figure 20.4 shows a statistically significant increase in the basal concentration of glutamate in the mPFC of B(a)P-exposed $Cpr^{lox/lox}$ offspring compared to WT C57BL, WT $Cpr^{lox/lox}$, or B(a)P aerosol–exposed brain-*Cpr*-null offspring. The basal glutamate concentrations obtained in our *Cpr* mouse model are identical to recent reports in the literature for dorsal hippocampus in a 129/SvEv mice KATII KO model (*45*) and for the PFC in a Wistar rat ethanol model (Chefer et al., 2011). These findings are consistent with the idea that increases in glutamate concentration promote altered inward currents via the NMDA receptor.

As a means of assessing the impact of *in utero* exposure to B(a)P aerosol on developmental NMDA-mediated processes, we evaluated developmental expression profiles for NMDA receptor subunits compared to those of Sp4 and Sp1 proteins with and without *in utero* B(a)P exposure. Inspection of the expression profile (P1–P15) in control $Cpr^{lox/lox}$ offspring (Figure 20.5A, left panel) revealed that Sp4 expression is constitutively low on P1, reaches peak expression levels on P7, and subsides to constitutive levels by P15, which is consistent with earlier reports (Li and Pleasure, 2005). Conversely, the Sp4 developmental expression profile in B(a)P-exposed $Cpr^{lox/lox}$ offspring (Figure 20.5B, right panel) indicates that as early as P1, Sp4 reaches near maximal levels and by P3, it reaches peak expression levels and remains at maximal levels through P7. The interpretation of this finding is that the leftward shift in peak Sp4 expression is in response to *in utero* B(a)P aerosol exposure occurring during the E14–E17 period. The consequence of this exposure is the mistiming of peak Sp4 expression in B(a)P-exposed $Cpr^{lox/lox}$ offspring, which could significantly affect downstream signaling processes, perhaps in a way similar to those reported for Sp4 null mice (Zhou et al., 2007).

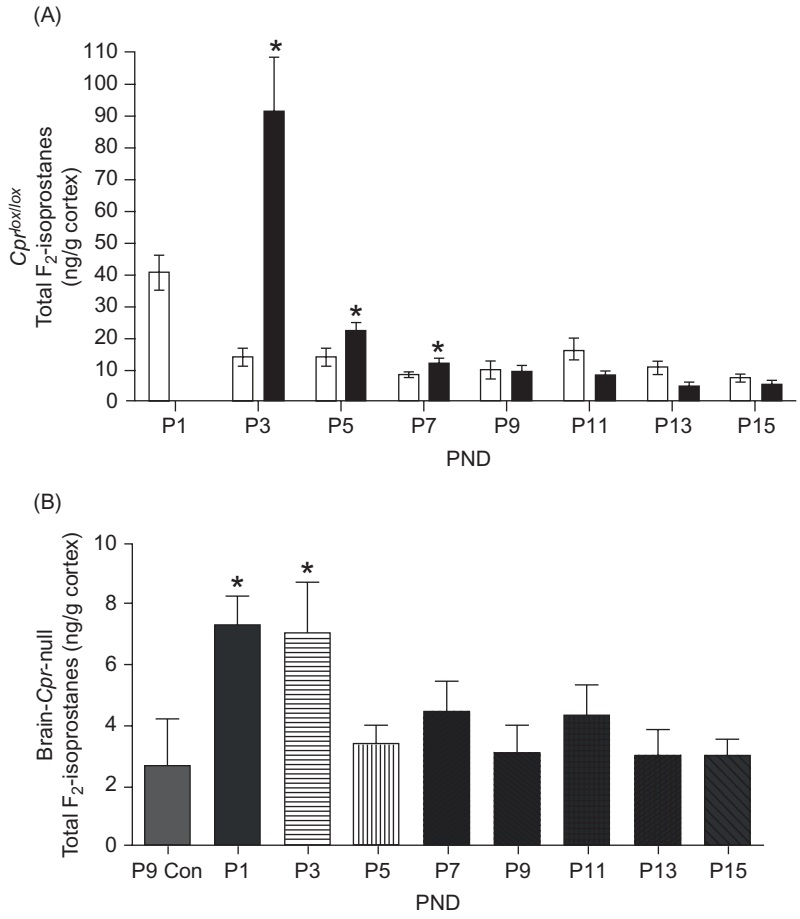

FIGURE 20.3 *In utero* **exposure to B(a)P aerosol (100 μg/m^3) produces an approximate 10× higher concentration of F_2-isoprostanes on P3 in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring as compared to B(a)P-exposed brain-*Cpr*-null offspring.** (A) F_2-isoprostanes levels derived from control (white bars) and B(a)P-exposed *Cpr*$^{lox/lox}$ (black bars) offspring neocortex. (B) F_2-isoprostane levels for derived from control PND9 (white bar) and B(a)P-exposed (black bars) brain-*Cpr*-null offspring neocortex. Due to the limited number of control offspring, only PND9 was used for time points in favor of reserving pups for behavioral determinations in later-life. *P = <0.05 versus Control.

Having demonstrated that B(a)P exposure shifts the Sp4 expression profile to the left and given that NR2A is a Sp4 target gene, it is not surprising that B(a)P exposure also modulated the temporal expression of NR2A. The Sp4 nontarget NR2B developmental expression profile, on the other hand, for control *Cpr*$^{lox/lox}$ offspring (Figure 20.5A, left panel) revealed that expression was low from P1–P3 and gradually increased until peak NR2B levels were present on P15. The NR2B developmental expression profile in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring (Figure 20.5B, right panel) is strikingly similar to that of controls. What is apparent is the significant change in the NMDA subunit ratio of NR2B:NR2A on P7 in control *Cpr*$^{lox/lox}$ offspring (NR2B:NR2A = 1/0.4, as seen in Figure 20.5A, lower-left panel) as compared to B(a)P-exposed *Cpr*$^{lox/lox}$ offspring (NR2B:NR2A = 1/0.78, seen in Figure 20.5B, lower-right panel). Reminiscent of Sp4 expression, the NR2A developmental expression profile in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring (right panel) is constitutively "on," beginning from P1–P5, and by P13, it reaches maximal expression levels compared to P15 in B(a)P-exposed offspring.

The developmental expression profiles for brain-*Cpr*-null offspring are shown in Figure 20.6. Inspection of the expression profiles from these *null* mice (P1–P15) in control brain-*Cpr*-null offspring (Figure 20.6A, left panel) revealed that Sp4 expression is constitutively low on P1 and reaches peak expression levels on P13. Conversely, the Sp4 developmental expression profile for B(a)P-exposed brain-*Cpr*-null offspring (Figure 20.6B, right panel) indicates that as early as P1, Sp4 expression is evident, reaches maximal levels by P3, and then drops to constitutive expression levels by P7. What is apparent from the brain-*Cpr*-null offspring data is that the NMDA subunit ratio of NR2B:NR2A on early postnatal days (P1, P3, P5, and P7) in B(a)P-exposed brain-*Cpr*-null offspring (NR2B:NR2A = 1/0.5, seen in Figure 20.6B, lower-right panel) is approximately the same as for P7 in control *Cpr*$^{lox/lox}$ offspring (NR2B:NR2A = 1/0.5, as seen in Figure 20.6A, lower-left panel). Based on the results of

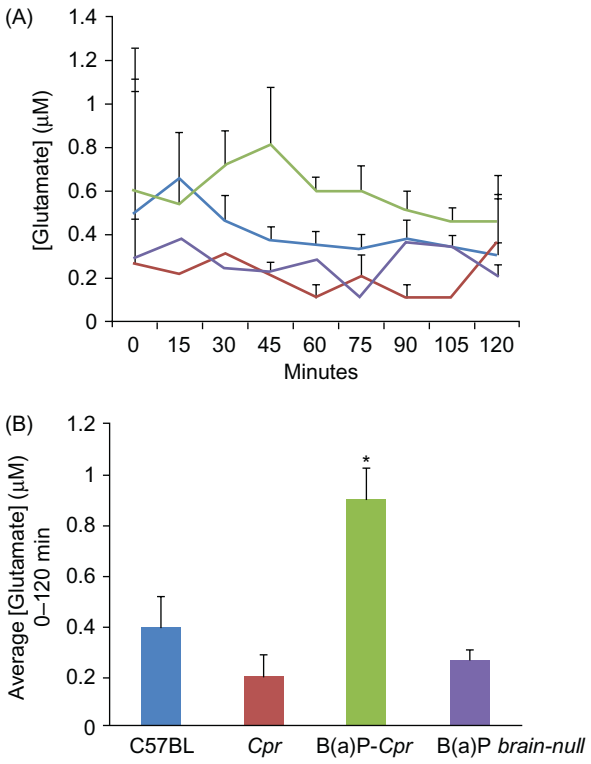

FIGURE 20.4 ***In utero*** **exposure to B(a)P aerosol (100 μg/m^3) results in a robust increase in prefrontal cortical glutamate concentrations as assessed in awake behaving *Cpr$^{lox/lox}$* offspring but not in brain-*Cpr*-null offspring (PND100).** (A) Glutamate concentrations assessed from medial PFC as a function of time. (B) Quantitation of average glutamate concentrations over this time period (μM) assessed in control C57BL, control *Cpr$^{lox/lox}$* offspring versus B(a)P-exposed *Cpr$^{lox/lox}$* and brain-*Cpr*-null offspring. See text for details (C57BL, blue; *Cpr*, red; and B(a)P-*Cpr*, green; B(a)P brain-*Cpr*-null, purple). *P = <0.05 versus *Cpr*.

a recent NR2A subunit knockout study in mice (Brigman et al., 2008), it would be reasonable to predict that in our null model, the absence of modulation in NMDA subunit ratios would mean that these mice would not exhibit a behavioral deficit phenotype. Such a finding would support our hypothesis that a significant deficit in behavioral learning results from the loss of proper temporal functioning of the NR2A subunit.

Rescue of Spatial Discrimination Deficit Phenotypes in Brain-*Cpr*-Null Offspring Subsequent to *In Utero* Exposure to B(a)P Aerosol

Novel object discrimination testing was used to measure B(a)P-induced behavioral effects as described in our recent report (Sheng et al., 2010). Data in Figure 20.7 show that control *Cpr$^{lox/lox}$* offspring mice were better able to discriminate between novel and familiar objects (8.1 ± 0.31), as compared to B(a)P-exposed *Cpr$^{lox/lox}$* offspring (2.0 ± 0.17). An analysis of variance revealed significant differences between the control *Cpr$^{lox/lox}$* offspring (observational, small zone, and large zone) with regard to entries into the novel zone compared to the B(a)P-exposed *Cpr$^{lox/lox}$* offspring. The exposed group exhibited a diminished ability to discriminate novel from familiar objects as indicated by a significantly reduced time as compared to controls ($P < 0.05$ for the 100 μg/m^3 group). Post hoc analysis using the Bonferroni's test revealed the significance at $P < 0.01$. Testing of control brain-*Cpr*-null offspring versus B(a)P-exposed brain-*Cpr*-null offspring in the 100 μg/m^3 group revealed no significant differences in object discrimination at 9.1 ± 1.4 versus 9.0 ± 0.8. Given that brain-*Cpr*-null offspring are incapable of producing significant levels B(a)P metabolites via CYP1B1, these data strongly implicate a sustained-oxidative neocortical tissue metabolite burden during the critical period from P0-P5 that contributes to the discrimination deficit phenotype observed in B(a)P aerosol–exposed *Cpr$^{lox/lox}$* offspring.

Figure 20.8 shows coronal sections of adult neocortical sections from *Cpr$^{lox/lox}$* offspring that were analyzed for cytoarchitecture by immunoshistochemical staining for NeuN. Neocortical neurons were counted in *Cpr$^{lox/lox}$* offspring that had not been exposed to B(a)P *in utero* (A, low magnification; B, higher magnification) and compared to *Cpr$^{lox/lox}$* offspring that were exposed to the B(a)P *in utero* (C, low magnification; D, higher magnification). Figure 20.8E shows the plot resulting from stereological analysis of neocortical neurons subsequent to application of a two-tailed *t*-test. As can be seen, no significant differences were seen in the density of neocortical neurons as a result of *in utero* exposure to B(a)P aerosol.

Negative modulation of cortical inward currents in neurons derived from B(a)P-exposed *Cpr$^{lox/lox}$* offspring. Finally, electrophysiology experiments were performed on *ex vivo* primary cortical neurons as a means of ascertaining potential B(a)P exposure–induced effects on cortical currents. On the seventh day in culture, *ex vivo* primary cortical neurons derived from control and B(a)P-exposed Cpr$^{lox/lox}$ offspring were voltage-clamped using the whole cell configuration and held at −60 mV, in a Mg^{2+} free external solution. The current–voltage (I–V) relationship of cortical neurons derived from control and B(a)P-exposed (100 μg/m^3) Cpr$^{lox/lox}$ offspring was nearly linear between −100 and −20 mV (Figure 20.9A). Figure 20.9B shows a representative current trace obtained from carbon black control and cortical neurons derived from 100 μg/m^3 B(a)P aerosol–exposed offspring at −80 mV using the same experimental configuration as in Figure 20.8A. Although there were no apparent differences between the current recorded results in control and B(a)P-exposed cortical neurons at positive membrane

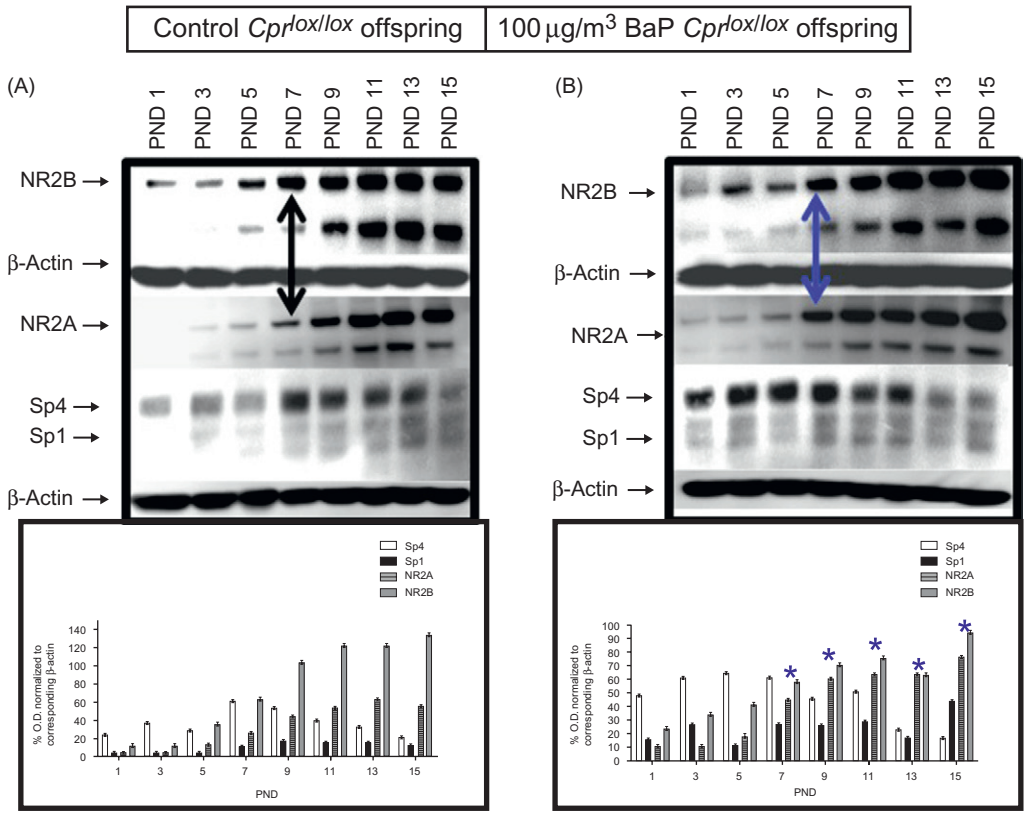

FIGURE 20.5 *In utero* **B(a)P aerosol exposure (100µg/m^3) induces a leftward shift in the peak temporal developmental expression of Sp4 and alters NMDA subunit ratios in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring (B, right panel) as compared to control *Cpr*$^{lox/lox}$ offspring (A, left panel).** Representative results from a typical experiment. The panels show developmental expression profiles from PND1 to PND15 for NR2B-Sp4 nontarget gene, NR2A-Sp4 target gene and Sp4-Sp1 proteins following SDS-PAGE of neocortical tissue from offspring gauged relative to internal β-actin controls. Lower panels represent quantitation of left and right upper panels. *Post hoc* analysis with the Bonferroni test with significance shown at $P < 0.05$.

potentials, there was a statistically significant, voltage-dependent decrease in the inward currents recorded at negative membrane potentials, as shown in Figure 20.8C ($t = -2.92789$, $P < 0.0429$).

IMPLICATIONS

We have presented complementary biophysical, molecular, neurochemical, and neurobehavioral approaches examining how the PAH component of combustion processes negatively affects gestational and developmental processes in our experimental model systems.

Our central findings are that *in utero* exposure to the PAH component of *c* (as B(a)P aerosol; Figure 20.1) in the *Cpr* mouse results in a substantial neocortical oxidative tissue burden of B(a)P metabolites (Figure 20.2) and F_2-isoprostanes (Figure 20.3) during a period when synapses are developing for the first time. Such a sustained neocortical tissue burden might be expected to contribute to an increased cortical oxidative load. The presence of three distinct categories of metabolites (diols, diones, and hydroxy-derivatives) indicate an active phase 1 biotransformation pathway in our control *Cpr* mouse model. Present in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring were the 4.5-, 7.8-, 9-, and 10-diols, the 3.6- and 6.12-diones and the 3-OH and 9-OH B(a)P metabolites. The important point in interpretation of this data is the identification of the developmental period over which there is formation and accumulation of 3-OH and 9-OH metabolites. These metabolites further oxidize to form B(a)P quinines that could undergo redox cycling and generate reactive oxygen species (Li et al., 2012). While the data from the brain-*Cpr*-null support the suggestion that accumulation of hydroxy-metabolites and their conversion into reactive intermediates in the *Cpr*$^{lox/lox}$ mouse likely contribute to the observed neurotoxicity, the levels may be below the threshold required to cause gross alterations in neuropathology (Figure 20.8). Additional experiments will be needed to gain a better mechanistic understanding on why there were no observable pathologies in the neocortical cytoarchitecture in B(a)P-exposed animals. While speculative, one possibility may be that the plastic state of these young brains allows for certain compensations—for example, these mice may have an

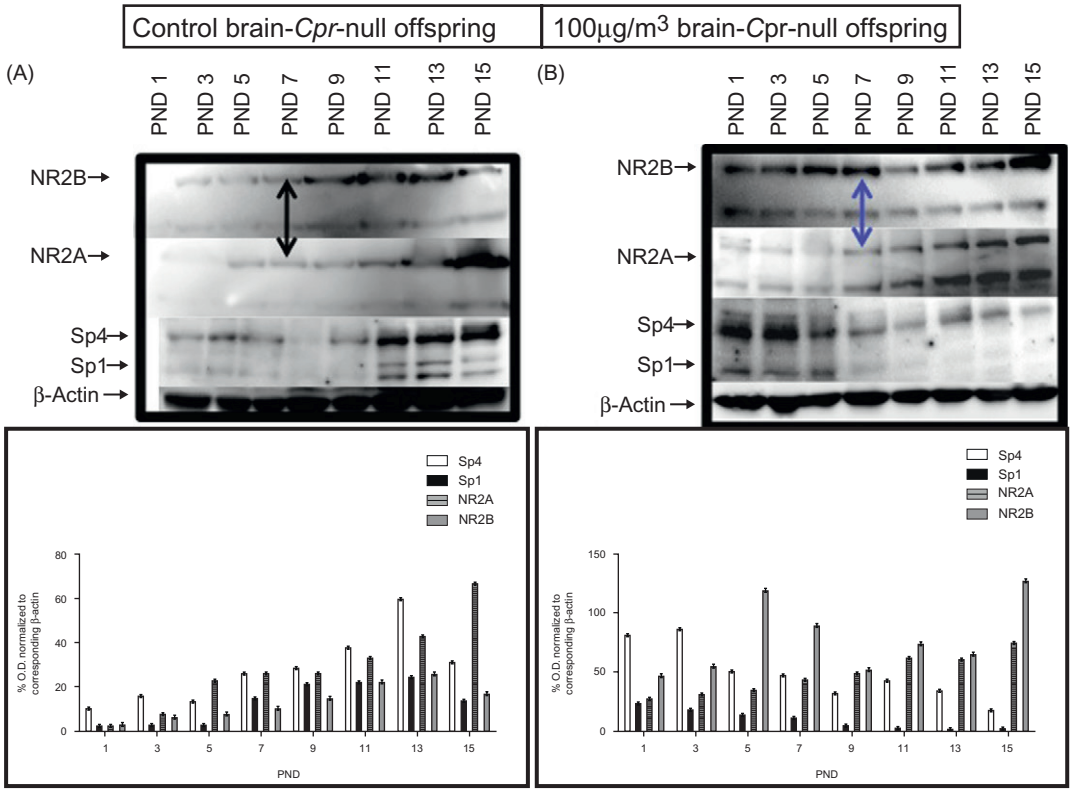

FIGURE 20.6 ***In utero*** **B(a)P aerosol exposure (100 μg/m^3) upregulates developmental expression of Sp4 in brain-*Cpr*-null offspring (right panel) with accompanying dysregulation of NMDA subunit ratios as compared to control *Cpr*$^{lox/lox}$ offspring (left panel).** Representative results from a typical experiment. The panels show developmental expression profiles from PND1 to PND15 for NR2B-Sp4 nontarget gene, NR2A-Sp4 target gene and Sp4-Sp1 proteins following SDS-PAGE of neocortical tissue from offspring gauged relative to internal β-actin controls. Lower panels represent quantitation of left and right upper panels. *Post hoc* analysis with the Bonferroni test with significance shown at $P < 0.05$.

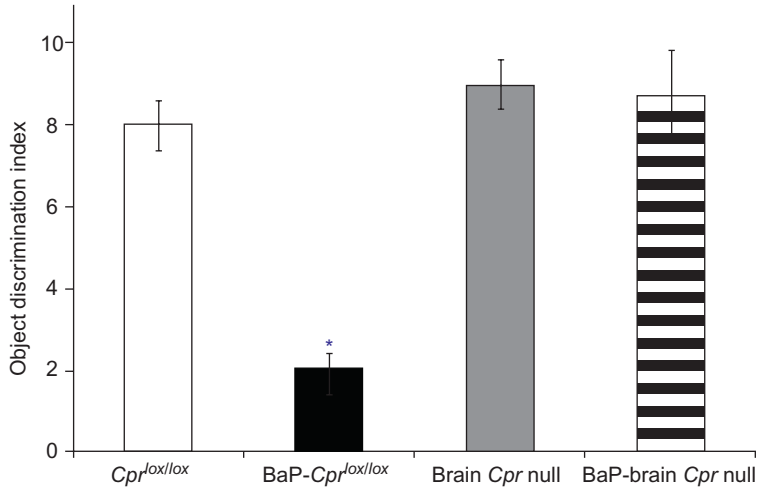

FIGURE 20.7 ***In utero*** **B(a)P aerosol exposure induces a significant deficit in object discrimination in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring as compared to control *Cpr*$^{lox/lox}$, WT brain-*Cpr*-null or B(a)P-exposed brain-*Cpr*-null offspring.** Object discrimination index for control *Cpr*$^{lox/lox}$ offspring mice was 8.1 ± 0.31 and 2.0 ± 0.17 for B(a)P-exposed *Cpr*$^{lox/lox}$ offspring. An analysis of variance revealed a significant difference ($P < 0.05$ for the 100 μg/m^3 group). Testing of control brain-*Cpr*-null offspring revealed an index of 9.1 ± 1.4 versus 9.0 ± 0.8 for the B(a)P-exposed brain-*Cpr*-null offspring. *Post hoc* analysis with the Bonferroni test failed to reveal a statistically significance difference at $P < 0.05$.

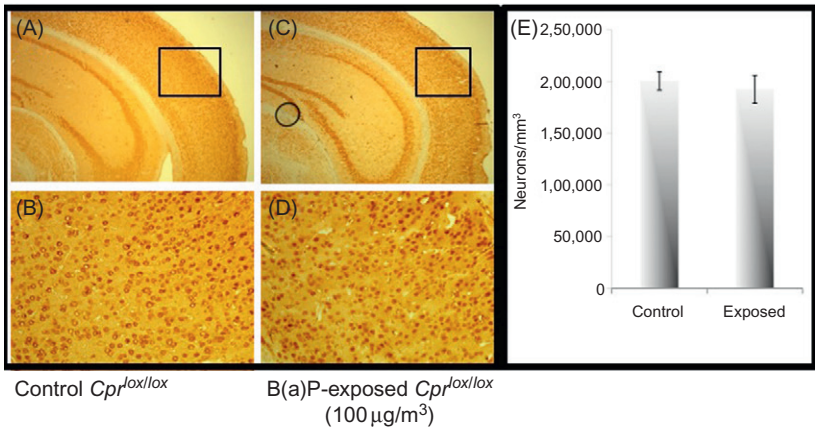

FIGURE 20.8 **Post-behavior characterization of neocortical cytoarchitecture.** Coronal sections of adult neocortical sections from *Cpr*$^{lox/lox}$ offspring were analyzed for cytoarchitecture by immunoshistochemical staining for NeuN. Neocortical neurons were counted in *Cpr*$^{lox/lox}$ offspring that had not been exposed to B(a)P *in utero* (A, low magnification; B, higher magnification) and compared to *Cpr*$^{lox/lox}$ offspring that were exposed to the B(a)P *in utero* (C, 4 × -low magnification; D, 20 × -high magnification). (E) It shows the plot resulting from stereological analysis of neocortical neurons subsequent to application of a two-tailed *t*-test. As can be seen, no significant differences in the density of neocortical neurons is observed as a result of *in utero* exposure to B(a)P aerosol.

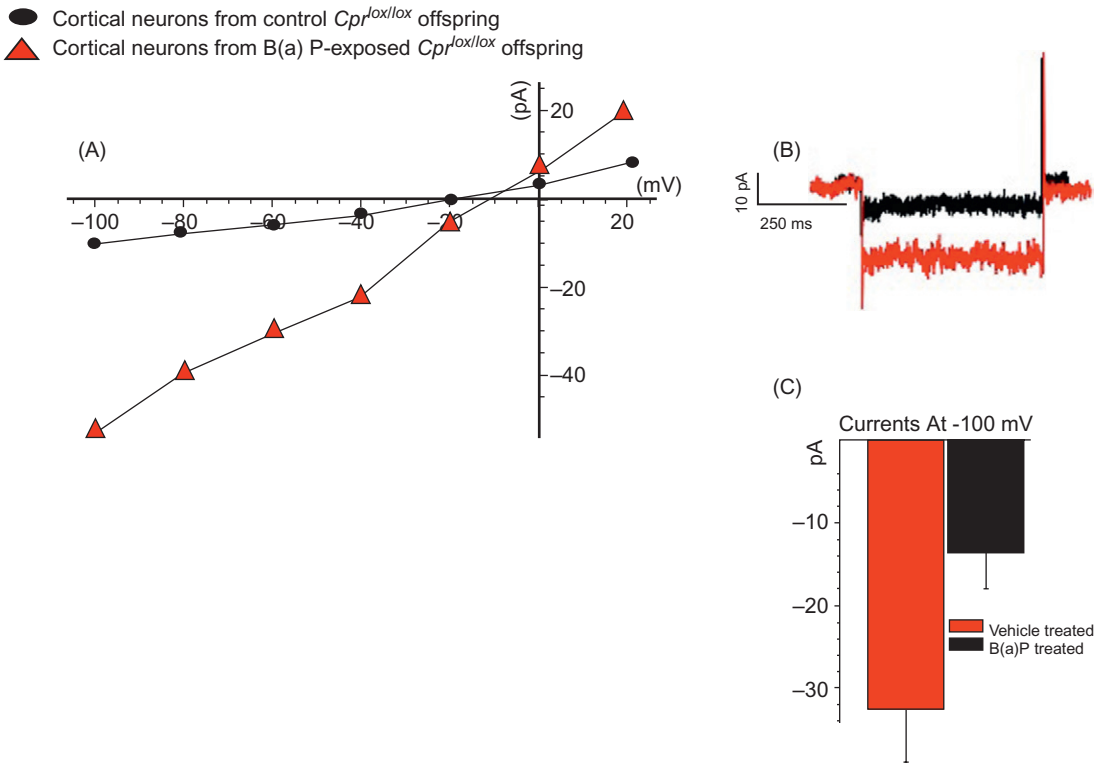

FIGURE 20.9 **B(a)P aerosol exposure (100 µg/m^3) induces a voltage-dependent decrease in the inward currents of cortical neurons derived from B(a)P-exposed *Cpr*$^{lox/lox}$ offspring.** Cortical neurons were voltage clamped using whole-cell configuration in a Mg^{2+} free external solution. Current–voltage relations were generated using a voltage step (1 s) protocol ranging from −80 to 20 mV separated by 20 mV from a holding potential of −60 mV. The experimenter was blind to the type of treatment. (A) Representative *I–V* currents for cortical neurons derived from carbon black and 100 µg/m^3 B(a)P aerosol–exposed *Cpr*$^{lox/lox}$ offspring. There is a voltage-dependent decrease in the magnitude of inward currents at negative membrane potentials in the cortical neurons derived from 100 µg/m^3 B(a)P-exposed *Cpr*$^{lox/lox}$ offspring. (B) Representative current traces obtained from carbon black control and cortical neurons derived from 100 µg/m^3 B(a)P aerosol–exposed offspring at −80 mV using the same experimental configuration as in (A). (C) Bar graph shows pA inward current recorded at −100 mV in control and B(a)P-exposed cortical neurons ($t = -2.92789$, $P < 0.0429$, $n = 3–5$).

improved mitochondrial capacity. Such an effect has already been noted in response to severe hyperglycemia and hyperinsulinemia. Also, no studies are available on the effect of B(a)P on the NRF2 transcription factor, a master switch that regulates an antioxidant pathway. We may find that the susceptibility-exposure paradigm inherent to the present study causes up-regulation of several or all antioxidant genes in response to Nrf2. Thus, despite dysregulation of glutamate homeostasis and ROS production, gross morphological alterations may be absent. Furthermore, it should be considered that the lack of change in total cell numbers would not take into consideration a more subtle change in cellular migration patterns and synaptogenesis in $Cpr^{lox/lox}$ offspring exposed *in utero* to a beno(a)pyrene aerosol.

The present study demonstrates that *in utero* exposure to a B(a)P aerosol in a *Cpr* experimental model system results in a temporal modification of upstream Sp4 transcription factor protein expression (Figure 20.5) during a time when synapses are forming for the first time. It is likely that this response has negative effects downstream as follows: (i) Sp4 target gene–subunit ratio protein expression (NR2A, Figure 20.5); (ii) homeostatic glutamate neurotransmitter concentrations (Figure 20.4); (iii) novel object discrimination phenotype (Figure 20.6); and (iv) the magnitude of inward currents in cortical neurons (Figure 20.9). The latter finding presents the potential for pharmacological augmentation of NR2A-mediated currents at cortical synapses as a means of modulating evoked activity in a structure-specific manner. The basal glutamate concentrations obtained in our *Cpr* experimental model system are identical to recent reports in the literature for dorsal hippocampus in a 129/SvEv mice KATII KO model and for the PFC in a Wistar rat ethanol model. These findings are consistent with the concept that increases in glutamate concentration promote alteration of inward currents via NMDA receptor expression. Whether these elevated concentrations of glutamate result from modulation in the activities of vesicular, glial, or astrocyte transporters (VGluT, GLT, or GLAST) remains to be seen.

That the mPFC integrity is, in part, important for the expression of object discrimination memory is supported by several studies where hippocampal lesions per se were found not to impair object discrimination (i.e., the ability to recognize a familiar object and discriminate it from a novel object). These studies would suggest that the hippocampus is not totally required for the type of discrimination utilized in the present study. We agree that there are examples in the literature demonstrating that hippocampal lesions can impair object discrimination when the memory for the sample object includes spatial information. This is to say that when an object is presented in a complex environment with many visual and tactile cues, it appears less salient to the animal and therefore can be encoded by the hippocampus as part of this "complex" environment. Conversely, when an object is placed in an impoverished environment, it might appear highly salient and therefore can be encoded by the perirhinal cortex (for example), separate from the environment. The data from these studies may facilitate a discussion regarding the currently accepted hypotheses as to why rodents with hippocampal lesions may or may not be impaired in the object discrimination paradigm under complex but not impoverished environment conditions.

Other pharmacological studies have investigated the role of the parahippocampal glutamate receptor within the context of performance on the object discrimination task, and those results support our findings. The parahippocampal region is located dorsal to the hippocampal formation, and the evidence argues against a role of hippocampal NMDA receptors in object discrimination. In one study, intraseptal infusions of a low (0.4 mg) dose of the NMDA receptor antagonist AP-5 (D,L-2-amino-5-phosphono-pentanoic acid) improved object discrimination at a delay of 24 h, but not 45 min in rats when the dose was given either prior to or after the sample, or prior to the test trial. Another study reported that a subcutaneous (s.c.) injection of kynurenic or 5,7-dichlorokynurenic acid (antagonists at the glycine-site of the NMDA receptor) given before the sample trial in doses of 0.6 or 30 mg/kg also improved one-trial object recognition in rats at a 1 h delay. These studies argue against a crucial role for the hippocampal NMDA receptor, thereby diminishing the necessity of performing of *in vivo* microdialysis to delineate a role for glutamate in hippocampal formations within the context of performance of the object discrimination task.

It has been previously demonstrated that subacute exposure to B(a)P (0–200 mg/kg) (1 intraperitoneal injection per day for 10 days) in adult mice modulated gene expression of NMDA-NR1 subunits in brain regions highly involved in cognitive function (Grova et al., 2007). Subacute exposure to B(a)P seemed to differentially affect NMDA-R1 expression in different parts of the brain. Cerebral regions, including the temporal cortex, showed no change in expression regardless of the B(a)P dose administered. In the hippocampus, exposure to B(a)P led to a 17-fold increase in a dose-dependent manner. In the frontal cortex, mRNA expression decreased 4–35 times with increasing doses of B(a)P. The results from these subacute studies in adult mice at relatively high doses in comparison to the present study suggest a link between *in utero* exposure to B(a)P aerosol, expression of functional obligatory NMDA-R1 mRNA, and impairments in short-term and spatial memory.

We know that postnatal brain development requires experience-dependent input that can induce the release of glutamate and thereby promote critical aspects of synaptic

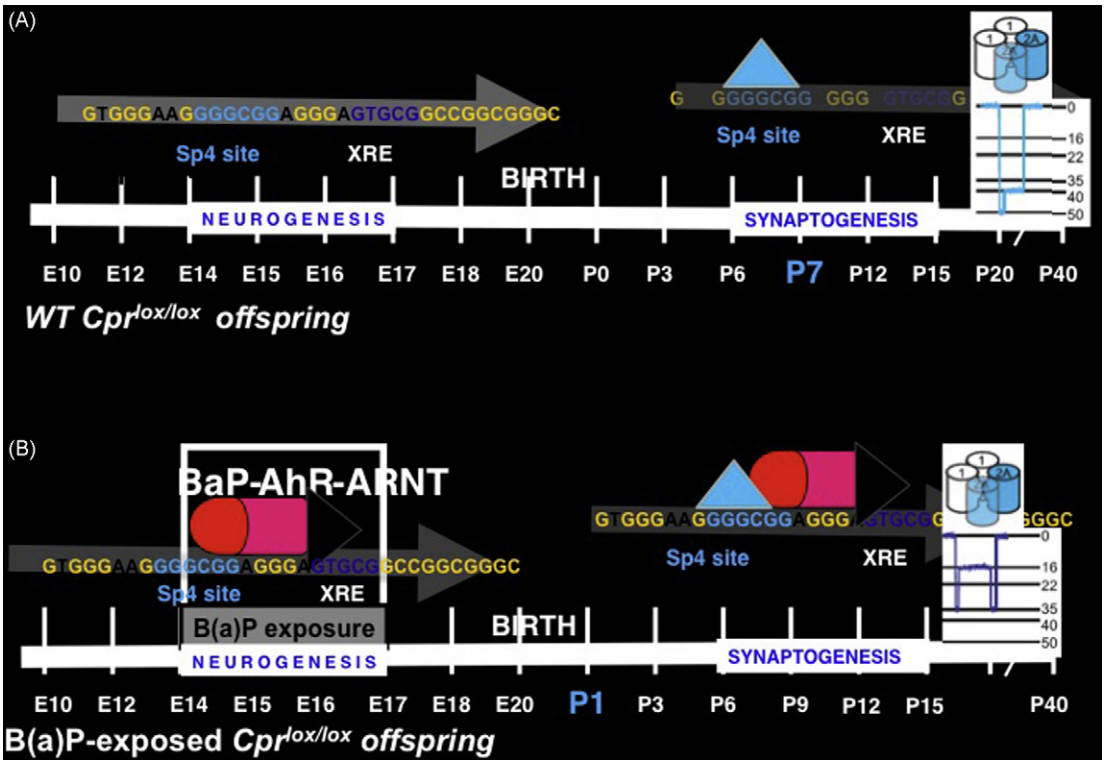

FIGURE 20.10 (A) Normal glutamate and NMDA-NR2A homeostasis. (B) Dysregulated (elevated) cortical glutamate and NMDA-NR2A homeostasis. (A) Normal temporal activation of Sp4 expression and of its target genes during embryonic development in timed-pregnant control $Cpr^{lox/lox}$ dams is depicted from E10-E20 (birth). During the early postnatal period, the Sp4 target gene-NR2A facilitates in establishing constitutive NMDA-NR2A-driven cortical currents. This occurs during the *critical period of synapse formation* (P7-P15) and contributes to a normal object recognition/discrimination phenotype in $Cpr^{lox/lox}$ offspring. (B) Premature activation of Sp4 expression and of its target gene, NR2A occurs subsequent to *in utero* exposure of timed-pregnant $Cpr^{lox/lox}$ dams to B(a)P in aerosol. During the early postnatal period in exposed $Cpr^{lox/lox}$ offspring, dysregulated Sp4 and target gene expression results in upregulated NMDA-NR2A-driven cortical currents. This occurs during the *critical period of synapse formation* when NR2A-NR2B subunit ratios are altered (P7-P15) thus contributing to an impaired object recognition/discrimination phenotype in exposed $Cpr^{lox/lox}$ offspring.

maturation. It is during this process of postnatal synaptogenesis that the effects of *in utero* B(a)P exposure on neural activity are most likely to alter the expression of genes, each with its unique temporal expression profile. In neurons of the neonatal brain, NR2A mRNA progressively increases during development and is dependent upon synaptic activity (Cull-Candy et al., 2001). In sensory pathways, Philpot et al. (2001) showed that the developmental shift from NR2B to NR2A can be postponed by sensory deprivation (Philpot et al., 2001). Studies such as these have predicted whether an alteration in the biophysical or molecular properties of NMDA receptors (NMDARs) from insertion of NR2A, loss of the NR2B, or both, places upper-limit constraints on the length of the critical period with respect to neural activity and experience-dependent fine-tuning of certain circuits.

A recent study by Zhou et al. (2007) reported on postnatal development of the hippocampus in the complete absence of the Sp4 gene. Notable observations were that the dentate granule cell precursors appeared to divide less during postnatal development in Sp4 *null* mice. Dentate granule cells from Sp4 *null* mice displayed less dendritic growth and arborization than those from wild-type mice placed into primary neuronal cultures. Additionally, Sp4 *null* mutant adult mice displayed both decreased neuronal cell density in the dentate granule layer and presented with a smaller dentate gyrus. The dentate gyrus is the primary gateway for hippocampal trisynaptic circuits to process the information received from the entorhinal cortex. The overall conclusion from this Sp4 *null* report is that abnormalities in circuitry exist in the *null* mouse characterized by a reduced hippocampal volume, representing a significant risk factor for some neurobehavioral deficit disorders. Collectively, results from genome-wide analyses predict an overlap of Sp protein transcription factor family members with the AhR network, and the Grin family of ionotropic glutamate receptor subunits (e.g., NR2A). Earlier studies have reported that knockdown of the transcription factor Sp4 in mice leads to increased numbers of highly branched dendrites during the maturation of cortical neurons in primary neuronal cultures (Ramos et al.,

2007). The results from this report suggest that the Sp4 transcription factor likely controls dendritic patterning during development by limiting branch formation and by promoting activity-dependent pruning during a time when synapses are forming for the first time.

The signaling events that regulate deactivation kinetics for the establishment of fast synapses are preceded by changes in the subunit composition of the NMDA receptor at the synapse. This occurs during the postnatal critical switch period and is represented by the preweaning, postnatal period from P1–P14. Results from a study by Brigman et al. (2008) illustrate the principle that NMDA receptor subunit proteins mediate certain forms of synaptic plasticity and learning. A touch-screen system was used to assess spatial discrimination learning in an NR2A subunit protein knockout (KO) mouse (Brigman et al., 2008). The study found that NR2A KO mice exhibited a significantly retarded discrimination learning pattern, supporting the currently accepted hypothesis that relative increases in the NR2A subunit protein during development ultimately serve to stabilize memories by constraining excessive synaptic plasticity.

Alternatively, NR2B-containing NMDARs appear to be the dominant form found during development, and their activity can initiate anatomical and functional plasticity, including LTP (Feldman and Knudsen, 1998). Paradoxically, NR2B does not contain GC-box elements within the 5′ promoter region, suggesting a potential mechanism for regulation of NR2A by the Sp4 transcription factor during the time when synapses are developing (Figure 20.10). The rationale for the Sp4 transcription factor and its target genes as viable targets for B(a)P exposure–induced modulation during critical periods of development, is based on the identification of canonical xenobiotic responsive element consensus sequences and GC-box elements within the 5′ promoter region (Figure 20.11A). This is thought to render this gene and its target genes susceptible to modulation by B(a)P during critical phases of development. Figure 20.11B shows the 5′ promoter region of the GRIN2B (NR2B) gene, which contains a single Sp4 binding site (GC box) and no xenobiotic response element (XRE) sequence in its 5′ promoter. The NR2B gene, however, does have seven XRE sequences in its 5′UTR (Figure 20.11B). Due to the absence of canonical XRE sequences and multiple GC-box elements in the 5′ promoter region, NR2B would not be classified as an Sp4 target gene. Conversely, the GRIN2A gene (NR2A) contains two Sp4 binding sites (GC-boxes) and six XRE sequences in its 5′ promoter (three in a forward orientation and three in a reverse orientation) (Figure 20.11C). There are two additional XRE sequences in the ORF of GRIN2A (one forward and one reverse), as is shown in Figure 20.11C. The NR2A receptor subunit thus qualifies as a Sp4 target gene and has been reported as such (Liu et al., 2003).

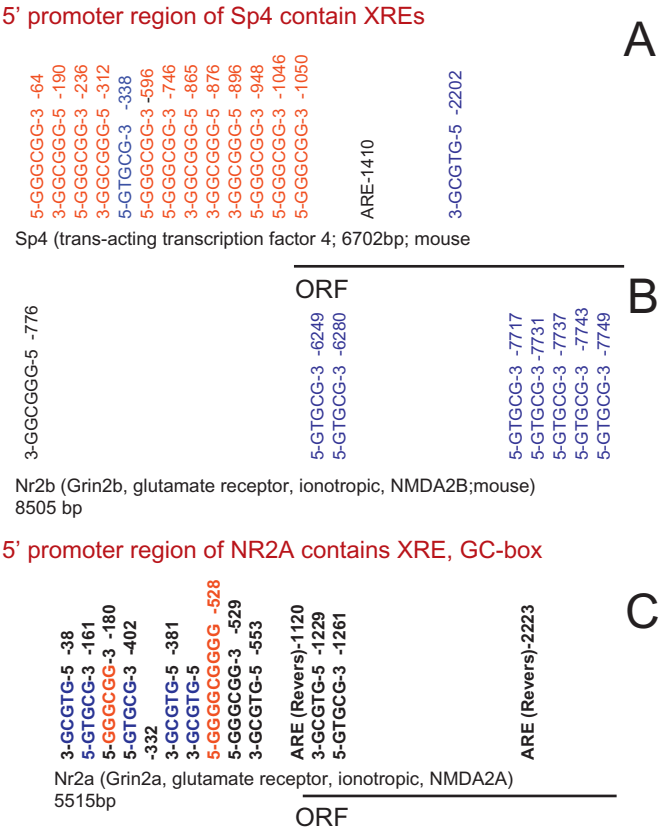

FIGURE 20.11 Promoter analyses for canonical XRE and GC-box consensus sequence requirements in Sp4 target gene versus nontarget genes. (A) Core Sp4 promoter; (B) Core GRIN2B (NR2B) promoter; (C) Core GRIN2A (NR2A) promoter. *Blue*—XRE, xenobiotic response element; *Red*—GC box/Sp4-binding site; *Black*—ARE, antioxidant response element.

Clearly, the novel object discrimination task is sensitive enough to detect deficits in response to object discrimination that are reflective of learning and memory impairments. PAH aerosol–induced negative modulation in the temporal developmental expression of Sp4 demonstrates a particular sensitivity to environmental exposure during a critical period of development. Studies in the immediate future will seek to elucidate the functional changes and mechanisms undergirding alterations in Sp4 target gene-driven neural activity (NMDA NR2A-mediated) and plasticity using our *Cpr*-null model. Translating these new concepts into animal studies offers the promise of advancing our ability to establish the presently absent mechanistic connections between exposure-induced diseased phenotypes, due to disturbances in temporal expression patterns during critical windows of development. In order for significant advances in the field to come to fruition, molecular-level hypotheses of *in utero* air pollution exposure effects on later-life phenotypes must continue to be investigated by multidisciplinary teams.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The research described in this chapter represents the contribution toward leading the development of novel methodology to systematically discover and develop therapeutic glutamate receptor/aryl hydrocarbon receptor antagonists that will mitigate the neurotoxicity associated with *in utero* exposure to B(a)P aerosol. A fundamental premise of future research takes into account the need for simultaneous temporal measurements, and integrative physiological analysis of critical signaling processes, to better understand the mechanisms of PAH exposure–induced neurotoxicity. Future experiments should also define the signatures of PAH exposure–induced neurotoxicity and novel therapeutics/protectants in experimental model systems.

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CHAPTER

21

Carbon Monoxide: From Public Health Risk to Painless Killer

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Carbon monoxide causes more health problems and death every year than any other poison or many together.

Lewin (1920) Translation from German, Pankow (2000)

INTRODUCTION

Colorless carbon monoxide (CO) is everywhere. Wherever there is combustion there is CO; it is the predominant product above 800°C. The concentration of CO might vary from 0.1 ppm in clean atmosphere to 5,000 ppm in the proximity of domestic wood fire chimneys (Fawcett et al., 1992) and is present in significant quantities in cigarette smoke (Hartridge, 1920; Hoffmann et al., 2001). The atmospheric lifetime of CO is 1–2 months, which allows its intercontinental transport (Akimoto, 2003).

The universal presence and unsuspecting inhalation of CO make it the most common source of poisoning in the modern world and the leading cause of unnatural death in the United States. However, CO is not on the list of chemicals used as chemical weapons in wars (Elsayed and Salem, 2006), although there are reports that the Nazis used CO gas against Jewish people during 1941 because CO was cheaper than bullets.

Mysterious illnesses, strange visions, and inexplicable deaths of entire households, generally attributed to haunted houses, are most likely due to malfunctioning chimneys, resulting in carbon monoxide poisoning. Even nonlethal concentrations of CO can exert dramatic effects in the presence of preexisting cardiac diseases. Continued exposure to relatively high, yet nonlethal, concentrations of CO can adversely affect a pregnant woman and her fetus. CO is a contributory factor in the adverse effects of smoking (United Nations Environment Program and WHO, 1979; Akrawi and Benumof, 1997); moreover, smokers occasionally set the entire home on fire, producing excessive quantities of CO (Sacks and Nelson, 1994).

CO is also endogenously generated. CO is a bit like other endogenous gases of great physiological and pathological significance such as O_2, NO, and hydrogen sulfide (H_2S). CO also shares some properties with hydrogen cyanide (HCN) in that both produce hypoxia.

Given the toxicological and epidemiological importance of CO, it has been the subject of several books and monographs since 1920 (Lewin, 1920; Hamilton, 1921; Nicloux, 1925; Drinker, 1938; Flandin and Guillemin, 1942; Von Oettingen, 1944; Grut, 1949; Raymond and Vallaud, 1950; Cooper, 1966; Bour and Ledingham, 1967; Gras, 1967; Bouletreau, 1970; Putz et al., 1976; Coburn et al., 1977; Buchwald et al., 1979; Tiunov and Kustov, 1980; Pankow, 1981; Shephard, 1983; Jain, 1990; Penny, 1996; IPCS, 1999; Mannaioni et al., 2006). CO has also been a subject of numerous reviews, some of which are referred to here (Beck, 1927; Killick, 1940; Lilienthal, 1950; Pugh, 1959; Root, 1965; Goldsmith and Landow, 1968; Fenn, 1970; Lawther, 1975; Winter and Miller, 1976; Morandi and Eisenbud, 1980; Thom and Keim, 1989; Penny, 1990; Ernst and Zibrak, 1998; Von Berg, 1999; Weaver, 1999; Blumenthal, 2001; Piantadosi, 2002; Gorman et al., 2003; Ryter and Otterbein, 2004; Kao and Nanagas, 2006; McGrath, 2006; Raub et al., 2006; Prockop and Chichkova, 2007).

During the past 20 years, however, the attention of researchers has shifted from the toxicity of CO to its physiological role (Snyder and Ferris, 2000; Wu and Wang, 2005). As a result, the toxicity of CO is sometimes not mentioned as much as it should be. For example, the

Handbook of Toxicology of Chemical Warfare Agents.
DOI: http://dx.doi.org/10.1016/B978-0-12-800159-2.00021-X

first edition of the textbook *The Pharmacological Basis of Therapeutics* by Louis Goodman and Alfred Gilman published in 1941 (MacMillan, New York) devoted five and one-quarter pages to carbon monoxide; the 11th edition of the same book edited by L.L. Brunton, J.H. Lazo, and K.L. Parker and published in 2006 (McGraw-Hill) mentions nothing under the heading of carbon monoxide; the term "carbon monoxide" appears only in relation to the therapeutic uses of oxygen. Similarly, the *Pharmacological Reviews* had the subject reviewed twice—first in 1950 (Lilienthal, 1950), when CO was known for its toxicity, and then 55 years later (Wu and Wang, 2005), but only dealing with its physiological role.

The pharmacology of CO spans a broad spectrum, from public health risk to endogenous modulator of important biochemical events to lethal poison. Given the theme of this book, this chapter mainly focuses on the toxicology of CO. However, for the sake of completeness, physiological roles of endogenous CO are also presented. CO relates to all forms of life; however, its roles in microorganisms (Frankland, 1927) and plants (Wilks, 1959; IPCS, 1999; Muramoto et al., 2002; K. Liu et al., 2007; Han et al., 2008) are not covered in this chapter.

HISTORICAL BACKGROUND

An adequate amount was known about carbon monoxide at the beginning of the twentieth century. Haldane (1922) devoted 14 pages to the history of carbon monoxide in his book titled *Respiration*; it occupied almost the same number of pages in the second edition 13 years later (Haldane and Priestley, 1935). An excellent description of the history of CO is contained in an article by Pankow (2000). Because most of the pioneering work regarding CO was performed in Germany, and because Pankow is based in Halle, Germany, he has been able to do justice to the celebrated first book about CO by Lewin (1920) and other related literature. There is another book called *Carbon Monoxide: The Silent Killer* by the University of Toronto Professor Roy J. Shephard. The title contains every ingredient of the history and toxicology of this mysterious poison—not invented by humans but made by nature itself, like arsenic or cobra venom.

CO has always been a part of the universe. However, atmospheric CO has increased over time. When volcanoes erupted, continents collided, and winds embraced the trees sparking fires millions of years ago, all this contributed to the stock of CO. However, when CO first made a significant presence in the air we breathe, humans lived in the open. A very long time must have passed by before humans inhabited caves or built enclosures for protection from the effects of the weather or the tyranny of predators. In the process, however, humankind invited the unwanted guest—carbon monoxide, the silent killer. So where there is smoke, there is not only fire but also CO; in terms of human cost, the latter is more dangerous than the former. However, it must have taken several thousands of years to tame the fire, and over those years CO has claimed many innocent victims who went to sleep after a hearty meal, never to wake up. The knowledge of these mysterious events has been unraveled over time.

Just as concoctions of cinchona, bark of willow, and leaves of the common foxglove plant were used during ancient times without knowing that they contained, respectively, quinine, aspirin, or digoxin, so is the story of CO. The toxicity of CO was recorded by Aristotle (384–322 BC) in the third century BC; by the first century BC, charcoal fumes were used for suicide and executions without any knowledge of the exact nature of the killer (Lewin, 1920; Shephard, 1983). Byzantine emperor Julian the Apostate and his successor, Jovian, were poisoned in AD 363 and 364, respectively, because coal was used to heat their braziers (Lascaratos and Marketos, 1998).

The industrial revolution in Britain and Europe and the discovery of the New World introduced coal for domestic heating and cooking. In 1700, Bernardino Ramazzini (1633–1714), a physician and professor at the University of Modena, published the book *De Morbis Arteficium Diatriba* (*Diseases of Workers*), which was translated from the original Latin text into Italian, French, German, English, and Dutch. Ramazzini recognized that fumes from burning coal by confectioners caused headache and dyspnea, and miners encountered "noxious vapors;" burning coals, unless vented out, could "kill a man on the instant" (Shephard, 1983; Pankow, 2000).

In their review, Prockop and Chichkova (2007) wrote "CO was first prepared by the French chemist de Lassone in 1716. Because it burned with a blue flame, de Lassone thought it to be hydrogen." In his book *Carbon Monoxide: The Silent Killer* (page 7), Shephard (1983) attributes this episode to the illustrious British chemist Joseph Priestley (1733–1804) who mistook this "noxious vapor" for hydrogen for the same reason—it burned with a bright blue flame. However, both Prockop and Chichkova (2007) and Shephard (1983) agree that the identity of this noxious vapor was evaded until Cruikshank demonstrated that the gas that burned with the blue flame was an oxide of carbon (CO) that could be converted into a dioxide of carbon (CO_2) by exploding it with oxygen.

Because the cause of CO poisoning that often resulted in death was unknown, myths emerged. Friedrich Hoffmann (1660–1742) of Halle, Germany, recounts that two farmers and a student used charcoal fire on a Christmas night. The farmers died and the student was found very sick; theologians, who claim to possess great knowledge, opined that it was a divine curse (Pankow, 2000).

French physiologist Claude Bernard (1857) was perhaps the first to describe the toxicity of carbon monoxide. In 1846, he forced a dog to inhale CO; of course, the dog

died and on autopsy he noted that the blood was crimson in color in all the heart chambers as well as in the veins (Bernard, 1865). Claude Bernard correctly assumed that the crimson color was due to excess of oxygen in the blood. It was left to the celebrated British physiologist J.S. Haldane (1860–1936), to whom this chapter is dedicated, to identify carboxyhemoglobin (COHb) and determine its chemical nature (Haldane, 1895a).

By the end of the nineteenth century, Haldane had not only described the effects of exposure to various concentrations of CO on himself and mice but also discovered that the symptoms of CO poisoning could be alleviated by oxygen (Haldane, 1895a; Haldane and Priestley, 1935), which remains the most effective antidote against CO toxicity to date (Sluijter, 1967; Tibbles and Edelsberg, 1996). Haldane (1895a) also noted that "symptoms of poisoning occur far more rapidly in mice than in man [himself]" because the relationship of the body mass to surface area is such that the mouse "produces [CO] in an hour 10 grams per kilo body weight, while man produces only about half a gramme." Haldane was the first to use mice for the detection of CO in mines until canaries took over this service in 1921. By 1987, chemical methods for the detection of CO replaced the fate of the canaries.

Haldane worked out the physical chemistry of the CO–hemoglobin interaction. His first law states: "When a solution containing hemoglobin is saturated with a gas mixture containing O_2 and CO the relative proportions of the haemoglobin which enters into combination with the two gases are proportional to the relative partial pressures of the two gases, allowing for the fact that the affinity of CO for hemoglobin is about 3 times greater than that of O_2." This law is valid today; however, Haldane underestimated the affinity of CO for hemoglobin.

EPIDEMIOLOGICAL CONSIDERATIONS

Morbidity and mortality from CO poisoning vary from country to country because of the different standards of surveillance, extent of urbanization, housing conditions, source of energy, and so on. The major source of CO in the modern era is from the burning of petrol gas. Naturally, megacities with a population of more than 10 million have a much higher level of atmospheric CO than smaller cities and rural areas.

Demography is changing rapidly in many countries, especially in the two countries with populations of more than one billion each—China and India. With surging economies in both countries, the number of automobiles is fast increasing. In China, for example, the number of automobiles has been increasing at a rate of 13% per year since the 1980s; there were 18–21 million automobiles in China in 2000 compared with 11 million barely 3 years earlier in 1997. It is estimated that 3,000 kg of CO is discharged from every 1,000 automobiles each day (Chen and Wang, 2000).

In most countries, CO accounts for the maximum number of unintentional deaths in peace time. The estimated annual number of emergency admissions due to unintentional CO poisoning is approximately 40,000 in the United States (Weaver, 1999), 5,000–8,000 in France (Annane et al., 2001), 6,000 in Italy (Gandini et al., 2005), and 523 in Poland (Sokal and Pach, 2000). Annual unintentional deaths from CO poisoning are approximately 600 in the United States (Cobb and Etzel, 1991) and 1,500 in the United Kingdom (Hamilton-Farrel and Henry, 2000).

Atmospheric CO is related to human civilization; consequently, its concentration varies markedly from east to west and from south to north. Also, CO in the atmosphere varies greatly within different regions of the same country, denoting uneven industrialization. To highlight these variables, we deal with this subject under two separate headings—the external source and the endogenous source of CO. Details can be found in an excellent article by Vreman et al. (2000).

It is common knowledge that death due to CO poisoning is painless. The generation of CO in an enclosed space is also well-known. Consequently, CO poisoning has emerged as the method of choice for suicides in favor of hanging and firearms.

TOXICOKINETICS AND TOXICODYNAMICS

Sources of CO

There are two main sources of CO, exogenous and endogenous. Although the atmospheric CO is the principal cause of CO toxicity, the endogenous source is physiologically very important (Marks et al., 1991; Maines, 1997; Wu and Wang, 2005) and, under certain conditions, may even become pathological (Nezhat et al., 1996).

External Sources of CO

Carbon monoxide is a product of incomplete combustion as encountered in the operation of vehicles, heating, coal power generation, and biomass burning (Godish, 2003). Natural geographical events such as volcanic eruptions, emission of natural gases, degradation of vegetation and animals, and forest fires all contribute to atmospheric CO. Approximately 40% of global CO comes from these natural sources. Human intervention such as fossil fuel consumption, garbage disposal, tobacco smoke, and charcoal fires contribute to the remaining 60% of global CO (Jain, 1990; Vreman

et al., 2000). Because human activity and density differ from place to place because of socioeconomic factors, atmospheric CO varies greatly from place to place. CO emission in the United States in 2001 was 120.8 million short tons, of which 74.8 million came from on-road vehicles (McGrath, 2006).

Apart from various other changes, the developing countries are characterized by increasing migration of rural population to slums and shanty towns on the outskirts of cities like São Paulo, Mexico, Johannesburg, Mumbai, Shanghai, and others; this is associated with, among other things, an increase in atmospheric CO. Fortunately, atmospheric CO has not exceeded safety levels globally or in any specific areas, including, for example, Mexico City and Los Angeles, but it can. It is reassuring that many efforts are being made by government agencies to reduce CO emissions.

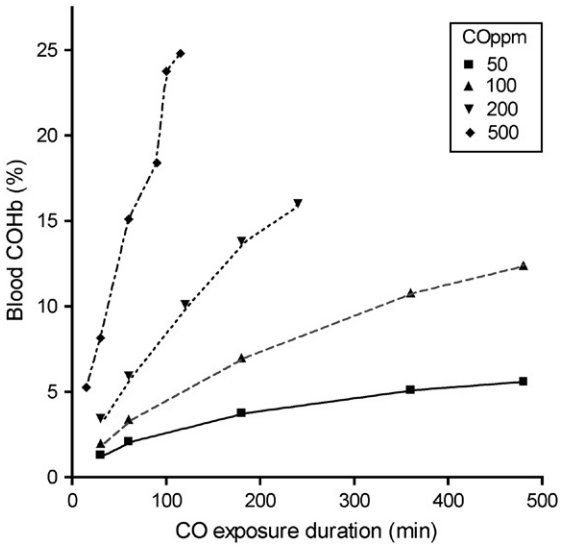

FIGURE 21.1 Relationship between the duration of exposure to different concentrations of carbon monoxide and blood carboxyhemoglobin (COHb) in healthy volunteers. The plot is based on the data of Stewart and Peterson (1970).

Endogenous Sources of CO

The knowledge that CO is normally present in the body dates back to 1894, when Grehant (1894) detected a combustible gas in blood. By 1898, this combustible gas was suspected to be carbon monoxide (De Saint-Martin, 1898; Nicloux, 1898). At the time, methods did not exist to ascertain if the CO in the blood was generated inside the body or if it was derived from the air. It was not until 1949 that the evidence for endogenous production of CO was firmly established (Sjorstrand, 1949). Tenhunen et al. (1968, 1969, 1970) elaborated on the role of heme oxygenase in the generation of CO.

The major source of endogenous CO in a healthy individual is from the degradation of heme by heme oxygenase (HO)—HO-1 and HO-2. The enzyme HO-1 is inducible and HO-2 is constitutive; heme oxygenase degrades heme into CO and biliverdin, and the latter is rapidly converted into bilirubin (Coburn et al., 1963, 1967; Coburn, 1979; Mores and Sethi, 2002). Current literature emphasizes only a physiological role for endogenously generated CO (Choi and Otterbein, 2002; Boehning and Snyder, 2003; Ryter and Otterben, 2004; Wu and Wang, 2005; Mannaioni et al., 2006), although it is quite likely that it could add to the toxicity of inhaled CO.

Heme oxygenase is the rate-limiting step in the production of CO and its activities account for 86% of endogenous CO production. The remaining 14% is derived from nonheme sources. The lifespan of red blood cells is approximately 120 days. The older the erythrocyte, the greater is its CO output. In neonates, red blood cells have a shorter lifespan and, relative to erythrocytes of adults, they produce two- to three-times more CO (Fallstrom, 1968). HO-2 is activated during neuronal stimulation by phosphorylation by the enzyme CK2 (Boehning et al., 2003).

The rate of COHb formation is a function of inhaled CO concentration and duration (Figure 21.1). The rate of CO production and excretion parallels the rate of bilirubin production; hence, a measurement of COHb serves as a measure of heme degradation and bilirubin production. A measurement of end-tidal CO in breath corrected for inhaled CO is used as a measure of assessing infants at risk for severe hyperbilirubinemia because CO and bilirubin are produced in equimolar amounts (Bartoletti et al., 1979).

A small fraction (20%) of endogenous CO is derived from other hemoproteins such as myoglobin and many other iron-containing enzymes (Coburn, 1970; Vreman et al., 2000). This serves as an example of the use of endogenous CO monitoring for diagnostic purposes.

In addition to the major exogenous and endogenous sources of CO described, certain medical procedures within the body can also generate CO. For example, laser and bipolar electrocautery during laparoscopy can generate more than 200 ppm CO in the body cavity, which finds its way into the circulation, increasing COHb levels (Wu et al., 1998) sufficient to induce toxicity (Nezhat et al., 1996). Anesthetic machines equipped with drying material like soda lime or barium hydroxide were found to generate CO (Moon et al., 1992; Baum et al., 1995) from several anesthetic agents (Fang et al., 1995). Likewise pulmonary function tests based on determining the CO diffusion capacity as a means of determining the alveolar-capillary diffusion capacity for gases can also elevate CO and, in turn, COHb (Vreman et al., 2000).

TABLE 21.1 Physicochemical Properties of Carbon Monoxide

Property	Description
Chemical structure	
Molecular weight	28.01
Critical point	−140°C at 3,495 kPa
Melting point	−199°C
Boiling point	−191.5°C
Fundamental vibration transition	2143.3 cm^{-1} (4.67 μm)
Density at 25°C, 101.3 kPa	1.145 g/L
Specific gravity relative to air	0.967
Solubility in water at 0°C	35.4 mL/L
Solubility in water at 25°C	21.4 mL/L
Explosive limit in air	12.5–74.2%

Physicochemical Properties of CO

CO is a colorless, odorless, tasteless, noncorrosive, stable diatomic molecule in the atmosphere. Unlike hydrogen peroxide (H_2O_2) or superoxide ($O^{\bullet}$), CO is not a radical. CO has low water solubility and negligible quantities dissolve in blood at normally encountered pressure (Shephard, 1983). CO has a shorter inter-atomic distance (~1.13 Å) than would be anticipated for a single bond. It has high-heat formation from the constituent atoms (bond strength 2.07 MJ/mol) and the electric charge is distributed rather uniformly between the carbon and oxygen atoms. Pauling (1960) postulated that under normal circumstances CO existed as a hybrid containing approximately equal proportions of three structures. According to the modern atomic theory, carbon atoms have the first shell filled with two electrons in S orbitals, whereas the second shell contains two electrons in the S orbital and two in the P orbital (Shephard, 1983). The general properties of CO are presented in Table 21.1.

Methods for CO Measurement

Appropriate techniques exist for the quantitation of CO in gaseous samples and tissue samples. In either case, caution needs to be exercised to ensure that CO concentration is not altered by interaction with the sample vessel during storage and transport. For example, Vacutainer tubes were found to greatly alter CO levels when used for storage of blood samples (Vreman et al., 1984). Furthermore, routine monitoring of air for public health requires a simple and yet reliable sample collecting system. Ambient CO levels at the workplace can be very low, requiring sensitive analytical techniques. With the recognition of the hazardous nature of CO and its almost universal presence, several sophisticated methods for quantifying CO have been developed (IPCS, 1999; Vreman et al., 2000).

Measurement of Blood CO

Haldane (1895b) wrote: "In view of the very poisonous nature of carbonic oxide, and the comparative frequency with which cases of poisoning by this gas occur, much attention has been given to its detection and estimation…The method now to be described is very simple, and depends on the fact that when a hemoglobin solution is well shaken with air containing carbonic oxide and the proportion of hemoglobin, which finally combines with carbonic oxide, varies with the percentage of carbonic oxide in the air. By determining colorimetrically the proportion of the hemoglobin which has combined with the carbonic oxide it is thus possible to infer the percentage of carbonic acid in the air."

"The bottle containing the blood solution and sample air must be covered with a cloth during the process of shaking, and (although this is by no means so important) very bright light should be avoided during the process of titration with the carmine solution" (Haldane, 1896).

The currently used method of quantifying blood CO and the precaution that is needed for accuracy are simply refinements of the method described by Haldane (1895b, 1896) more than a century ago; colorimetric detection was soon replaced by the spectroscopic detection method (Hartridge, 1912) and has undergone further sophistication since then. Both gas chromatography and spectrophotometry are considered appropriate, although the former is favored (Coburn et al., 1964; Collison et al., 1968; Vreman et al., 1984, 1998; Kane, 1985; Constantino et al., 1986). However, blood CO is still measured by determining COHb levels, which are normally undetectable but might be involved in the transfer of CO between cells. The measurement of COHb is useful not only in the quantification of CO but also to detect neonatal hemolysis (Necheles et al., 1976; Ostrander et al., 1982).

COHb is quite stable and its concentration does not change over a long period (up to 6 months) if the blood sample is stored in the dark and under sterile conditions. Blood levels of COHb are not expected to exceed 5% at ambient levels of CO. IPCS (1999) focuses on methods that can accurately measure COHb less than 10%. A method that simply requires finger-prick blood is convenient for mass screening and is described in detail by Commins and Lawther (1965). In this method, the sample is diluted in ammonia solution, which is divided into two parts; from one of these, CO is displaced by oxygen and the COHb-containing part is placed in the sample beam of a spectrophotometer, so that the instrument records the difference between the absorbance of COHb and oxyhemoglobin.

Several techniques for measuring COHb exist (IPCS, 1999). The sensitive techniques require the release of CO from hemoglobin into a gas phase. CO can then be detected directly by a number of methods such as infrared absorption, difference in thermal conductivity between CO and the carrier gas, amount of ionization after conversion of CO to methane, or the release of mercury vapor resulting from interaction of CO with mercuric oxide.

The conventional method of expressing CO in blood samples is as percent COHb, and it is determined by a formula where CO content is the CO concentration in mL/100 mL blood, Hb is hemoglobin in g/100 mL blood, and 1.389 is the combining capacity of CO for Hb in mL of CO per gram Hb.

The blood level of COHb as a percent of total hemoglobin is directly related to the exposure duration at any concentration of CO (Figure 21.1).

Blood COHb measurements have been used to monitor exposure of populations to atmospheric CO, which also functions as a measure of air pollution. Using this approach, New Hampshire, Vermont, and the city of St Louis (Missouri) were found to have blood COHb levels in the range 1–1.5% in the early 1970s (Davis and Ganter, 1974; Kahn et al., 1974).

Ambient Air CO

Because CO concentrations in ambient air and at workplaces are usually quite low, reliable methods for sample collection and transport as well as highly sensitive methods for measurement are needed (Smith and Nelson, 1973; IPCS, 1999). The sampling method recommended by WHO (IPCS, 1999) comprises a sample introduction system consisting of a sampling probe, an intake manifold, tubing, and air remover. Known gas concentration is periodically collected to verify the method.

According to IPCS (1999), the analyzer system consists of an analyzer as well as sample preconditioning components fitted with a moisture-control system such as the nondispersive infrared (NDIR) analyzer. The infrared absorption near 4.6 μm, characteristic of CO, is used to measure its concentration. The most sensitive analyzers can detect CO concentrations as low as 0.05 mg/m^3 (0.044 ppm). The NDIR analyzer designed by Luft (1962) is considered appropriate because it is little affected by flow rate, requires no wet chemicals, has a short response time, and is sensitive over wide concentration ranges.

Other analyzers include gas chromatography, which is a sensitive, automated, and semi-continuous technique in which CO is separated from water, CO_2, and hydrocarbons (other than methane) by a stripper column and CO is passed through a catalytic reduction tube where it is converted to methane. The converted CO is passed through a flame ionization detector; its sensitivity range is 0.026–43.7 ppm (IPCS, 1999). Other methods such as small personal exposure monitors can measure CO concentrations on a continuous basis and store data on internal digital memories (Ott et al., 1986).

Home Detectors

Residential CO detectors are designed like smoke detectors and provide protection from excessive CO concentrations inside homes by sounding alarms. They are based on an interactive-type sensor, such as tin oxide, artificial Hb that relies on an interaction between CO, and the sensitive element to generate an alarm. The alternate current–powered home detectors have a metallic sensor that reacts with CO. The battery-powered ones have a chemically treated gel that darkens on exposure to CO. They are designed to sound the alarm within 90 min at CO concentrations of 100 ppm, within 35 min at 200 ppm, or within 15 min at 400 ppm (IPCS, 1999). CO concentration of 400 ppm can increase COHb to 10%, and CO toxicity is observed at levels higher than 10%.

CO in Expired Breath

Measurement of CO in the expired breath is based on the assumption that CO in alveolar air is in equilibrium with the partial pressure of CO in blood, which, in turn, is in equilibrium with CO bound to Hb, that is COHb (Douglas et al., 1912).

Absorption, Distribution, and Elimination of CO

Atmospheric carbon monoxide can travel thousands of kilometers from its source and has a half-life of 1–2 months (Akimoto, 2003). Although the biological effects of CO differ depending on whether it is inhaled or endogenously produced, the ultimate fate of CO is the same regardless of its source.

Exogenous CO reaches the body solely by pulmonary absorption. Once inhaled, CO combines reversibly with hemoglobin and, to a smaller extent, with myoglobin and other iron-containing macromolecules. Because most of the CO in the body is bound to hemoglobin, the relative affinities of CO and oxygen for hemoglobin are of critical significance in terms of both its toxicity and excretion. This relationship was first described by Haldane (1922) in what is known as Haldane's first law, represented by an equation where M is the affinity constant, thought to be between 208 and 245 at 37°C, and PCO and PO_2 are the partial pressures of CO and oxygen, respectively.

Haldane had conducted the experiments on himself and found that he needed to inhale 0.07% CO in the air containing 20.9% oxygen for Hb to achieve 50% oxygen and 50% CO saturation. On this basis, he calculated the relative affinities of CO and oxygen for Hb as 1.299. Although more refined techniques have estimated the affinity of CO for Hb to be much higher (Sendroy

and Liu, 1930; Roughton and Darling, 1944; Ernst and Zibrak, 1998), the fundamental relationship proposed by Haldane (1922) remains valid and has been discussed in detail by others (Forster, 1970; Roughton, 1970). This relationship between CO, oxygen, and hemoglobin explains both the high toxic potential of CO and the need of hyperbaric oxygen to treat CO overdose (Pace et al., 1950; Peterson and Stewart, 1970; Jay and McKindley, 1997).

Under normal physiological states there is almost always a certain amount of COHb even when breathing CO-free air, because the breakdown of Hb results in endogenous production of CO, which can result in 0.5–0.8% COHb in normal blood (Lawther, 1975). At any CO concentration in the air, COHb will attain an equilibrium (16% at 100 ppm ambient CO and 1.7% at 10 ppm CO). It follows that if CO in the air is lower than that required for a given COHb % at equilibrium, then CO will leave COHb and be exhaled until a new equilibrium is established. This has practical implications. For example, a smoker with a relatively high COHb at equilibrium may exhale CO. Also, if the basal COHb is high, then an equilibrium at a high CO concentration in the air will be achieved more quickly than it would be at an initial low COHb.

The reaction of CO with ferrous iron is relevant to its pharmacology. Shephard (1983) provides the following description of this reaction. When reacting with ferrous iron, carbon atoms of CO form a sigma bond, also involving a D-orbital electron from the third shell of Fe^{2+}. In the body, Fe^{2+} also has four nitrogen linkages that contribute a substantial electron density. The effective valency is less than Fe^{2+}, enabling a more readily reversible reaction with both oxygen and carbon monoxide. The solubility of CO in water is approximately 20% less than that of O_2, such that it is practically absent from the solution in blood.

Reaction of CO with hydroxyl radicals ($OH^{\bullet}$) is the major method of removing CO from the atmosphere (IPCS, 1999). The cycle of hydroxyl radicals is coupled to cycles of CO, methane, water, and ozone. They are produced by the photolysis of ozone followed by the reaction of the excited oxygen atoms with water vapor to produce two hydroxyl radicals: ($O(^1D) + H_2O \rightarrow OH^{\bullet} + OH^{\bullet}$). A small proportion of atmospheric CO is removed by the soil.

Carbon monoxide is eliminated from the body almost exclusively via the lungs. When respiring room air, the elimination half-life of CO in a healthy adult is approximately 4 h, which can be reduced to 1 h when breathing oxygen at normal atmospheric pressure (Roughton and Root, 1945; Pace et al., 1950; Bartlett, 1968). High concentration of COHb can shorten the elimination half-life of CO (Haldane, 1895a; Henderson and Haggard, 1921) and low concentration can increase it (Peterson and Stewart, 1970). However, half-life for a drug is a meaningful feature only if it is not dose-independent, which is not the case with CO (Jay and McKindley, 1997).

MECHANISM OF TOXICITY

Wu and Wang (2005) chose to name endogenous CO as "Dr. Jekyll" and not "Mr. Hyde" because it is good rather than bad. If they are correct, then this mysterious gas proves more than any other agent the correctness of the dictum of Paracelsus (1493–1541) that: "All substances are poisonous. Only the dose differentiates a poison from a remedy." If Paracelsus had known about the views of Wu and Wang (2005) regarding CO, he might well have added not only the "dose" of the substance but also its source.

There are very few agents like the anticoagulant warfarin whose toxicity and therapeutic effects are produced by the same mechanism. In most cases, beneficial and harmful effects recruit different mechanisms of action. As far as CO is concerned, its mechanisms of toxic and physiological actions differ and the source from where it is derived matters, too. So, the mechanisms of action of inhaled-generated and heme-generated CO also seem to differ. Because the focus of this chapter is CO toxicity, we attempt to elaborate the mechanism of its toxicity more than the mechanism of its physiologic actions.

The French physiologist Claude Bernard (1857) and the British physiologist Haldane (1895a) inferred that the toxicity of CO was caused by its interaction with hemoglobin. In his review of carbon monoxide, Lilienthal (1950) wrote: "It has been said many times that the effect of CO on man may be attributed to two actions and, in essence, to these two actions alone: (a) occupation of the Hb molecule by CO, with a resultant decrease in the O_2 transport capacity of the blood; and (b) alteration of O_2Hb dissociation characteristic produced by COHb, with a resultant impaired unloading of O_2 at the tissues." The O_2Hb dissociation curve is shown in Figure 21.2.

Both features described by Lilienthal (1950) boil down to hypoxia as the principal mechanism of CO toxicity, thereby confirming the basic observations of Claude Bernard and J.S. Haldane made 150 years earlier, and working out details of CO–hemoglobin interaction. Haldane (1894a, 1896) knew that the affinity of CO for hemoglobin was greater than that of oxygen, even if he underestimated it. The color of COHb is red, which explains the cherry-like discoloration of CO victims. This is generally but erroneously believed to be a specific characteristic of cyanide poisoning. Obviously, any time oxygen is not fully extracted from the arterial side, the venous blood will become cherry red.

More sophisticated techniques than the ones used by Haldane have established that CO binds to hemoglobin with an affinity 200-times greater than that of oxygen

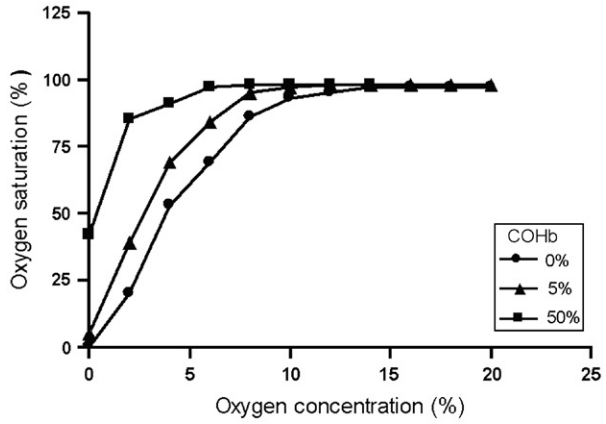

FIGURE 21.2 Effect of carboxyhemoglobin (COHb) on the shape of the oxyhemoglobin dissociation curve in healthy males. Based on the data of Haldane and Priestley (1935) as presented by Shephard (1983).

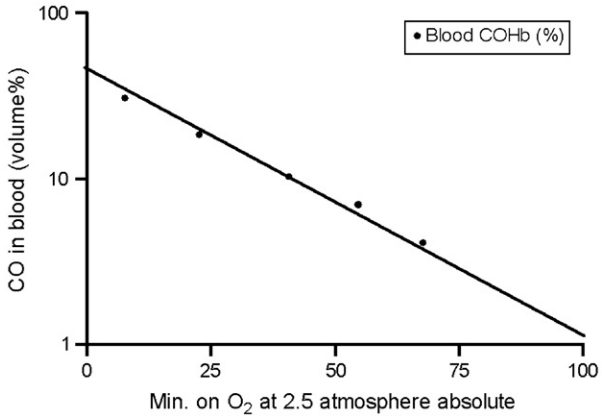

FIGURE 21.3 Data are from a healthy male volunteer who breathed a low concentration of carbon monoxide and then oxygen at 2.5 atmospheric pressure, absolute. Blood was collected at different times after starting oxygen. Data points are taken from Pace et al. (1950) and may not be exactly the same as in their figure. Each point is the mean of triplicate determination and intercept indicates CO level at time zero before oxygen therapy.

(Sendroy et al., 1930; Roughton and Darling, 1944; Ernst and Zibrak, 1998). Carbon monoxide diffuses from the alveoli to the blood in pulmonary capillaries across the alveoli–capillary membrane, which is composed of pulmonary epithelium, the capillary epithelium, and the fused basement membranes of the two. The uptake of CO by Hb is very rapid and the transfer of CO is diffusion-limited (Prockop and Chichkova, 2007).

The greater the duration of the exposure to CO, the concentration of CO in the inhaled air, and the alveolar ventilation, the greater the total amount of COHb. Although the uptake of CO by Hb is very rapid, the release of CO from COHb complex is extremely slow. Because the concentration of COHb increases, the formation of oxyhemoglobin at any concentration of oxygen in the inhaled air decreases. There is a strong case for using COHb concentration as a predictor of CO toxicity (Renwick and Walton, 2001).

There is yet another mechanism by which CO produces hypoxia. COHb shifts the dissociation curve of the remaining oxyhemoglobin to the left, which further decreases the release of oxygen (Douglas et al., 1912; Roughton and Darling, 1944), which causes headaches when it is mild and death when it is extreme. Douglas et al. (1912) also observed that "the presence of a small proportion of oxygen may greatly increase the formation of CO–haemoglobin, and *vice versa*" and this "paradoxical effect explains the favorable physiological effect sometimes produced by carbon monoxide in conditions of great anoxhaemia." The studies of Pace et al. (1950) of human volunteers demonstrated that the release of CO bound to hemoglobin and its expiration can be increased by inhalation of hyperbaric oxygen (Figure 21.3).

However, the data of Goldbaum et al. (1975, 1976) imply that formation of excess of COHb might not account for the lethal effects of CO. These researchers found that dogs breathing 13% CO died within 1 h after achieving COHb levels between 54% and 90%, but transfusion of blood containing 80% COHb, which resulted in 57–64% COHb in the recipient dog, did not cause marked toxicity. It would thus appear that the toxicity of CO results from a combination of tissue hypoxia due to COHb plus other effects of CO, which would explain why COHb levels do not always correlate with the severity of clinical effects (Myers, 1984; Norkool and Kirkpatrick, 1985; Rottman, 1991; Brown and Piantadosi, 1992).

Besides hemoglobin, CO binds to many heme-containing proteins such as myoglobin, guanylyl cyclase, and cytochrome oxidase (Chance et al., 1970; Hill, 1994; Omaye, 2002; Kao and Nanagas, 2006). However, the affinity of CO for cytochrome oxidase is very low and this interaction would require a dose far exceeding the lethal dose of CO (Prockop and Chichkova, 2007). However, even at sublethal concentrations, binding of CO to cytochrome may lead to the generation of superoxides (Zhang and Piantadosi, 1992; Hardy and Thom, 1994) and interfere with cellular respiration. Cellular metabolism remains inhibited even after COHb levels decline to normal range (Olson, 1984; Brown and Piantadosi, 1992; Piantadosi et al., 1995).

The interaction of CO with myoglobin can explain some of its toxic effects. It might impair oxygen supply to the mitochondria, which can result in myocardial ischemia. Patients with underlying cardiac conditions are therefore at high risk for cardiac arrhythmias after exposure to CO (DeBias et al., 1976; Olson, 1984; Sangalli and Bidanset, 1990) as well as chest pain and even death. A study of Swiss soldiers accidentally exposed to CO

noted a substantial number had development of chest pain some time after exposure to CO (Henz and Maeder, 2005). The interaction of CO with skeletal muscle myoglobin can cause muscle weakness (Herman et al., 1988; Wolf, 1994; Richardson et al., 2002).

Nitric oxide (NO) and NO donors as well as phosphodiesterase inhibitors increase gyanylyl cyclase and, hence, cGMP, causing marked vasodilatation. Patients using nitrates for angina are well aware that the drug may cause dizziness and loss of consciousness. CO also activates guanylyl cyclase, and there is always a possibility that the resulting hypotension and cerebral vasodilatation may lead to a loss of consciousness. It is therefore prudent to also treat an increase in cGMP as a potential toxic effect, and not only as a physiological activity of CO or proof of a neurotransmitter role for CO, as suggested by many (Verma et al., 1993; Snyder and Ferris, 2000; Wu and Wang, 2005).

Any event that leads to hypoxia can lead to reperfusion and secondary hyperoxia, especially if vascular autoregulation is not fully developed such as in premature births and certain animal models (Chemtob et al., 1991; Hardy et al., 1997, 2000). Hyperoxia leads to generation of superoxides and other radicals, which are involved in a number of pathologies (Kowluru et al., 2001; Pryor et al., 2006; Brault et al., 2007). Recent studies suggest that hypoxia caused by the formation of COHb may also be followed by reperfusion injury in addition to that produced by the initial hypoxia. The oxidants generated during reperfusion can oxidize essential proteins and nucleic acid and can cause lipid peroxidation, leading to demyelination of CNS lipids and other effects (Ernst and Zibrak, 1998; Prockop and Chichkova, 2007).

In addition, CO can promote neutrophil adhesion to the microvasculature, resulting in the activation of xanthine oxidase and generation of oxidative radicals, which ultimately culminate in brain lipid peroxidation, which is the likely cause of delayed neurological sequelae (Thom, 1990, 1993; Hardy and Thom, 1994; Thom et al., 1994, 1997, 2001; Ischiropoulos et al., 1996; Gilmer et al., 2002). The observed brain lipid peroxidation in CO poisoning is likely to be caused by changes in cerebral blood flow plus oxidative damage (Thom, 1990, 1992; Zhang and Piantadosi, 1992; Hardy and Thom, 1994; Ischiropoulos et al., 1996; Thom et al., 1997).

CO might also alter myelin basic protein, which may react with lipid peroxidation products to produce immunologic cascade (Thom et al., 2004). Other events that could contribute to CO toxicity include glutamate-induced neuronal injury (Ishimaru et al., 1992; Penny and Chen, 1996; Piantadosi et al., 1997), atherogenesis (Lightfoot, 1972; Thom et al., 1999), and apoptosis (Piantadosi et al., 1997).

The model of carbon monoxide toxicity proposed by Kao and Nanagas (2006) combines the cascade of changes resulting from three primary events—binding to HB, direct cellular injury, and increased NO activity. CO is not a radical but many of the injuries produced by it are those that are caused by oxidative stress, which is secondary to hypoxia. In the model of Kao and Nanagas (2006), the oxidant is NO, which contributes to oxidative damage to the brain and produces the clinical syndrome of delayed neurologic sequelae (Thom et al., 1997).

TOXICITY OF CARBON MONOXIDE

Acute Toxicity

All chemicals produce toxicity. However, only a few of them cause death every time if taken in overdose; carbon monoxide is one of them. The toxicity of CO has been extensively reviewed (Hamilton, 1921; Drinker, 1938; Lilienthal, 1950; Putz et al., 1976; Thom and Keim, 1989; Robkin, 1997; Gorman et al., 2003; Ryter and Otterbein, 2004; Kao and Nanagas, 2006; Mannaioni et al., 2006; McGrath, 2006; Prockop and Chichkova, 2007; Samoli et al., 2007) or contained in monographs (Shephard, 1983; IPCS, 1999; Penny, 2000).

The acute toxicity of CO spans from dizziness to death. Symptoms of CO poisoning begin at approximately 20% COHb and death occurs between 50% and 80% COHb (Ryter and Otterbein, 2004). The relationship between blood COHb and toxic signs and symptoms is presented in Table 21.2.

Haldane (1895a) provided the first description of the relationship of CO in inhaled air to its toxic effect on humans. In the first experiment on himself and a

TABLE 21.2 Relationship of Carboxyhemoglobin (COHb) to Toxicity in Humans following Exposure to Carbon Monoxide

COHb %	Signs and Symptoms
<10	No effects in healthy individuals[a]
10–20	Mild headache, exertional dyspnea, cutaneous vasodilation
20–30	Throbbing headaches, nausea
30–40	Severe headaches, dizziness, visual disturbance, fatigue
40–50	Tachypnea, tachycardia, collapse, syncope
50–60	Coma, convulsions, Cheyne–Stoke's respiration
60–70	Cardiorespiratory depression, possible death
>70	Respiratory failure and death

Source: Based on data from Goodman and Gilman (1941); Renwick and Walton (2001).

[a]*Symptoms may appear in subjects with cardiovascular disease. Concentration of COHb depends upon the duration of exposure to any concentration of CO in the air and therefore not included in the table. Exposure to approximately 200 ppm for 2 h results in 10% COHb; ~12,000 ppm CO would produce lethal concentrations of COHb within 2–3 breaths.*

mouse, Haldane inhaled 0.5% CO for 11.5 min with no obvious symptoms except "hyperapnea after running upstairs." In the second experiment, Haldane and his mouse inhaled 0.39% CO. At 15 min his COHb was 23%, but he had no symptoms; however, the mouse started panting after 1.5 min and lay helplessly on its side by 15 min. At 29 min his pulse was 120 beats/min and he felt distinctly "abnormal," while the mouse lay on its back with hyperapnea. At 30.5 min the COHb was 39% and he stopped inhaling CO but felt giddy and had palpitations after running up 24 steps and could not see well. On resting for 2–3 min, Haldane felt better and hyperapnea disappeared; 45 min after stopping the inhalation of CO, COHb was 23%, at 105 min it was 18%, and at 186 min it was 5%.

Not satisfied with the data Haldane had so far collected, he performed nine more experiments (total of 11) with his companion mouse. Aside from all other facts, Haldane came to the conclusion that mice are more sensitive than humans to CO toxicity and COHb remains elevated for a long time.

The higher sensitivity of mice than of humans led to the introduction of canaries in the coal mines. To our knowledge, CO toxicity on humans was again experimentally determined 75 years after Haldane's experiments (Hosko, 1970; Peterson and Stewart, 1970; Stewart et al., 1970). Stewart et al. (1970) exposed 25 human volunteers to CO concentrations from less than 1 to 1,000 ppm for variable time periods and determined COHb as well as subjective symptoms and objective signs (EEG recording, EECG, hand and foot reaction). The main conclusions of these researchers was that even exposure up to 100 ppm CO for up to 24 h did not produce detectable subjective or objective changes; exposure to 200 ppm CO caused mild frontal headache in 4 h and headache was experienced within 1 h at 500 ppm. Full recovery occurred in all cases when subjects were transferred to a hyperbaric oxygen chamber. COHb strongly correlated with inhaled CO concentration reaching approximately 20% at 100 ppm and 30% at 200 ppm CO (Peterson, 1970; Peterson and Stewart, 1970). Hosko (1970) found that at COHb more than 20%, visually evoked responses were altered in human volunteers; at high altitudes, even a much smaller increase in COHb (5–10%) can exert similar effects (Lilienthal and Fugitt, 1945).

The data cited and the relationship of COHb to CO toxicity presented in Table 21.2 clearly show that the acute toxicity of CO is concentration-dependent. This is of great relevance in setting permissible or desirable atmospheric CO concentration standards. However, the binding affinity of CO for Hb is 200- to 300-times greater than that of oxygen to Hb; therefore, the effects of CO must be considered cumulative. Prolonged exposure to CO would increase COHb more than can be accounted for by its ambient concentration; likewise, a long period of CO-free atmosphere would be needed for COHb to return to normal levels, as also revealed by the data of Haldane (1895a). As a caution, it should be noted that toxic symptoms of CO poisoning do not necessarily correlate with COHb levels; for example, seizures in a 4-year-old child were noted at COHb levels of approximately 25%, but 40% is the usual level for such symptoms (Herman, 1998). Putz et al. (1976) exposed 30 volunteers for 4 h to 5–70 ppm CO and found that 5% COHb (achieved after exposure to 70 ppm CO) impaired coordination, response time to light, and auditory acuity. However, exposure to CO during sleep, which increased COHb to 12.5, did not impair performance after awakening, although it had some effect on sleep pattern (O'Donnell et al., 1971).

Air pollution, which consists of particulate matter, CO, sulfur oxide, nitrogen oxide, hydrogen sulfide, acid gases like HF and HCl, volatile organic solvents, and radiation, is estimated to be responsible for 800,000 annual deaths worldwide (Cohen et al., 2005). A review of the health effects of these pollutants found that respiratory and cardiovascular mortality was consistently associated with particulate matter and CO (Curtis et al., 2006). A large study of the effect of air pollution on mortality was investigated in 19 European cities under the APHEA (Air Pollution and Health: A European Project) project. Samoli et al. (2007) found a significant association of CO both with total and cardiovascular mortality. However, because CO is not the only noxious air pollutant, such epidemiological studies fail to categorically establish a role of CO because it is also possible that CO toxicity is aided by the presence of other pollutants. Purser (1996) analyzed scenarios in the event of fire in aircrafts that release a host of toxic gases including CO, and concluded potentially serious consequences in terms of performance of the crew and the survival of passengers.

It is of interest that big cities like Mexico City and Los Angeles harbor not only high concentrations of CO in the air but also an excess of noise, and there are both clinical and experimental data (Cary et al., 1997; Fechter et al., 1988) to show that noise increases the toxicity of CO as well as of other noxious chemicals.

Edmund Hillary and Sherpa Tenzing conquered Mount Everest on May 29, 1953. This sparked great enthusiasm about mountain climbing. However, at high altitudes the partial pressure of oxygen is low and even a small amount of CO can be fatal. For example, two healthy men died at a height of approximately 6,000 m in Alaska from exposure to CO generated just by a cooking stove in their tent (Foutch and Henrichs, 1988). Even a 5–10% increase in COHb can cause appreciable deterioration in flicker fusion frequency at high altitudes (Lilienthal and Fugitt, 1945). Also, altitude exaggerates CO toxicity in subjects with coronary artery disease (McGrath, 2006).

Atmospheric CO is the main source of exposure to the population. For this reason, WHO has set the

following limits for CO exposure (IPCS, 1999): 100 mg/m^3 (87 ppm) for 15 min; 60 mg/m^3 (52 ppm) for 30 min; 60 mg/m^3 (26 ppm) for 60 min; and 10 mg/m^3 (9 ppm) for 8 h (the usual working period per day).

Delayed Toxicity

Delayed CO toxicity is also referred to as the interval form of toxicity (Mannaioni et al., 2006) with significant effects on the central nervous system (Raub and Benignus, 2002). The interval form refers to neuropsychiatric symptoms within several days or even weeks after asymptomatic existence after recovery from unconsciousness caused by exposure to CO. The most common symptoms of the "interval form" of delayed CO toxicity are lethargy, behavior changes, forgetfulness, memory loss, and Parkinsonian symptoms (Yoshii et al., 1998). These symptoms are attributed to diffuse demyelinization of cerebral white matter in specific regions of the brain (Okeda et al., 1981; Yoshii, et al., 1998; Hsiao et al., 2004; Mannaioni et al., 2006). Neurological symptoms were found to accompany lesions in the brain as detected by magnetic resonance imaging in several other studies (Parkinson et al., 2002; Hsiao et al., 2004).

Daily exposure of dogs for 11 weeks to 100 ppm CO resulted in persistent ECG changes as early as 2 weeks and cardiac muscle degeneration at the end of the study. Observed disturbances in gait and posture were attributed to lesions in the cerebral cortex (Hamilton and Hardy, 1974). Visual disturbances after recovery from a period of CO-induced unconsciousness have also been reported (Kelly and Sophocleus, 1978). Moderate to severe CO poisoning can shorten lifespan because of myocardial injury (Henry et al., 2006).

A lack of close correlation between COHb levels and the severity of CO toxicity is well-recognized (Mannaioni et al., 2006); this clearly suggests the involvement of other factors. These other factors most probably include xanthine oxidase–derived reactive oxygen species, which are produced due to hyperperfusion after the initial hypoxia. This inference is supported by several animal studies (Sokal and Kralkowska, 1985; Thom, 1992; Zhang and Piantadosi, 1992).

CO and cyanide have certain similar toxicities that are a consequence of hypoxia such as disruption of cochlear functions (Tawackoli et al., 2001). CO has been found to cause hearing loss in humans (Sato, 1966; Morris, 1969; Goto et al., 1972; Makishima et al., 1977) and in animal models (Douglas et al., 1912; Fechter et al., 1988; Liu and Fechter, 1995).

Cardiovascular Toxicity

CO can cause and exacerbate underlying cardiovascular diseases (Atkins and Baker, 1985; Thom and Keim, 1989). CO seems to affect each step in the course of ischemic heart disease (Goldsmith and Aronow, 1975; Turino, 1981). CO has been found to exacerbate the atherogenic potential of cholesterol in monkeys (Webster et al., 1970). Inhalation of CO during treadmill exercise in patients with coronary artery disease hastens the onset of anginal symptoms and causes a prolongation of ECG changes (Anderson et al., 1973; Aronow and Cassidy, 1975). A number of deaths from myocardial infarction have been recorded at COHb levels much lower than the levels required to produce neurological effects (Scharf et al., 1974; Atkins and Baker, 1985). The potential of CO to aggravate myocardial pathology is understandable because the disease is associated with relative decrease in oxygen supply to the myocardium. At any concentration, CO can only further increase hypoxia as well as interfere with any compensatory mechanism. Given that cardiac disease is common in the developed world, which also has more than the usual level of CO in the atmosphere, important public health measures would be required to deal with this issue.

Anemia will exert a profound effect on CO saturation and its toxicity because most, if not all, of the CO in the blood is bound to hemoglobin. This subject assumes special importance because anemia is quite common in the general population, particularly in slum dwellings in developing countries. Anemic subjects approach equilibrium levels of COHb more rapidly than those with normal hemoglobin at any level of exposure to CO. For example, a 4 h exposure of subjects with hemoglobin levels of 7 g/100 mL could attain COHb concentrations of 4–5% compared with an anticipated level of 2.5% for normal individuals. This fact coupled with greater production of endogenous CO would pose a greater threat of toxicity in subjects with anemia than in normal subjects (United Nations Environmental Program and WHO, 1979).

Maternal, Fetal, and Infant Toxicity of CO

Carbon monoxide crosses the placenta freely. Blockage of maternal and fetal hemoglobin by CO places the fetus at serious risk, especially in the final weeks of gestation (Shephard, 1983). Maternal and fetal hemoglobin are not identical. Fetal hemoglobin has a higher affinity for CO than maternal hemoglobin. Almost 2 days are needed to achieve equilibrium in maternal and fetal hemoglobin, and at equilibrium fetal COHb is slightly higher than maternal COHb (Longo, 1970; Hill et al., 1977).

Smoking is a well-recognized risk factor during pregnancy (Rogers, 2008). Norman and Halton (1990) analyzed 60 cases of exposure of pregnant women to CO and concluded that severe acute exposure to CO can lead to fetal deaths as well as anatomical malformation and functional alterations. Cigarette smoke has also been

found to decrease fetal weight in rats (Carmines and Rajendran, 2008). Cigarette smoke contains 4,800 identified chemicals, including CO, HCN, and, of course, nicotine; there is no difference in the toxicities of different brands of cigarettes (Hoffmann et al., 2001). The well-established adverse effects of smoking on the fetus may be contributed by CO. Although effects of chronic exposure to CO at levels that can be delivered by smoking CO have not been clearly documented, several animal studies and studies with embryos reviewed by Robkin (1997) suggest that the adverse effects of smoking on fetal development are due to factors other than CO.

CO poisoning of pregnant women can cause fetal death and malformation (Norman and Halton, 1990; Rogers, 2008). Several reports document that exposure to CO from ambient air pollution during pregnancy can decrease fetal weight and lead to preterm births (Ritz et al., 2002; Gilboa et al., 2005; Mannes et al., 2005; Wilhelm and Ritz, 2005; Ziaei et al., 2005; Leem et al., 2006; S. Liu et al., 2007). However, a large study conducted in 96 counties in the United States between 1999 and 2002 examined the effect of air pollution on pregnancy outcome. The study analyzed 3,538,495 births, including 6,639 infant deaths, and air composition of particulate pollutants more than 2.5 μm as well as CO, SO_2, and O_3. The study concluded that infant mortality was contributed to by particulate matters and not by CO (Woodruff et al., 2008).

In animal models, exposure to low levels of CO during pregnancy, which does not reveal obvious signs of fetal toxicity at birth, results in neurological and other abnormalities in later life (Annau and Fetcher, 1994; Carratu et al., 1997; Giustino et al., 1999; Benagiano et al., 2005). The commonly encountered behavioral abnormalities in children could be due to prenatal exposure to low concentrations of CO. This possibility is very much real and, if so, then prophylaxis is a monumental task and a major public health challenge because people have no choice but to breathe the air around them.

Tolerance to CO Toxicity

Hypoxia is the principal mechanism of CO toxicity. Hypoxia can also be produced by the low partial pressure of oxygen at high altitudes. The fact that people living at high altitudes do not exhibit signs of hypoxia implies adaptation to low oxygen pressure. It is thus no surprise that continuous exposure to CO also leads to adaptation (Killick, 1948). In one of the earliest studies on adaptation to CO, mice were exposed to successively higher concentrations of CO for 6–15 weeks; by the end, CO concentration was nearly 3,000 ppm and COHb 60–70%. Symptoms of this very high COHb were far less in the adapted than in the nonadapted mice (Killick, 1937). The underlying mechanism of this adaptation seems to be increased synthesis of hemoglobin (IPCS, 1999). These data have been confirmed by other researchers (Clark and Otis, 1952; Wilks et al., 1959).

Adaptation to CO toxicity also seems to occur in humans. Performing experiments on herself, Killick (1940) found diminished symptoms and lower COHb on chronic exposure to CO than in the beginning, which is in accord with the data of Haldane and Priestley (1935). Adaptation to hypoxia is the reason why people living at high altitudes feel perfectly normal while a visitor from the plains may feel quite unwell. Indian and Pakistani soldiers are facing one another in Siachen of Kashmir, the highest place for any military confrontation in the world. Unless the soldiers are acclimatized before they go to Siachen, many develop fatal pulmonary edema. If they are acclimatized, then the incidence of pulmonary toxicity is considerably reduced.

PHYSIOLOGICAL ROLES OF CARBON MONOXIDE

The primitive earth atmosphere was composed of CO_2, CO, nitrogen, and water, and somehow radiation, heat, and sparks contributed to the generation of amino acid from these basic materials (Kobayashi et al., 2002; Miyakawa et al., 2002). If this is true, then it should be possible to experimentally prove this. We are not aware whether this has been done. However, if it is true, then nothing could be physiologically more important than CO, nitrogen, and H_2O.

Furchgott and Zawadzki (1980) made the seminal observation that vascular endothelium was essential for the vasorelaxant effect of acetylcholine. Soon after, this vasorelaxant substance was identified as nitric oxide (Palmer et al., 1987). It was subsequently found that NO increases guanylyl cyclase activity and cGMP and performs numerous physiological functions (Moncada et al., 1991; Culotta and Koshland, 1992). The study of the physiological role of CO is relatively recent, but it would not be surprising that in years to come CO may be on a par with NO. The excellent and extensive review of the physiological role of CO by Wu and Wang (2005) most certainly paints a rosy picture of CO. One can interpret the review to imply that the infamous CO is about to become a cure for many maladies.

NO and CO have several common properties. Both are gases, both are putative neurotransmitters, both cause vasorelaxation, both increase cGMP, both inhibit platelet aggregation, both increase cGMP, albeit from different pools, and both are synthesized endogenously, although CO also had an exogenous source (Snyder and Ferris, 2000; Hartsfield, 2002). Being gases, neither CO nor NO directly depends on plasma membrane transporters or channels for its entry into cells. Also, certain

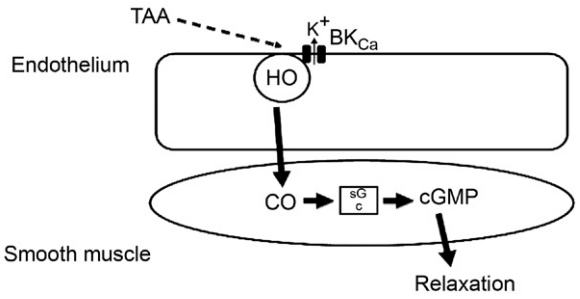

FIGURE 21.4 A model depicting the involvement of CO in mediating relaxation of brain pial microvessels of rats by trans-arachidonic acid (TAA). In this model, TAA acts on calcium-dependent potassium cannels (BKca), leading to activation of hemeoxygenase-2 (HO-2), which releases carbon monoxide (CO). CO activates guanylyl cyclase (sGC) activity. The resulting increase in cGMP causes dilation of rat brain pial microvessels. *Source: Reproduced from Kooli et al. (2008) with permission from* Free Radical Biology & Medicine.

interdependence exists between CO and NO. NO augments HO-1 gene expression and, consequently, CO formation (Hartsfield et al., 1997). Heme oxygenase can decrease NO synthesis (Maines, 1997). On the basis of data from our laboratory, we have proposed that *trans*-arachidonic acid increases the activity of heme oxygenase-2, leading to the generation of CO, which increases cGMP and thereby causes relaxation of cerebral microvasculature of rats, as depicted in Figure 21.4 (Kooli et al., 2008).

At the same time there are differences between CO and NO. For example, NO is an unstable gas and a radical, whereas CO is a stable gas and not a radical. CO binds only ferrous heme but NO binds to both ferrous and ferric hemoprotein (Hartsfield, 2002). The combination rate of NO with hemoglobin is faster and dissociation is slower than that of CO (Sharma and Ranney, 1978), so that the affinity of NO for hemoglobin is ~1,500-times that of CO (Foresti and Motterlini, 1999). Formation of endogenous CO in a variety of tissues has been demonstrated (Marks et al., 2002). Given the complexity of colocalization and activities of heme oxygenase and nitric oxide synthase, it has been speculated that "CO and NO could function in a synergistic, compensatory, and/or counterregulatory way" (Hartsfield, 2002).

The source of endogenous CO and NO and the enzymatic mechanisms in their synthesis differ (Hibbs et al., 1987; Marletta et al., 1988; Moncada et al., 1991; Wu and Wang, 2005). There is no common mechanism of coordinating the release and activities of these two molecules; therefore, it is difficult to conceptualize what would be the ultimate physiological effect of any interaction between the two molecules. Usually, if a substance has physiological effects, its excess or deficiency manifests in some physical disorder. Although there is strong evidence that decreased formation of NO can affect blood pressure homeostasis, no condition has yet been identified that can be attributed to an excess or deficiency of endogenous CO. In contrast, the toxic effects of exogenous CO are well-characterized and there is no reason to assume that the source of this molecule makes a fundamental difference in its pharmacology. In any case, this chapter focuses on CO toxicity. Putative physiological functions of endogenous CO have been extensively reviewed (Marks et al., 1991; Baringa, 1993; Snyder and Ferris, 2000; Choi and Otterbein, 2002; Wu and Wang, 2005; Mannaioni et al., 2006). A brief account of the postulated physiological roles of CO is presented.

CO as a Putative Neurotransmitter

The colocalization of heme oxygenase-2 (HO-2), the enzyme responsible for the release of CO from COHb, and guanylyl cyclase in discrete brain areas lacking NOS, coupled with changes in cGMP by agents and procedures that alter CO, has been advanced as evidence for a putative neurotransmitter role for CO (Verma et al., 1993). However, these workers (Verma et al., 1993) did not test whether changes in cGMP were associated with any changes in neuronal activity, nor did Baringa (1993) feel any need for such details in a commentary in the same issue of *Science*. In further support for a neurotransmitter role for CO, Boehning et al. (2003) demonstrated that HO-2 is activated by phosphorylation of CK2 (casein kinase 2) during neuronal depolarization, and that CK2 activation is essential during CO-mediated nonadrenergic noncholinergic relaxation of the smooth muscle. Also, a role of heme oxygenase and CO has been demonstrated in olfactory neurons in culture (Ingi and Ronnett, 1995; Ingi et al., 1996), myenteric plexus (Zakhary et al., 1996), and vas deferens (Burnett et al., 1998).

The researchers cited have provided strong evidence that CO is released during heme breakdown and that liberated CO can increase cGMP. However, according to the established criteria, a neurotransmitter, among other things, must transmit a message across a synapse. Unless clear evidence is presented for an effect of CO on neuronal transmission, CO should be treated only as a putative, but not a proven, neurotransmitter.

Other Physiologic Effects of CO

Brune and Ullrich (1987) demonstrated that CO increases the activity of guanylyl cyclase–like NO and inhibits platelet aggregation triggered by a variety of agents. CO was found to dilate coronary arteries and increase coronary blood flow in rats (McGrath and Smith, 1984; McFaul and McGrath, 1987). These researchers did not examine if this was associated with an increase in cGMP, but they excluded involvement of an adrenergic system. Similar vasodilator effects of CO have been

found on porcine coronary artery (Graser et al., 1990), rabbit cerebral arteries (Brian et al., 1994), rabbit aorta (Furchgott and Jothianandin, 1991), and rat aorta (Lin and McGrath, 1988). CO produces similar effects on cerebral blood flow. This subject has been reviewed by Koehler and Traystman (2002). CO has been shown to relax cerebral microvasculature (Leffler et al., 1999) and pulmonary as well as systemic vessels (Villammor et al., 2007) of newborn pigs. The vasorelaxant effect of CO is endothelium-independent (Vedernikov et al., 1989; Wang et al., 1997) and exerted via K^+ channels (Graser et al., 1990; Wang and Wu, 1997) in a manner similar to the oxidant OCl^- (Varma et al., 2006), which is also endogenously produced (Weiss et al., 1985, 1986). Also, CO can increase cerebral blood flow in rats (Macmillan, 1975), rabbits (Meyer-Witting et al., 1991), and fetal sheep (Rosenberg et al., 1986).

Heme oxygenase activation as well as CO induce the synthesis of vascular endothelial growth factor (Dulak et al., 2002) but suppress its induction by hypoxia (Liu et al., 1998). Heme oxygenase-1 plays a protective role against vasoconstriction and proliferation (Lee et al., 1996; Sammut et al., 1998; Duckers et al., 2001), regulates vascular cGMP (Morita et al., 1995), suppresses endothelial cell apoptosis (Brouard et al., 2000), and activates mitogen-dependent protein kinase pathway (Otterbein et al., 2000). Paradoxically, CO can prevent ischemic lung injury by suppressing fibrinolysis (Fujita et al., 2001).

A number of studies have examined the anti-inflammatory activities of CO (Abraham et al., 1988; Mannaioni et al., 2006). An activation of the heme oxygenase system and exposure to CO *in vitro* (Otterbein et al., 1999, 2000; Sethi et al., 2002; Gibbons and Farrugia, 2004; Pae et al., 2004; Chauveau et al., 2005) and *in vivo* (Otterbein et al., 1999; Nakao et al., 2003; Dolinay et al., 2004; Neto et al., 2004) revealed anti-inflammatory activity in animal models but not in humans (Mayr et al., 2005). CO can inhibit apoptosis of endothelial cells (Soares et al., 2002). There is some evidence that upregulation of HO-1 might be beneficial in inflammatory diseases (Willis et al., 1996). In any case, the ability of CO to inhibit platelet aggregation and inflammation has important therapeutic implications and needs to be explored.

Liver is a site for cytochrome P450 synthesis as well as degradation of senescent erythrocytes and, consequently, catabolism of hemoglobin and heme oxygenase, which mediates the generation of endogenous CO. Because oxyhemoglobin can capture CO, it would reduce available CO, which could and has been shown to increase hepatic vascular resistance (Suematsu et al., 2000). There is some evidence that endogenous CO formation in the hypothalamus might modify hypothalmo-pituitary-adrenal axis (Mancuso et al., 1997).

In conclusion, there is definite evidence that endogenously produced CO exerts biological effects. If these are important physiological effects, then one can assume that a deficiency in endogenous CO generation might manifest as a disease state or worsen existing diseases. Vascular NO is thought to be of relevance in maintaining vascular tone and its deficiency can cause hypertension or platelet aggregation. Diseases caused by a deficiency of hormones or neurotransmitters are ameliorated by substitution therapy. There is no reason to think that exogenous CO cannot substitute for a deficiency of endogenous CO. For example, endogenous CO deficiency increases acid-dependent bile flow; this can be corrected by exogenous CO (Shinoda et al., 1998; Suematsu et al., 2000). A lower incidence of Parkinson's disease among smokers than in nonsmokers is attributed to nicotine in cigarettes (Quick, 2004). Fortunately, the beneficial effects of nicotine in cigarettes have not led to promoting smoking. Hopefully, any useful role for CO would also not lead to promoting the therapeutic value of cigarettes, which is a rich source of CO.

TREATMENT OF CARBON MONOXIDE OVERDOSE

Oxygen

When Humphrey Bogart rescued Audrey Hepburn from a garage where she had turned on the ignition keys of several cars in the film *Sabrina*, she knew that exhaust fumes could kill and he knew that fresh air could save life. The treatment of CO intoxication by oxygen was elaborated by Haldane (Haldane, 1895a, 1922; Haldane and Priestley, 1935). Perhaps the general public is aware of this therapy.

It was implicit in Haldane's studies that oxygen at high pressure would be more effective than at atmospheric pressure. However, there was a reluctance to resort to this practice because of the fear of oxygen toxicity. End and Long (1942) successfully demonstrated the value of oxygen at high pressure to treat CO overdose in laboratory animals. To our knowledge, the first controlled study of the value of hyperbaric oxygen against CO poisoning was performed by Pace et al. (1950) on 10 human volunteers (five men and five women). Data of these workers show that changing ambient pO_2 from 0.2 to 2.5 atmospheric pressure, absolute, increased the rate constant (k) of elimination of CO from 0.0028 to 0.0315 min, which would alter the half-life of CO elimination from 246 to 21 min.

Other Therapeutic Measures

CO poisoning leads to several abnormalities such as generation of oxidants, lipid peroxidation, disturbance in glucose metabolism, and so on, which compound the toxic

effect of CO caused by hypoxia. Measures other than oxygen are intended to deal with these secondary changes.

Allopurinol and N-Acetylcysteine

There is strong evidence that some of the toxic effects of CO poisoning are produced by release of reactive oxygen metabolites and xanthine oxidase plays a major role in these processes (Sokal and Kralkowska, 1985; Thom, 1992; Zhang and Piantadosi, 1992). Xanthine oxidase is a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase, which is converted to oxidase under ischemic conditions utilizing molecular oxygen in place of NAD and, in the process, generates superoxide radicals and hydrogen peroxide. Allopurinol is a xanthine oxidase inhibitor and *N*-acetylcysteine is a sulfhydryl donor. The efficacy of these agents against neuronal injury caused by CO has been documented (Howard et al., 1987; Thom, 1992).

Insulin

Hyperglycemia, which follows stroke or myocardial infarction, worsens the resulting neurological insult. Acute CO poisoning is also characterized by hyperglycemia, which has been found to worsen brain dysfunction in rats (Penny et al., 1990). Similar observations have also been made in CO-poisoned patients (Pulsinelli et al., 1980). Moreover, neurological complications of CO poisoning in diabetic patients seem worse than in healthy individuals. There is evidence that insulin treatment is effective in reducing neuronal damage caused by stroke or cardiac arrest, and similar encouraging results have been found after CO poisoning in humans (White and Penny, 1994).

Other Measures

Because of the role of excitatory amino acids in ischemic neurodegeneration, Ishimaru et al. (1992) studied the effect of several agents including NMDA receptor antagonists against CO poisoning in mice with encouraging results. We are not aware of any clinical studies of the efficacy of NMDA receptor antagonists in reducing neurological toxicity in humans after CO poisoning. Cerebrolysin, produced by enzymatic breakdown of lipid-free proteins of porcine brain, have been found to offer protection against certain neurological changes after CO poisoning in animal models. Hypothermia has also been found to be of benefit in CO poisoning (Peirce et al., 1972).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We have attempted to deal with most, though not all, aspects of the pharmacology, toxicology, and physiology of carbon monoxide. The literature on CO is immense and dates back to 1857 or even earlier. The omnipresence of this colorless, odorless, tasteless, and nonirritating gas, generated by incomplete combustion and ever present in megacities, has traveled a long way from being a leading cause of unintentional death and an agent of choice for suicide to finding a physiological role as big as a neurotransmitter like nitric oxide.

Many currently used drugs like acetyl salicylic acid, digitalis, nitrates, and quinine have existed for a long time, and so have many poisons like cyanide and neurotoxins. However, their mechanism of action was not known until the middle of twentieth century or even later. CO is unique. The details of its mechanism of toxicity were published by Haldane as early as 1895, and subsequent work has only elaborated these details while affirming their correctness. The generation of COHb is the principal cause of CO toxicity; however, it is not the only one. Much research has been performed to elaborate these additional mechanisms of CO toxicity, but we have not discussed them in detail, just as we have not gone into detail about the physiological role of endogenous CO as one of the many warfare chemicals.

If detected in time, CO poisoning can be treated with hyperbaric oxygen plus other supplementary measures. However, safeguarding against chronic toxicity, if it results from chronic exposure to low levels of CO, is a difficult challenge. Many megacities are highly polluted with CO. Burning petroleum in automobiles as the chief source of CO in the modern world would, in most probability, increase rather than decrease atmospheric CO, ozone, nitrogen, and hydrogen sulfide. This poses one of the great challenges of modern times to public health care. It is the responsibility of the environmental toxicologists, other scientists, and a socially conscious population to do whatever they can to ensure that measures are taken to protect the citizenry from hazardous chemicals in the air we breathe.

Acknowledgments

We thank Ms. Emily Gibson for compiling and checking references. This article is dedicated to the memory of Professor John Sanderson Haldane, FRS (1861–1936), who conducted experiments on himself with poisonous carbon monoxide to save the lives of miners, and whose laws on the exchange of gases remain valid even today.

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CHAPTER

22

Methyl Isocyanate: The Bhopal Gas

Daya R. Varma and Shree Mulay

INTRODUCTION

Methyl isocyanate (MIC), the smallest, most reactive, and most toxic member of the isocyanate family, was unheard of until December 2–3, 1984, when nearly 40 metric tons of this deadly chemical leaked out of the Union Carbide India Limited (UCIL) pesticide plant at Bhopal within a period of 45–60 min. Bhopal became a "city of death," in the words of *India Today* (December 30, 1984). The journal *Nature* (Opinion, 1984) wrote: "…. the anguish vividly carried round the world by the television cameras seems not to have matured into the anger, even hysteria, there would have been had the accident occurred on the edge of a European city—or in Connecticut [site of Union Carbide's U.S. headquarters]."

Prior to the Bhopal disaster, there had been only one scientific report on MIC toxicity (Kimmerle and Eben, 1964); this led *Lancet* (Editorial, 1984) to comment: "In a year's time we will have learnt a lot more about methyl isocyanate—at an appalling price."

The Bhopal disaster evoked immense interest amongst journalists, scientists, the corporate world, lawyers, social activists, and the Indian government (Chemical and Engineering News, 1985; Varma, 1986). This chapter mainly focuses on how the disaster took place in Bhopal, as well as the physicochemical characteristics of MIC and its toxicity; how such disasters can be prevented in developing countries like India, which lack rigorous safety protocol against hazardous chemicals. Legal implications are not elaborated in this chapter.

The population of Bhopal in 1984 was 800,000. The Union Carbide plant was within 1 kilometer of the Bhopal railway station and not too far from the city's two large hospitals; densely populated slums lay across the road just a few hundred meters from the factory. The toxicity of a chemical, barely tested in animal models, was now suddenly being observed as it acted on 200,000 people, as well as on animals and plants living in Bhopal. There is no precise information on how many people died in the immediate aftermath of the disaster; usually a figure of 2,500 is quoted, but our estimate based on projecting deaths in a sample survey (Varma, 1987) is that about 8,000 people died. Most of the deaths happened between 24 and 48 h after the discharge of MIC, which would not have been the case if the culprit had actually been hydrogen cyanide (HCN), as was thought initially (HCN acts within minutes). A British medical student on her elective at a Bhopal hospital wrote: "The dead and dying arrived by the truckload, others came by rickshaw or were carried by relatives. For some, the effort of the journey itself proved too much, and they died soon after arrival." (Sutcliffe, 1985).

Soon after the Bhopal accident, the government of India promised to conduct a comprehensive study on the acute and long-term effects of exposure to MIC. The Indian Council of Medical Research (ICMR) released approximately $2 million for research; a cohort of 80,021 gas-exposed people and 15,931 controls were registered, but only 16,860 exposed subjects and 5,741 controls could be contacted in 2010 (Sharma, 2013). Researchers were not recruited to study all aspects of toxicity. No definite criteria were set to make a quantitative assessment of the exposure to MIC; the best estimate has remained the distance from the Union Carbide factory (Dhara et al., 2002). A number of laboratories in the United States and United Kingdom initiated research on MIC (Environmental Health Perspective, 1987), but their interest faded quickly. The twentieth anniversary of the disaster drew the attention of the media, but not of researchers.

THE MAKING OF A DISASTER

For days after the disaster, no one was sure how the accident had happened. A detailed account of how MIC escaped from Tank E-610 of UCIL was ultimately

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provided by Stewart Diamond in *The New York Times* on January 28, and this report was corroborated by others as well (Varadarajan et al., 1985; Varma, 1986). Although it is highly unlikely that an accident would occur exactly in the way it happened in Bhopal, the accidental escape of hazardous chemicals is not uncommon. The US Environmental Protection Agency (EPA) had recorded 28 instances of minor leaks of MIC from the Union Carbide plant in Institute, West Virginia, between 1980 and 1984, and a leak of aldicarb oxime from the same plant on August 11, 1985, sent 200 people to hospitals.

According to Diamond's article, water entered the pipes on the floor of the factory during routine cleaning, which took place without placing safety slips at the joints. It would seem that the floor of a chemical factory was cleaned with no more precautions than are taken when cleaning the platform of an Indian railway station. The water reached Tank E-610. The exothermic reaction between the water and MIC increased the temperature of the tank, converting liquid MIC into gas. The increase in pressure forced open the vent valve, letting most of the MIC escape as gas. Various safety measures to neutralize MIC, such as caustic soda scrubbers, were either inadequate or completely nonfunctional. Even if safety measures had been in perfect working order, they were not designed to handle such a big leak. MIC gas, being almost twice as heavy as air, settled on the ground, which affected all living beings in the area. Undoubtedly, the lack of rigorous safety procedures in the maintenance was the cause of the accident.

Fortunately, MIC stored in the other two tanks (E-611 and E-619) was not affected. The Indian Council of Scientific and Industrial Research (CSIR) decided that the safest way to dispose the remainder of MIC was to convert it into carbaryl, the pesticide for which the factory had been set up to produce. This exercise was termed "Operation Faith," and it was extensively covered by Indian and international media. Operation Faith ended safely on December 22, 1984, without any further mishap. Nonetheless, the disaster frightened the people of Bhopal so much that despite all assurances by the Indian government, almost half of the population left the town; some never returned to Bhopal.

An examination of the causes of the Bhopal disaster clearly indicates that the accident would not have occurred if all necessary precautions had been taken in the maintenance and operations of UCIL. Union Carbide had sent a team of American experts in May 1982, and they pointed out many lapses in the operation of the plant. However, the company failed to act on their suggestions. In addition, after making a number of visits to the plant, the local journalist Rajkumar Keswani warned of the impending disaster 4 months before the accident.

MIC was used in the United States, Germany, and Japan, but it was stored in Bhopal in much greater quantities than in any of these other places. The question of whether the operation of hazardous industries (like the chemical industry) can be both safe and profitable is debatable. What is not debatable, however, is that safety must remain the top priority. Following a cyclohexane explosion in Flixborough in 1974, an Advisory Committee on Major Hazards was set up in the United Kingdom, and the European Economic Council Directive of 1982 was triggered by the Sevesco accident of 1976 in Italy. However, the Bhopal disaster of 1984, which was far worse than the Flixborough and Sevesco accidents (in fact, it was the worst in history), has not led to additional regulations outlining corporate and state responsibilities, despite the strong case for global monitoring of potentially toxic materials (Sriramachari and Chandra, 1997).

TOXICOKINETICS OF ISOCYANATES

As stated previously, MIC is a member of the isocyanate family of chemicals. The high chemical reactivity of isocyanates is central to their commercial use, but it is also a key element of their toxicity. No clinical use of isocyanates has so far been demonstrated.

Chemistry of Isocyanates

Organic isocyanates were first synthesized in 1849. Isocyanates (Table 22.1) are highly reactive heterocumulene chemicals. The general structure of isocyanates is R—N═C═O, which is distinct from that of cyanate (N═C—O—H). The reactivity of organic isocyanates is due to the strain in the cumulative double bonds (—N═C═O) of isocyanates (Varadarajan et al., 1985).

TABLE 22.1 Commonly Used Isocyanates

Isocyanates	MW	LC_{50} (ppm)[a]	Ceiling (ppm)[b]
Methyl isocyanate (MIC)	57	5.1	0.02
Hexamethylene diisocyanate (HDI)	168	55.9	0.02
Toluene diisocyanate (TDI)	174	49.0	0.02
Isophorone diisocyanate (IPDI)	222	28.5	0.02
Diphenylmethane diisocyanate (MDI)	250	36.0	0.02
Dicyclohexylmethane diisocyanate (SMDI)	262	–	0.01
1,5 Naphthalene diisocyanate (NDI)	210	–	0.02

[a] *LC_{50} (lethal concentration killing 50 of experimental animals) values are after 4 h exposure.*
[b] *To convert ppm to mg/m^3, divide it by (24.4/MW); see, review by Varma (1986).*

Most of the commercially used isocyanates are diisocyanates, and R is an aromatic ring. MIC is an exception; its structure is $H_3C{-}N{=}C{=}O$. The physicochemical properties of MIC differ from those of other isocyanates (Lowe, 1970). Because of the high chemical reactivity of MIC with alcohols, it serves as an intermediate in the production of carbaryl, a pesticide. Diisocyanates are primarily used for the manufacture of polyurethanes.

Synthesis of MIC

MIC ($CH_3N{=}C{=}O$) can be synthesized using different reactions. The commercial synthesis of MIC by Union Carbide, Bayer, and Dupont is described next:

At Union Carbide Corporation, Bhopal, India:

$$CH_3NH_2\,(\text{monomethyl amine}) + COCl_2\,(\text{phosgene}) \rightarrow CH_3N{=}C{=}O + 2HCl$$

At Bayer, Germany:

$$CH_3NH{-}CO{-}NHCH_3\,(\text{dimethyl urea}) + (C_6H_5O)_2CO\,(\text{diphenyl carbamate}) \rightarrow CH_3\,N{=}C{=}O + \text{by products}$$

At Dupont, the United States:

$$CH_3{-}NH{-}CHO\,(\text{methyl formamide}) + O_2 \rightarrow CH_3\,N{=}C{=}O + H_2O$$

MIC was produced and stored in Bhopal, whereas CO and phosgene, which also are required for the production of carbaryl, were not stored; rather, it was produced and utilized right away. In the United States and Germany, MIC is produced as needed; it is not stored.

Various steps in the production of carbamate pesticide at Bhopal were as follows (Varadarajan et al., 1985):

1. Petroleum coke (2C) was reacted with oxygen to produce 2CO.
2. CO and chlorine were reacted to produce phosgene ($COCl_2$).
3. Phosgene and methylamine (CH_3NH_2) were reacted to form methylcarbamoyl chloride ($CH_3NHCOCI$) plus HCl.
4. Methylcarbamoyl was then pyrolyzed to yield MIC ($CH_3N{=}C{=}O$) and HCl.
5. Finally, MIC was reacted with a slight excess of α-naphthol in the presence of a catalyst in carbon tetrachloride solvent to produce the desired pesticide carbaryl.

In the system used in Bhopal, the stored liquid MIC was transferred through pipes to charged pots weighing 1 metric ton and under 16 psi of pressure of nitrogen. These charged pots were connected to two reactors, where MIC and α-naphthol reacted to produce carbaryl. The charging of α-naphthol was done by dissolving it in carbon tetrachloride at approximately 50°C in the presence of a trimethylamine catalyst. The reaction between MIC and α-naphthol is exothermic. The temperature was maintained at 70°C for efficient production of carbaryl.

$$CH_3N{=}C{=}O + \alpha\text{-naphthol} \rightarrow \text{1-naphthyl-}N\text{-methyl carbamate (carbaryl)}$$

The alternative way to make carbaryl involves reacting α-naphthol with phosgene to generate α-naphthol chloroformate. Then, α-naphthol chloroformate reacts with methylamine to produce carbaryl.

Physicochemical Reactions with MIC

MIC can interact with a large number of molecules, as well as with itself. Indeed, 21 products were identified (Varadarajan et al., 1985) and almost 10 unidentified chemicals (Sriramachari, 2004) were detected leaking from Tank E-610. An MIC trimer, as well as other metabolites of MIC such as dimethyl isocyanurate and 2, 4-dione of MIC, were identified in autopsies of Bhopal victims. Reaction of MIC with water is important because it will occur whenever MIC comes into contact with the body or the environment, as happened in Bhopal. It is important to note that while excess of water can neutralize MIC, only small quantities of water are enough to generate heat during the reaction, which leads to the vaporization of MIC, and this actually happened in Bhopal. Some important interactions of MIC are enumerated next.

1. Polymerization (self-addition of many MIC molecules).
2. Trimerization: 3 ($CH_3N{=}C{=}O$)→trimethyl isocyanurate.
3. Dimerization is common with aromatic isocyanates, but is rare with aliphatic isocyanates like MIC.
4. Additive reactions with molecules containing reactive hydrogen species, which migrate to the nitrogen of MIC.
5. General reaction of MIC with molecules containing hydroxylic groups:

   $CH_3N{=}C{=}O + ROH \rightarrow CH_3NHCOOR$ (urethane)

6. Reactions with water:

   $CH_3NCO + H_2O \rightarrow CH_3NH_2$ (MMA) + CO_2
   $2(CH_3NCO) + H_2O$ (excess)→$CH_3NHCONHCH_3$ $(DMU)_+CO_2$
   $3(CH_3NCO)$ in excess + $H_2O \rightarrow CH_3NHCON\,(CH_3)$ $CONHCH_3$ (TMB) + CO_2
   $4(CH_3NCO) + H_2O \rightarrow DMI + (CH_3)2NH$ (DMA) + CO_2

7. Reaction with DMA:

   $CH_3NCO + DMA \rightarrow (CH_3)2N\text{-}CO\text{-}NH\text{-}CH_3$ (TMU)

8. Reaction with alcohols and phenols (used by UCIL to produce carbaryl pesticide):

   CH_3NCO + α-naphthol→1-naphthyl-*N*-methyl carbamate (carbaryl)

9. Reaction with primary and secondary amines:

   $CH_3NCO + R\text{-}NH_2 \rightarrow CH_3\text{-}NH\text{-}CO\text{-}NH\text{-}R$

10. Reaction with nitrates and nitrites:
    The reaction of MIC with nitrates and nitrites, which are normally present in water, can yield carcinogenic nitrosamines.
11. Decomposition into HCN at temperatures in the range of 427–548°C at 55–300 torr (Blake and Ijadi-Maghsoodi, 1982).
12. Reaction with HCN at normal temperatures, leading to the formation of other cyanides (Slotta and Tschesche, 1927).
13. Reactions with body constituents:
    In general, interactions between isocyanates and endogenous molecules are reversible (Tse and Pesce, 1978). MIC has been shown to cause greater interaction with macromolecules than aryl isocyanates (Brown et al., 1987). Reversible conjugation of isocyanates with glutathione (Slatter et al., 1991), which occurs both spontaneously and enzymatically, may have been the mechanism of distributing MIC molecules to different parts of the body and the reason behind its diffuse toxicity profile (Baillie and Slatter, 1991; Pearson et al., 1991). MIC can act as a hapten, which leads to the generation of antibodies in both animals and humans (Karol et al., 1987). MIC can carbamylate macromolecules (Segal et al., 1989). MIC has been shown to be an effective anti-sickling agent *in vitro*; it combines with α-amino groups of hemoglobin and thus increases its oxygen binding affinity (Lee, 1976).

Quantification of MIC

Measurement of MIC in the workplace requires the collection of samples with special tubes and then reacting it with an amino-based reagent such as 1-(2-methoxyphenyl) piperazine (2MP) or other similar substances (von Zweigbergk et al., 2002). Sampling under controlled experimental conditions can be done using gas-tight syringes. Several techniques have been used for the quantitative analysis of MIC. Methods used by Ferguson et al. (1986) and by ourselves (Varma et al., 1987) used a Perkin-Elmer Model 3920 gas chromatograph equipped with a nitrogen-phosphorus detector. The absolute retention time for MIC under these conditions was approximately 1 min and sensitivity of the method was 0.8 ng MIC.

MECHANISM OF DEATH FOLLOWING EXPOSURE TO MIC

MIC exerts a wide spectrum of toxic effects. The immediate effect of exposure to MIC in Bhopal was lacrimation, choking sensations, and difficulty in breathing, followed in many cases by death. An important element of the fatalities in Bhopal was a lag period of several hours between the exposure to MIC and death (Paintal, 1986; Varma, 1986). Most deaths occurred more than 24 h after the exposure. Delayed death was also observed in experimental animals exposed to MIC (Alarie et al., 1987; Bucher et al., 1987; Varma et al., 1988). It would thus appear that the lethal effects of MIC were caused by pulmonary complications. It was also found that a single exposure to MIC can produce long-lasting pulmonary complications (Ferguson and Alarie, 1991; Kamat et al., 1992; ICMR, 2004).

The pattern of death in experimental animals (i.e., rats, mice, and guinea pigs) following exposure to MIC was biphasic and similar to that observed in Bhopal. Even excessive concentrations (3506 ppm for 15 min) of MIC were not lethal to rats in a 10 min period. Exposure to MIC caused a significant decrease in body weight within 24–48 h (Varma et al., 1988), suggesting substantial loss of body fluid. In the only controlled experiments on humans, Kimmerle and Eben (1964) noted that subjects could not tolerate 21 ppm MIC even for a few seconds.

THE CYANIDE CONTROVERSY

In the chaos that prevailed in Bhopal following the disaster, a number of journalists, social activists, and even some scientists contended that the culprit was HCN, not MIC. HCN poisoning leads to cherry-red venous blood (which showed up in the autopsies of the victims) because oxygen is not being used by the tissues. Sodium thiosulfate is a known antidote for HCN, and because there were reports that the victims benefited from this type of treatment, that further supported the theory of HCN involvement. Also MIC can get converted into HCN at high temperatures and pressure (Sriramachari, 2004). But this was not borne out by the sum total of evidence that emerged. The physicochemical properties and toxicity profiles of MIC and HCN are shown in Table 22.2.

Cyanide is an instant killer at a certain dose level. However, deaths by pulmonary edema after a lag period (Paintal, 1986) and other toxicities observed in Bhopal victims (Varma, 1987) are not known to be caused by cyanide. Some cyanide is present in most individuals, and more so if there is smoke and fire. Any beneficial effect of sodium thiosulfate could be explained by environmental exposure to cyanide, especially because a

TABLE 22.2 Properties of MIC and HCN

Property	MIC	HCN
Molecular weight	57	27
Appearance	Liquid	Liquid/gas
Boiling point (°C)	39.1	25.7
Vapor density (air = 1)	1.97	0.95
Threshold limit value (ppm)	0.02	10.0
Lethal level 1 h exposure (ppm)	3.0	100
Concentration immediately fatal (ppm)	Undetermined	>270
Antidote	None	Sodium thiosulfate
Long-term effects	Many	None identified

majority of the victims were slum dwellers who may have been using fire to keep their homes warm, and many were chronic smokers. Cherry-red blood could have resulted from the formation of carboxyhemoglobin from exposure to atmospheric carbon monoxide.

Under controlled experimental conditions, sodium thiosulfate did not reduce the toxicity of MIC (Alarie et al., 1987; Bucher et al., 1987; Varma et al., 1988). Finally, HCN is a fast killer at concentrations >100 ppm (Goldstein et al., 1968), and the concentrations of MIC that were achieved in Bhopal would not have killed anyone if the gas actually were HCN. Moreover, HCN could not descend to the ground because, unlike MIC, it is lighter than air. Moreover, there is definite evidence of long-term effects in survivors of the Bhopal disaster (Bucher, 1987; Dhara and Dhara, 2002; Dhara et al., 2002; Sriramachari, 2004), whereas a single dose of cyanide is not known to produce such effects (Goldstein et al., 1968). Sodium thiosulfate is well known to be an effective antidote against cyanide poisoning, and it should be administered almost immediately for that purpose, rather than several days or even months later (Chen and Rose, 1956; Goldstein et al., 1968).

TOXICITY OF ISOCYANATES

Commonly used isocyanates include toluene diisocyanate (TDI), methylenediphenyl diisocyanate (MDI), naphthalene diisocyanate (NDI), and hexamethylene diisocyanate (HDI). All isocyanates are toxic (Rye, 1973) to varying degrees; TDI seems to be the most toxic.

The National Institute of Occupational Safety and Health in the United States projected as early as 1978 that approximately 50,000 to 100,000 workers would be exposed to these chemicals within 2 years (NIOSH, 1978). The routine method of exposure of workers to isocyanates is by inhalation, and their toxicity is greater following inhalation than following oral ingestion; isocyanates that produce both pulmonary and sensory irritation are more toxic than those that cause only sensory irritation (Weyel et al., 1982).

Toxicity of MIC

Experimental research on the toxicity of MIC vapor on rats, mice, rabbits, and guinea pigs, as well as on human volunteers, was first reported in 1964 (Kimmerle and Eben, 1964). For the next 20 years, though, no follow-up studies were done. It is to the credit of Kimmerle and Eben (1964) that their stringent observations were confirmed by all the studies that followed the Bhopal disaster of 1984.

It is tragic that the toxicity of poisons is tested on humans during wars, more often than not by the most developed countries. It is equally unfortunate that one finds out about human toxicity of chemicals during industrial accidents or as a consequence of environmental neglect. In this sense, Bhopal offered the most expansive opportunity to observe and investigate the toxicity of MIC on such a large scale on humans, as well as livestock and vegetation. Surprisingly, however, most of the obvious questions raised in the aftermath of the Bhopal disaster have not been answered.

Although human toxicity to MIC has only been observed following inhalation, animal experiments reveal that it is also toxic following injection; this contradicts the prediction by the visiting American team soon after the disaster that MIC is so reactive that it will be destroyed upon contact with the body. Metabolites of MIC are also toxic (Varma and Guest, 1993).

For the sake of simplicity, data on the toxicity of MIC on humans and animals are presented separately in the rest of this chapter. It is worth mentioning, however, that almost all the data derived from animal studies seem to confirm what has been observed in humans in Bhopal.

Toxicity of MIC in Animal Models

Mortality

Barely 4 h after the disaster on December 3, 1984, the streets of Bhopal were littered with dead animals—790 buffalo, 18 bullocks, 84 calves, 270 cows, 483 goats, 90 dogs, and 23 horses (Varma, 1986). According to autopsy reports, the dead animals showed swollen livers and lymph nodes, bloated digestive tracts, engorged blood vessels, edema, necrosis in lungs with blood clots, and congested hearts and kidneys (Varma, 1986). House flies survived, however, but it is not known why.

Kimmerle and Eben (1964) estimated an LC_{50} value of 5 ppm in rats following a 4 h exposure, and 21 ppm following a 2 h exposure. Unlike with cyanide, death

followed several hours after MIC exposure and continued for up to 18 days. In later studies, it was found that 10 min exposure to as much as 3506 ppm was not immediately lethal (Dodd et al., 1986), although guinea pigs died during exposure to high concentrations (i.e., greater than 500 ppm). In general, deaths following exposure to MIC occur 1–2 days later, and a second phase of mortality follows after a week or more (Alarie et al., 1987; Bucher et al., 1987; Varma et al., 1988). Guinea pigs are more sensitive to MIC toxicity than rats (Dodd et al., 1986).

Pulmonary Toxicity

Kimmerle and Eben (1964) reported that MIC caused lacrimation, mucosal irritation, and pulmonary edema in rats, mice, rabbits, and guinea pigs. Other studies also found that MIC causes both sensory and pulmonary irritation; if death did not ensue, the recovery from these pulmonary effects was very slow to occur (Ferguson et al., 1986; Alarie et al., 1987). Exposure to MIC caused concentration-dependent degenerative changes in bronchiolar and alveolar epithelium in rats and guinea pigs, resulting in the plugging of major airways and atelectasis (Nemery et al., 1985; Fowler et al., 1987), increase in lung weight (Bucher et al., 1987; Stevens et al., 1987), pulmonary (Bucher et al., 1987) and olfactory epithelial necrosis, airway obstruction, and compromised cardiopulmonary function (Tepper et al., 1987) in surviving animals.

In a retrospective study of 4,782 Bhopal gas victims and 1,190 control subjects, De (2012) found a much higher risk of developing obstructive pulmonary complications in younger subjects (age 10–29 years) exposed to MIC than older ones (age 30–60 years).

Ocular Toxicity

As was the case in humans, lacrimation has also been found to be one of the earliest effects of MIC vapor in experimental animals (Bucher et al., 1987; Varma et al., 1988). However, exposure of rats to 3, 10, or 30 ppm MIC for 2 h (which approximates the situation in Bhopal) was not found to cause any damage to the cornea, although copious lacrimation was observed up to 3 months. Similar findings have been reported in mice (Boorman et al., 1987). On the other hand, exposure of lens explants to MIC *in vitro* has been shown to cause opacity (Harding and Rixon, 1985). It is very likely that profuse lacrimation acted as a protective mechanism, by chemically inactivating MIC.

Reproductive Toxicity

Exposure of mice on day 8 of gestation (gestation period 19 days) to 2, 6, 9, and 15 ppm MIC for 3 h or 1–3 ppm for 6 h on days 14–17 of gestation caused concentration-dependent fetal loss and maternal mortality (Varma, 1987; Varma et al., 1987); lengths of different fetal bones were significantly reduced in mice following exposure to 9 and 15 ppm MIC (Varma, 1987). MIC also caused maternal and fetal toxicity in rats; pregnancy loss accompanied a sudden decrease in progesterone, although it could not be determined which of the two events occurred first (Varma et al., 1990). Given the extensive nature of MIC toxicity, it is difficult (if not impossible) to determine if MIC-induced reproductive toxicity is a direct effect on the conceptus or a consequence of general toxicity; however, several observations indicate the possibility of a direct effect.

Radio-labeled MIC rapidly reaches the fetus (Ferguson et al., 1988). Intraperitoneal injection of MIC also caused reproductive toxicity of a similar magnitude as with inhalation (Varma et al., 1990). Moreover, MIC metabolite methylamines also produced reproductive toxicity without other obvious effects on pregnant mice. Of the three amines tested, monomethylamine, dimethylamine, and trimethylamine, the last was most toxic *in vivo*, as well as in mouse embryos in culture (Varma and Guest, 1993). Interestingly, administration of trimethylamine during mouse pregnancy resulted in stunting of male but not female progeny (Guest and Varma, 1993), similar to the effect reported years later in Bhopal victims (Ranjan et al., 2003). Another metabolite of MIC, *S*-(*N*-methylcarbamoyl) glutathione (GSH) and MIC metabolite trimethylamine exerted marked toxicity on cultured mouse embryos, as well as yolk sac and limb bud (Guest et al., 1992).

Immunotoxicity, Genotoxicity, and Carcinogenic Effects

MIC has been found to generate specific antibodies in guinea pigs following both inhalation and subcutaneous injections (Karol et al., 1987). MIC was found to be genotoxic in rats (Dutta et al., 1988) and caused dose-dependent increases in sister chromatid exchange, as well as chromosomal aberrations in hamster ovary cells in addition to cell cycle delay in mice (Shelby et al., 1987). MIC has also been reported to be mutagenic in mammalian and bacterial cell cultures (Caspary and Myhr, 1986; Meshram and Rao, 1988); MIC has been estimated to have a 76.6% probability of being a genotoxic carcinogen, but only in tests with low specificity (Ennever and Rosenkranz, 1987).

Mishra et al. (2009) examined the carcinogenic potential of MIC using cultured human lung fibroblasts and found that MIC induced inflammatory response, resulting in extensive DNA damage and genomic instability.

Other Toxic Effects

MIC caused dose-dependent necrosis of rat brain cells in culture (Anderson et al., 1990); these findings show that MIC can exert its effects even in liquid media.

Exposure of mice to 1–3 ppm MIC was found to inhibit erythroid precursors, pluripotent stem cells, and granulocyte-macrophage progenitor; recovery from this inhibitory effect was found within 3 weeks after 1 ppm but not after 3 ppm (Hong et al., 1987). At higher concentrations of 6–15 ppm, MIC inhibited cell cycling in bone marrow, alveolar cells, and T lymphocytes (Shelby et al., 1987); similar data were reported by others (Tice et al., 1987). MIC can inhibit bone marrow cell proliferation in mice (Meshram and Rao, 1988).

Exposure of rats, mice, and guinea pigs to MIC vapor caused dramatic body weight decrease in the first 2 days, which was followed by incomplete to complete recovery (Dodd et al., 1986; Bucher et al., 1987; Varma, 1987). The most likely cause of the rapid decrease in body weight is fluid loss, which may also explain the increase in hematocrit.

MIC also caused an increase in creatinine kinase, hemoglobin, hematocrit, reticulocytes, neutrophils, and blood PCO_2 in rats and guinea pigs (Dodd et al., 1986), as well as a decrease in blood pH and PO_2. MIC can cause hyperglycemia, lactic acidosis, and hypothermia in rats (Jeevaratnam and Vaidyanathan, 1992). Mishra et al. (1991) exposed rats to different concentrations of MIC vapor for 8 min and measured drug metabolizing enzymes in lungs and found that aminopyrene demethylase and aniline hydroxylase activities were inhibited, but glutathione-*S*-transferase activity was increased.

Toxicity in Humans

The human toxicity of MIC has been reviewed by several investigators (Mehta et al., 1990; Dhara and Dhara, 2002; Sriramachari, 2004). Toxicity in Bhopal consisted of minor eye ailments; throat irritation and cough; severe conjunctivitis, keratitis, acute bronchitis, and drowsiness; severe pulmonary edema; convulsions followed by cardiorespiratory arrest (Kamat et al., 1985; Misra et al., 1987).

Acute Toxicity

Nonlethal Effects Eye irritation, lacrimation, choking sensations, and difficulty in breathing were first reported by Kimmerle and Eben (1964), who exposed human volunteers to MIC vapor; the observation of these workers was confirmed by thousands of the victims of the Bhopal disaster. In addition, many of the victims lost consciousness; some, but not all, regained it (Varma, 1986).

Fatal Effects Although the precise number of people who died after being exposed to MIC is still not known; our estimate, based on fatalities in 3,270 households surveyed to determine effects on pregnancy (Varma, 1987), would suggest 6,000–8,000 deaths within 24–72 h after the gas leak in Bhopal. Deaths can be attributed to pulmonary edema. As mentioned earlier, deaths did not occur as quickly as is characteristic of cyanide poisoning; rather, it happened after a delay of several hours.

Subacute and Chronic Toxicity

Mortality. Anecdotal reports suggest that the Bhopal disaster resulted in approximately 20,000 deaths over approximately 2 years. Since late deaths have been observed by several workers in animal models (various articles in EHP, 1987), it is reasonable to assume that there will be reports of late deaths in humans as well. It is very likely that severe lung damage accounted for most of these late deaths, although a contributory role of dehydration, internal hemorrhage, and other complications cannot be ruled out.

Pulmonary Complications

Examination of 500 exposed people within 3 days of the Bhopal disaster (Sharma and Gaur, 1987) identified alveolar edema and destructive lesions in 8%. A retrospective study of 978 patients found mortality in 7.14%, breathlessness and cough in 95%, irritation and choking in the throat in 46%, and chest pain in 25% (Misra et al., 1987). Evidence of necrotizing lesions in respiratory tract, as well as radiological changes and compromise in lung function, has been documented (Gupta et al., 1988; Misra and Nag, 1988). Since the prevalence of compromise in lung function was higher in the population closer to the Union Carbide plant than in the population farther away, it is very likely that this occurred as a result of exposure to the toxic gases rather than preexisting bronchitis, tuberculosis, or emphysema. A follow-up of 113 exposed patients revealed worsening of pulmonary symptoms 2 years later, forced expiratory flow (FEF) between $FEF_{25\%}$ and $FEF_{75\%}$ of forced vital declined progressively over a 2-year period (Kamat et al., 1985, 1992; Patel et al., 1987), a 1- to 7-year period (Vijayan et al., 1989; Vijayan and Kuppurao, 1993; Vijayan and Sankaran, 1996) and a 10-year period (Acquilla et al., 1996; Dhara et al., 2002). Likewise, other researchers have found a direct relationship between pulmonary function compromise and inflammatory alveolitis and the severity of exposure (Vijayan et al., 1989). A causative relationship between the intensity of exposure to toxic gases and a decrease in $FEF_{25–75\%}$ is also suggested by another follow-up study of 454 adults conducted 10 years after the disaster (Cullinan et al., 1997).

Persistent airway hyperreactivity after a single exposure to chemical irritant has been termed *reactive airways dysfunction syndrome (RADS)* (Brooks et al., 1985). There is a strong likelihood of RADS among the victims of the gas exposure in Bhopal (Nemery, 1996); however, the question of whether the exposed Bhopal population suffers from RADS has not been carefully studied.

Ocular Toxicity

There are reports that eye irritation and some level of lacrimation was a common experience of workers at the UCIL pesticide plant. Indeed, because of these frequent episodes of eye irritation, workers initially did not suspect that something unusual was happening in the early morning of December 3, 1984. It would seem that the eyes are most sensitive to MIC toxicity since eye irritation was experienced even by people who lived quite far from the plant and seem not to have experienced pulmonary and other symptoms (Varma, 1986). Exposure to MIC produced ocular burning, watering, pain, and photophobia (Dwivedi et al., 1985), as well as conjunctivitis and corneal opacity (Maskati, 1986). Within the first 2 weeks of the disaster, Andersson et al. (1988) found no case of blindness in a community-based survey; surprisingly, the incidence of photophobia and interpalpebral erosion were highest in areas where the death rates were lowest.

Follow-up studies up to 2 years after the incident revealed persistent eye watering, itching, redness, photophobia, burning, Bitot's spots, and even corneal opacity (Khurrum and Ahmad, 1987; Andersson et al., 1988). It is noteworthy that in a gas-exposed cohort of 232 children admitted to the Pediatric and Eye Ward of the Hamidia Hospital, respiratory and cardiac complications were not accompanied by equally serious eye injuries (Dwivedi et al., 1985). It is very likely that poor living conditions, which favor infection (especially in children), further worsened ocular toxicity (Dhara and Dhara, 2002). On the other hand, it was feared at the time of the accident that a large number of survivors might be left with severe visual impairment; fortunately, this does not seem to be the case, which does indicate that profuse watering, a toxic effect of MIUC, also had the effect of minimizing ocular toxicity.

Reproductive Toxicity

A follow-up study of 865 pregnant women living close to the UCIL pesticide plant at the time of the Bhopal disaster found that 379 (43.8%) did not give birth to live babies (Varma, 1987). Another follow-up of 2566 pregnant women from 18,978 households also found that 23.6% of the exposed women suffered miscarriages, as compared to 5.6% of 1218 control cohorts (Bhandari et al., 1990). Kanhere et al. (1987) found that exposure to toxic gases resulted in decreased placental and fetal weights. In addition, approximately 14% of the subjects experienced increased loss of pregnancy and infant deaths within 1 month, 2 years, and 5 years after birth, compared with 2.6–3% within the pre-accident period (Varma et al., 1990). Other effects of exposure to MIC in women include leucorrhea, suppression of lactation, pelvic inflammatory disease, and irregular menstruation (Varma, 1986). No effect on spermatogenesis was detected within 6 months after the Bhopal disaster (Daniel et al., 1987).

Genotoxicity

In a study involving 43 gas-exposed women and 40 gas-exposed men 3 years after the disaster, a significant increase in chromosomal aberration was reported; these aberrations included breaks, gaps, and dicentric rings, which were more marked in females than in males (Ghosh et al., 1990). Chromosomal aberrations (Goswami, 1986; Ghosh et al., 1990) and cell cycle abnormalities have been identified in Bhopal victims (Deo et al., 1987).

Carcinogenicity

The possibility of cancer in the Bhopal population exposed to MIC was raised by journalists. Usually, multiple contacts with a carcinogen are needed to produce cancer, but a single dose of a chemical can have the same effect (Calabrese and Blain, 1999). A cancer registry was initiated by the ICMR. However, no conclusive evidence of increase in cancer in the exposed population has been documented. Senthikumar et al. (2011) have reported a total of 1,261 cancer patients in the long-term Bhopal survivors, but they do not provide information about the size of the population examined; it is, therefore, difficult to infer whether exposure to MIC increased cancer.

Immunotoxicity

Saxena et al. (1988) studied 31 exposed adults and found a significant increase in abnormal lymphocytes; however, they saw no compromise in humoral and cellular immunity in exposed populations in Bhopal. Anti-MIC antibodies were detected in blood samples from gas-exposed subjects, but the clinical implications of this discovery are not clear (Karol et al., 1987).

Neurotoxicity and Psychological Effects

Soon after the Bhopal disaster, there were displays of bizarre drawings by the surviving children, almost all of whom depicted gusts of flames going upward—starkly different from the usual pictures painted by children. Many of these drawings reflected their loss of parents or other family members or friends. While these drawings are unlikely to be specific to MIC, they probably reflected the children's response to unanticipated horror. Psychological trauma was experienced by adults, which shared many similarities with that experienced by soldiers returning from combat missions. One study categorized post-disaster psychological impact into four categories: (i) posttraumatic stress disorder characterized by anxiety, restlessness, and sleep disorder; (ii) pathological grief reactions expressed as suicidal tendencies and helplessness at not being able to save family members; (iii) emotional reaction to physical problems imposed upon them; and (iv) exacerbation of preexisting

problems (Murthy and Isaac, 1987). A survey of 164 gas-exposed children 105 days after the disaster found them apprehensive and jittery (Irani and Mahashur, 1986).

The authors of this chapter encountered a volunteer at Sambhavana Trust Clinic, which is located near the now-decommissioned pesticide plant. This young man, whose parents died during the disaster, was perfectly normal on the many occasions we saw him, but other workers at the clinic told us that from time to time, he would become very depressed. One day in 2007, we received a message from Bhopal that he had committed suicide. There are reports of other such cases, although it is never possible to causally link such tragic events with exposure to MIC.

Sethi et al. (1987) reported that a large number of survivors suffered from organic neurological problems, including neuroses, anxiety states, and accentuation of previous psychological problems. Bharucha and Bharucha (1987) also observed neurological and neuromuscular abnormalities in both adults and children and concluded that the incidence was lower than expected following a disaster of such magnitude as occurred in Bhopal; 24 of the 47 children examined by these authors experienced coma lasting for a maximum duration of 24 h. Neurological problems have also been reported by others (e.g., Kamat et al., 1985; Gupta et al., 1988; Misra and Kalita, 1997). Raphael and Middleton (1988) have suggested that 30–59% of those exposed to a disaster may suffer from traumatic neuroses.

Other Toxic Effects

Soon after the disaster, Bhopal was flooded with people hoping for a cure, as well as vendors selling all kinds of medicines that they claimed would provide one. Some did not know what to expect in days following the accident and hoped that pills would safeguard against existing ailments like loss of appetite, weakness, and breathlessness and prevent complications. This scenario is typical of developing countries.

At present, 25 years later, victims of the disaster still occupy hospital beds and clinics and go to private practitioners of all branches of medicine, with complaints ranging from vague to very specific. In general, however, it is difficult to relate many of these symptoms, such as weakness, loss of appetite, anxiety attacks and menstrual problems, to exposure to MIC. But many subjects continue to suffer from pulmonary dysfunctions and some compromise in visual functions.

As mentioned before, most of the victims of the disaster belonged to economically disadvantaged groups and lived in poor housing with nonexistent sanitary facilities. Therefore, they already probably suffered from many chronic diseases, including bronchitis, tuberculosis, and malaria. Even so, the Bhopal disaster certainly worsened their maladies.

TREATMENT

By their very nature, disasters involving chemicals pose special problems because they concern a large number of people in a state of panic, all at the same time. Most places are not equipped to deal with such situations, especially if there is confusion about the nature of the chemical. If the chemical is a pulmonary irritant, as was the case with MIC, there is a good likelihood of suspecting cyanide poisoning, as happened in Bhopal. The other reason for mass confusion is the erroneous belief that antidotes exist for every poison.

Laypeople are not expected to know that supportive therapy is the cornerstone of managing drug overdose or poisoning, rather than antidotes; a few exceptions include cyanide, narcotic analgesics, acetaminophen, methanol, organophosphates, digitalis, and carbon monoxide. It was legitimate for the media and people in Bhopal to demand an antidote. Therefore, conveying accurate information to the panicked population was vital. In the case of Bhopal, nondisclosure of the nature of the chemical for some period of time, inaccurate information on the cause (like the mistaken belief that it was cyanide), and lack of proper treatment proved as harmful as the poison itself. For example, if people had been warned not to run away, but instead encouraged to stay still and cover their faces with wet cloth, the benefits would have been significant.

In the midst of all the confusion, the doctors in Bhopal worked out as rational a treatment as possible, which comprised of atropine, antibiotic eyedrops, and antispasmodics. Treatment of pulmonary edema requires hospitalization and positive pressure respiration; Bhopal neither had enough beds nor equipment to provide this.

Long-term treatment is also supportive and is unrelated to the initiating factors. For example, the treatment of pulmonary, ophthalmic, or neurological complications has nothing to do with whether these occur due to MIC or phosgene poisoning or due to some other cause. Cyanide, unless ingested on a regular basis from the environment or food, does not produce long-term disability if it fails to cause death.

A redeeming feature of the Bhopal tragedy was the overwhelming response of the Indian people. Hundreds flocked into Bhopal on the morning of December 3 from nearby villages, and some came from far away. In addition, doctors did a commendable job working for long hours without a break. In contrast, the medical team dispatched by Union Carbide arrived 10 days later and tried to assure the public that MIC would be destroyed rapidly and that no long-term effects were to be expected. The government of India could not summon a high-level medical team to deal with the disaster and failed miserably in following up as well. Voluntary groups and nongovernmental organizations shared a major burden

of reassuring people and helping with both treatment and rehabilitation.

TOXIC POTENTIAL OF MIC BEYOND THE BHOPAL DISASTER

The Bhopal incident was the first case of mass exposure of humans, animals, and vegetation to MIC. Several factors influenced the toxicity of MIC, such as the living conditions of the victims. Under identical conditions in a developed country, the consequences most likely would have been different. At the same time, the exposure of the Bhopal population was to a specific concentration of MIC and for a specific duration.

The accidental release of MIC can happen wherever the chemical is stored. A minor leak occurred from the Union Carbide plant in West Virginia only a few months after the Bhopal disaster, and a school in the area had to be evacuated. Exposures to MIC at higher concentrations and for longer durations than in Bhopal can also occur, and these can be fatal to a substantially greater percentage of population regardless of where they happen. The fact that MIC is heavier than air makes it a potentially highly hazardous chemical, both in enclosed and open spaces.

If animal experiments were solely directed to answer questions relating to the Bhopal disaster, the use of excessively high concentrations of MIC (Dodd et al., 1986; Fowler et al., 1987) and repeated exposures would not have been very relevant. However, workers are likely to encounter repeated exposure to MIC; indeed, anecdotal reports suggest that the impending disaster was not suspected because workers were used to minor leaks (and consequently, eye irritation) in the Union Carbide plant.

No workers died inside the plant in Bhopal because MIC spewed outside the factory; however, an accident worse than Bhopal cannot be ruled out, especially if the space is enclosed.

The confusion caused by the lack of transparency about the identity of the poison by the concerned authorities at the Union Carbide headquarters and conflicting instructions only worsened the tragedy. It can be assumed that whenever a disaster of this kind of magnitude occurs, rumors are likely to come out. To counter this, it is vital that the facts about such incidents be made public by management as soon as possible.

BENZYL CHLORINES AND OTHER CHEMICALS AT BHOPAL

MIC and many other chemicals were stored in Bhopal. The disaster in 1984 led to the closure of the UCIL factory. While the remaining MIC was disposed of during Operation Faith, Union Carbide closed its Bhopal location, and several hundred metric tons of hazardous waste stored in open areas and sheds were not destroyed. The first study on this topic, by Dikshith et al. (1990), demonstrated the presence of 1-napathol in the soil and water in Bhopal. In all, 15 other studies were conducted between 1990 and 2010 by groups like Greenpeace International, Centre for Science and Environment (CSE), and National Environmental Engineering Research Institute (NEERI), to address the issues of whether the stored pesticides and chemicals on the factory grounds, solar evaporation ponds, and soil surrounding the factory contaminated the drinking water, and if so, which chemicals were present in levels that exceeded permissible levels. There is close agreement about the extent and type of contamination among several studies. In studies by CSE (Johnson et al., 2009; CSE, 2013), the maximum concentration found in soil samples from specific locations like the storage, processing, and dump areas recorded contamination with carbaryl (51,003 ppm), aldicarb (7,876 ppm), dichlorobenzene (2,049 ppm), HCH isomers (99,700 ppm), and α-naphthol (9,914 ppm), as well as heavy metals like mercury (128,000 ppm), lead (406 ppm), and chromium (1,065 ppm). The NEERI report downplayed contamination of groundwater, stating that it most likely occurred due to runoff from the material dumped in the pesticide plant; but the CSE report (2013), along with several other studies, concluded that the groundwater was contaminated significantly with chemicals. All the reports acknowledged that there was very little information on the contaminants in the solar evaporation ponds where factory waste was discharged during the entire operation of the UCIL plant (CSE, 2013). Both the CSE and the NEERI reports documented the type and amount of chemicals dumped within the factory premises from 1969 to 1984. What is most surprising is that none of these reports were published in mainstream journals.

Why remediation of the site has not occurred is no secret. The cost of incineration of over 1 million metric tons of contaminated soil would be well over 10,000 million rupees, and no one—neither the Indian government nor Dow Chemical, which bought Union Carbide in 2001—was prepared to pay the bill. So to date, very little has been done to get rid of the contaminated soil, let alone treat the contaminated water. In addition, the area surrounding the old UCIL plant has gained in population (now over 40,000 people), and these people have been drinking the chemical-laced water for 5–10 years, or even longer (up to 2 decades). There is concern that these chemicals (some known to be teratogens) might increase the incidence of birth defects. Indeed, a systematic study by the authors is underway to document the effect on people of exposure to contaminated water, gas, or both, as compared to people who were not exposed to any toxic substances. We anticipate the completion of this study by the end of 2014.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We have described the chemistry, pharmacology, and toxicology of MIC in the context of more commonly used diisocyanates. Combatants and civilians have been subjected to lethal and debilitating chemical agents during war. However, no poisonous chemical other than MIC has the dubious distinction of killing nearly 8,000 people within 72 h, and many more in the subsequent years and maiming civil society on a scale as large as what happened in Bhopal in peacetime. Unlike the September 11, 2001 terrorist attack in the United States, where the total number of casualties is accurately known, no one is still certain about the exact death toll from the Bhopal incident, whether immediately afterward and in the subsequent months and years; unfortunately, the same can be said about most disasters in the developing part of the world. While acute deaths were most likely caused by pulmonary edema, only a well-planned epidemiological study coordinated by official agencies could have determined the nature and the magnitude of long-term effects. Unfortunately, such a study was not done, and many of the long-term effects cannot be identified retroactively. Carcinogenicity and genotoxicity requires long-term follow-up of a large population. So far, the results have been disappointing.

MIC toxicity amply demonstrates that the full dimension of the pharmacology of a chemical cannot be predicted from its chemical structure, but it can be approximated by careful and painstaking research. Such an inquiry into MIC would be advisable.

India may be the 12th-largest economy in the world; however, the victims of Bhopal do not benefit from that fact. Just in the period of February to May 2008, hundreds of women, men, and children marched a distance of 800 km to the Indian capital of Delhi to demand clean water, appropriate therapy, and rehabilitation. The last time such a march happened in 2006, some promises were made, but they were not fulfilled. What will happen this time? Only time will tell.

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CHAPTER

23

Cyanide Toxicity and its Treatment

Rahul Bhattacharya and Swaran J.S. Flora

INTRODUCTION

Cyanide refers to a highly toxic chemical compound containing one atom of carbon and nitrogen each. Deaths caused by cyanide poisoning are relatively rare, largely because of its limited availability (Musshoff et al., 2002). However, the name cyanide and its toxic characteristic have long been a source of dread for humans. The first reported isolation of hydrocyanic acid from Prussian blue was by the Swedish chemist C.W. Scheele in 1782, and he was reported to be the first victim of this poison in 1786 when he accidentally broke a vial of the chemical in the laboratory and died from vapor poisoning (Ballantyne, 1987).

Since the days of ancient Rome, cyanide and its derivatives were used in weapons (Sykes, 1981). Nero used cherry laurel water, which contained cyanide as its main toxic component, to poison members of his family and others who displeased him. Napoleon III proposed the lacing of soldiers' bayonets with cyanides during the Franco-Prussian War. Hydrogen cyanide (HCN) was not very successful as a chemical warfare agent during World War I because it was not persistent. During the same period, the French army used artillery shells under the name Vincennite (50% HCN, 30% arsenic trichloride, 15% stannic chloride, and 5% chloroform) but because of high volatility of HCN, these munitions could not be used effectively. Also, German soldiers were adequately equipped to protect themselves from HCN exposure. In 1916, the French experimented with cyanogen chloride, which was heavier and less volatile than HCN. The effect of cyanogen chloride was similar to HCN. Additionally, it caused marked lacrimation, rhinorrhea, and bronchial secretions similar to phosgene. However, these effects were considered to be of little military importance compared with its tissue effects. During World War II, the Nazis used Zyklon B (briquettes of calcium sulfate impregnated with 40% HCN) made by IG Farben, Germany, to exterminate millions of civilians and enemy soldiers in the death camps (Robinson, 1971; Baskin, 1998). It is also reported that in the late 1980s, there was the possible use of cyanide against the inhabitants of the Syrian city of Hama, the inhabitants of the Kurdish city of Halabja, Iraq, and in Shahabad, Iran, during the Iran–Iraq war (Baskin and Rockwood, 2002). Cyanide gas precursor compounds were found in several subway restrooms in Tokyo after the release of sarin in Tokyo in 1995 (Sidell, 1996). Allegedly, cyanide was added to the explosives used in the first attack on the World Trade Center in New York (Brennan et al., 1999).

The use of cyanides for murder and suicide and accidentally use have been passably reviewed by Gee (1987). One hundred nine passengers in 1973 and 303 pilgrims in 1980 were feared killed in Paris and Riyadh, respectively, because of inhalation of HCN from smoldering plastic inside aircrafts that accidentally caught fire (Mohler, 1975; Weger, 1983). In 1985, the explosion of a Boeing 737 during take-off at Manchester, England, revealed that 20% of the 137 victims who escaped had dangerously high levels of carbon monoxide, whereas 90% had significantly elevated levels of cyanide. Similarly, short circuits in electrical wires also caused fire on ships that led to the combustion of plastic materials releasing HCN, which resulted in severe poisoning (Levine et al., 1978). The murders of two Ukrainians, Rebet (1957) and Bandera (1959), were committed by a Soviet agent in Munich using a gas pistol containing cyanide (Anders, 1963). In September 1982, seven people in Chicago died when cyanide was illicitly placed in bottles of Tylenol (acetaminophen manufactured by McNeill Consumer Products Co., Fort Washington, PA) (Wolnick et al., 1984). In 1978, 912 members of a religious sect in Port Kaituma, Guyana, committed mass suicide after consuming a grape-flavored drink laced with cyanide (Thompson et al., 1987; Sidell et al., 1997). Some other incidents of illicit cyanide use include cyanide

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gas–producing devices in Tokyo subway and railway station restrooms in 1995 (Okumura et al., 2003), ingestion of cyanide tablets by Michael Martin on receipt of a guilty verdict for arson in June 2012 (Davenport, 2012), and the death of Urooj Khan, a lottery winner, in Chicago in July 2012 (Keyser, 2013). The first case of illegal euthanasia in Spain was reported when a tetraplegic patient whose repeated legal requests for euthanasia were refused consumed potassium cyanide (KCN) (Blanco and Garcia, 2004).

The toxicology of cyanide and its treatment modalities have been extensively discussed by many authors (Vennesland et al., 1981; Way, 1984; Ballantyne and Marrs, 1987; Gonzales and Sabatini, 1989; Borowitz et al., 1992; Salkowski and Penney, 1994; Marrs et al., 1996; Baskin and Brewer, 1997). Additionally, over the years thousands of research articles on cyanide have appeared. Cyanide, being such an old poison, and its effects seem to be known universally. But most of the crucial molecular mechanisms underlying cyanide toxicity have been elucidated only in the recent past (Gunasekar et al., 1996; Sun et al., 1997; Borowitz et al., 2001). Cyanide is a very interesting molecule; it has always fascinated researchers and is the reason for further study. This chapter attempts to enlighten the reader with the known facts about cyanide and some of the recent advances made in its toxicology and antagonism.

SOURCES OF EXPOSURE

The use of cyanide for military purposes has been very insignificant and most of the cases of poisoning have involved civilians, for example, in the case of fires and industrial accidents. The human–cyanide interaction is very common because of its wide industrial applications. Worldwide industrial consumption of cyanide is estimated to be 1.5 million tons per year, and occupational exposures account for a significant number of cyanide poisonings (Cummings, 2004; Logue et al., 2010; Coentrão and Moura, 2011). Cyanide poisoning may occur from a broad range of exposures and is summarized in Table 23.1. Some of the major sources of poisoning are as follows.

TABLE 23.1 Various Possible Sources of Cyanide Poisoning

Fire smoke	Smoke generated after combustion of silk, polyurethanes, polyacrylonitriles, nylon, melamine resins, plastics, etc., in accidents including industrial, residential, car, aircraft, ship fire
Industrial exposure	Plastics production, dyeing, printing and photography, fumigation of pesticides/rodenticides, synthetic rubber production, fertilizer production, metal polish, tanning in leather industry, electroplating, metallurgy, paper and textile manufacture
Drugs	Sodium nitroprusside, Laetrile, Succinonitrile
Dietary	Cassava, lima beans, linseed, bamboo sprout, macadamia nuts, hydrangea, Rosaceae family (plum, peach, pear, apple, bitter almond, cherry), *Sorghum* species (Johnson grass, sorghum, Sudan grass, arrow grass), *Linum* species (flax, yellow pine flax)
Others	Cigarette smoking, phencyclidine synthesis, ingestion of nail polish remover, suicide, homicide, terrorist attack, chemical warfare, capital punishment

Fire Smoke

Residential fires may result in life-threatening poisoning (Alarie, 2002; Megarbane et al., 2003). In a closed space, fire induces a combination of oxygen deprivation directly related to combustion and simultaneous intoxication by asphyxiant and irritant gases (Baud, 2007). Cyanide can be liberated during combustion of products containing carbon and nitrogen. These products include wool, silk, polyurethane, polyacrylonitriles, melamine resins, and synthetic rubber (Vogel et al., 1981; Bismuth et al., 1987; Homan, 1987). It is estimated that significant levels of cyanide are present in almost 35% of all fire victims (Clark et al., 1981; Barillo et al., 1994; Sauer and Keim, 2001). Carbon monoxide is also an important component of fire smoke and its concomitant exposure with HCN is known to exacerbate the toxicity because both cause tissue hypoxia by different mechanisms (Barillo et al., 1994).

Industrial Exposure

Occupational exposures to cyanide in industry have caused serious problems (Blanc et al., 1985; Peden et al., 1986). Cyanide is used in many chemical syntheses, plastics and rubber processing, gold and silver extraction, tanning, metallurgy, photography, and fumigation of pesticides/rodenticides (ATSDR, 1997). Cyanide poisoning can also occur after contamination of the skin with cyanide solution or inhalation of released HCN during electroplating in jewelry polishing (Sullivan and Krieger, 2001; Megarbane et al., 2003). Exposure to silver brightener, which contains cyanide, has also caused death. Subsequent to initial lethal inhalation of cyanide, an extensive postmortem transcutaneous diffusion of cyanide was also shown to occur (Seidl et al., 2003). Cyanogens are complex nitrile-containing materials that can generate free cyanide of toxicological significance. Many of the cyanogens occur naturally or are produced synthetically. The synthetic cyanogens have a variety of industrial, domestic, and therapeutic uses (Ballantyne, 1987). Some of the synthetic cyanogens include acetonitrile, acrylonitrile, adiponitrile, malononitrile, propionitrile, succinonitrile, sodium nitroprusside, and

tetremethyl succinonitrile. Fires involving nitrogen-containing polymers, often found in fibers used in fabrics, upholstery covers, and padding, produce HCN (Tsuchiya and Sumi, 1977).

Drugs

Cyanide is a metabolic product of amygdalin (Laetrile®) that was introduced as an anti-neoplastic agent in the 1950s and was responsible for several cyanide poisoning cases (Hall et al., 1986; Bromley et al., 2005). Intestinal beta-D-glucosidase digests the amygdalin, releasing HCN. Also, iatrogenic exposure to cyanide may result after the use of sodium nitroprusside, an anti-hypertensive agent (Vesey and Cole, 1985), and succinonitrile, an anti-depressant (Ryan, 1998). Sodium nitroprusside is used medicinally as Nipride®, and its intravenous infusion is used to lower blood pressure in hypertensive emergencies. This application of sodium nitroprusside occasionally causes classical cyanide toxicity (Kurt, 1983). Death caused by mercuric cyanide or mercuric oxycyanide poisoning was reported because of possible ingestion of an antiseptic or a hair lotion commercialized in France (Labat et al., 2004).

Dietary

Cyanide occurs naturally in cassava (*Manihot esculenta* Crantz) as linimarin, a cyanogenic glycoside. Cassava roots are a major source of energy for millions of people in the tropics. However, chronic ingestion of cassava-based foods accounts for several toxicological effects, including various neuropathies and amblyopia (Rosling, 1989; Kamalu, 1995). Several common plants also contain cyanogenic glycosides and their ingestion can result in death attributable to improper processing (Conn, 1988). The family Rosaceae, which includes plum, peach, pear, apple, bitter almond, and cherry, is responsible for many cases of cyanide poisoning (Ryan, 1998).

Other Sources

There are numerous other instances of cyanide poisoning that include illicit synthesis of phencyclidine, ingestion of nail polish remover (acetonitrile), use in terrorist attacks, use as suicidal, homicidal, and chemical warfare agents, and use in capital punishment. HCN was considered to be a notorious chemical warfare agent because of its rapid lethal actions. It was used as a war gas in World War I (Gee, 1987), in World War II (Williams and Wallace, 1989), and in the Iran–Iraq War (Lang et al., 1986; Heylin, 1988). Because of poor persistence of HCN in the environment and lack of delivery system, cyanides were not optimistically considered for future use in wars. However, its possible use in local terrorist actions cannot be overlooked. Because cyanide can be released from both synthetic and natural materials, terrorist acts with explosives or incendiaries, which could cause fire in enclosed spaces, make cyanide and cyanogenic compounds potential threats for terrorism (Rotenberg, 2003). During the past decade, Japan has experienced the largest burden of chemical terrorism-related events in the world, which included the Nagano cyanide incident in 1998 and two intentional cyanide releases in Tokyo subway and railway station restrooms, which were thwarted in 1995 (Okumura et al., 2003). Because of the natural cyanide found in tobacco, cigarette smokers show a significant increase in blood cyanide levels as compared with nonsmokers. Visual abnormalities of tobacco amblyopia are usually associated with heavy smoking and vitamin B_{12} deficiency (Wilson, 1983; Homan, 1987).

TABLE 23.2 Toxicity of Hydrogen Cyanide by Inhalation

Concentration		
mg/m^3	ppm	Effects
20–40	18–36	Slight symptoms after several hours
50–60	45–54	Endurable for 20–60 min without effect
120–150	110–135	Very dangerous (fatal) after 30–60 min
150	135	Lethal after 30 min
200	181	Lethal after 10 min
300	270	Immediately lethal

TOXIC LEVELS OF CYANIDE

It is not easy to determine the lethal doses of cyanide in humans. Morbidity or mortality depends on the magnitude of poisoning, which varies with the dose and form of cyanide and the route of poisoning. The maximum permissible concentration for HCN in human is 11 mg/m^3 (Ballantyne, 1974). Taken orally, the fatal dose of HCN to adults is estimated at 50–100 mg, and for KCN it is approximately 150–250 mg (DuBois and Geiling, 1959). Figures estimated in terms of body weight for HCN vary from 0.7 to 3.5 mg/kg (Hallstrom and Moller, 1945). However, victims ingesting as much as 3.0 g of KCN have been saved with immediate therapy (Van Heijst et al., 1987). Table 23.2 shows that inhalation of HCN at a concentration of 300 mg/m^3 (approximately 270 ppm) will be immediately fatal, whereas at 20–40 mg/m^3 mild symptoms will appear after several hours of exposure (Rumack, 1983; FOA, 1992). Victims with a blood cyanide level of 2.5–3.0 µg/mL frequently succumb to respiratory cessation within 20–30 min of exposure or may survive up to 3 h (Ballantyne, 1974;

TABLE 23.3 Acute Lethal Toxicity of KCN by Oral Route for Different Species of Animals

Species	Sex	LD_{50} (mg/kg)	Reference
Mouse	Male	8.50	Sheehy and Way (1968)
	Male	12.5	Bhattacharya et al. (2002)
Rat	Male	10.0	Hayes (1967)
	Female	7.49	Ballantyne (1984)
	Female	14.1	Bhattacharya and Vijayaraghavan (2002)
Rabbit	Female	5.82	Ballantyne (1984)

Van Heijst et al., 1987). Oral ingestion of cyanide may also produce rapid onset of symptoms because many human doses far exceed the minimal lethal dose. Studies of experimental animals have shown that absorption of cyanide decreases with a more alkaline stomach and that normally most cyanide is absorbed within 2–3 h of ingestion (Ryan, 1998). However, substances such as nitrile compounds and amygdalin from plants require enough time for conversion to cyanide before they can produce symptoms of toxicity (Ryan, 1998). Laetrile is synthesized from amygdalin. One gram of laetrile contains the equivalent of 60 mg of cyanide, and each laetrile tablet may contain up to 100 mg of laetrile. A 12-table to 18-tablet laetrile overdose is sufficient to produce severe metabolic acidosis and convulsions (Ellenhorn et al., 1997). The lethal toxicity of HCN and its alkali salts by different routes for different species of animals and sexes has been largely discussed elsewhere (Ballantyne, 1984, 1987). A study performed using rabbits revealed the following order of decreasing toxicity of KCN administered by different routes: intravenous > intramuscular > intraperitoneal > per oral > instillation into conjunctival sac > percutaneous (Ballantyne, 1984). The comparative per oral LD_{50} values of KCN for different species of animals are given in Table 23.3. Rabbits were found to be more susceptible than rats and mice.

DETECTION AND ESTIMATION OF CYANIDE

Determination of cyanide or its metabolites in biological fluids is necessary for forensic, clinical, military, research, and veterinary purposes. The choice of analytical methods depends on a variety of factors like sensitivity, specificity, rapidity, convenience, facilities, and expertise. The selection of biological sample, time of sampling, time to analyze and storage conditions, and interfering substances are other factors that influence the choice of analytical methods (Troup and Ballantyne, 1987; Jackson et al., 2014). There are several convenient and sensitive methods for measuring cyanide in biological fluids, but many of them have limitations. Some of the methods are summarized here.

One of the most common procedures includes diffusion and trapping of cyanide in the alkaline media before colorimetric analysis in pyridine–pyrazolone mixture (Epstein, 1947). This method was subsequently modified for microdiffusion analysis of cyanide (Feldstein and Klendshoj, 1954). This procedure is widely used during the treatment of cyanide intoxication and thiosulfate is known to interfere in the colorimetric estimation, which was subsequently resolved (Morgan et al., 1979). A rapid (approximately 20 min), specific, and sensitive spectrophotometric method for whole blood cyanide assay has also been developed (LaForge et al., 1994). All these procedures are based on the König reaction, which starts with the production of cyanogen chloride. A spectrofluorometric determination of cyanide and thiocyanate based on modified König reaction in a flow-injection system was also reported with detection limits of 30 nM for both anions (Tanaka et al., 1992). Spectrophotofluorometry is also a convenient, sensitive method provided that previous microdiffusion has been performed to isolate and concentrate the cyanide. The fluorometric methods using pyridoxal are more sensitive and require fewer and more stable reagents than the colorimetric method (Takanashi and Tamura, 1970). However, sodium thiosulfate is known to interfere with the chemical conversion of the fluorophore and it is possible to circumvent the interference by using acetate buffer (pH 5.2) as the acidifying agent. Another fluorescent method with an advantage over pyridoxal uses parabenzoquinone, and this method is not known to have any extraneous interference (Guilbault, 1976).

The potentiometric determination of cyanide using ion-selective electrodes has become yet another very popular technique because it is a convenient, rapid, and sensitive method of analysis (Frant et al., 1972). Microdiffusion of biological samples containing cyanide is recommended before potentiometric determination. The use of a cyanide ion–selective electrode in combination with the Conway microdiffusion method for the measurement of cyanide concentrations in human red blood cells (RBCs) and plasma was reported with remarkable recovery of cyanide (Yagi et al., 1990). Ion chromatographic determination of sulfide and cyanide in real matrices by using pulsed amperometric detection on a silver electrode was reported by Giuriati et al. (2004).

The measurement of HCN directly by gas chromatography has also been reported, but this method lacks sensitivity with most detectors (Valentour et al., 1974). Gas chromatographic techniques are not widely used for measuring cyanide because other methods are more convenient. A simple and sensitive method was devised for determining cyanide and its major metabolite,

thiocyanate, in blood using an extractive alkylating agent (pentafluorobenzyl bromide). The detection limits of cyanide and thiocyanate were 0.01 and 0.003 µmol/mL, respectively (Kage et al., 1996). Rapid quantitation of cyanide in whole blood by automated headspace gas chromatography was performed on clinical samples from fire victims. This method could detect a wide concentration of blood cyanide (30–6,000 µg/L) in approximately 17 min (Calafat and Stanfill, 2002).

A direct and sensitive isotope dilution–mass spectrometry determination of blood cyanide by headspace gas chromatography was developed with the detection limit of 0.3 µmol/L (Dumas et al., 2005). This method was also compared with other techniques in a round-robin exercise. Cyanide can also be measured by indirect atomic absorption spectrometry where a metal–cyanide complex is formed and is then extracted in organic solvent to determine the metal content (Manahan and Kunkel, 1973). An original high-performance liquid chromatographic–mass spectrometric (HPLC-MS) procedure was developed for the determination of cyanide in whole blood. The limits of detection and quantitation were 5 and 15 ng/mL, respectively (Tracqui et al., 2002). Also, several other methods including HPLC, using post-column derivatization with *o*-phthalaldehyde (Sumiyoshi et al., 1995), capillary electrophoresis with fluorescence detection (Chinaka et al., 2001), polyphenol oxidase/clay biosensors (Shan et al., 2004), capillary electrophoresis microchip (Lu et al., 2004), ICT-based probes (Badugu et al., 2005), and microchemiluminiscence (Lv et al., 2005) have been reported for different environmental or biological samples. However, their utility to detect cyanide in blood samples is yet to be ascertained. A technique to detect cyanide currently utilized by water treatment facilities was used to rapidly detect concentrations of cyanide in the clinically important range (Rella et al., 2004). The CYANTESMO test strips accurately and rapidly detect cyanide more than 1 µg/mL. A paper test for cyanide (CYANTOSNO) in whole blood is now commercially available in the United States (Ellenhorn et al., 1997).

TOXICOKINETICS OF CYANIDE

Absorption

Oral absorption of cyanide is rapid, and toxic effects can occur within minutes. When salts of cyanide are ingested, hydrochloric acid in the stomach causes the release of HCN, which is readily absorbed as cyanide ion (CN^-). Cyanide (hydrocyanic acid [HCN]) is a small molecule with good lipid and water solubility. It is rapidly absorbed irrespective of route of exposure because of its low molecular weight (Borowitz et al., 2001). It is mainly absorbed by the respiratory and gastrointestinal mucosa and through skin and eyes on prolonged contact (Ballantyne, 1974; Ellenhorn et al., 1997; Ryan, 1998). In experimental rabbits, cyanide introduced into the conjunctival sacs was quickly absorbed in significant quantities to produce systemic toxicity (Ballantyne, 1983). Dermal exposure is rare, but the large surface area of the skin facilitates absorption of toxic consequences. Nitriles are more readily absorbed through the skin but the onset of toxicity is delayed.

Distribution

After absorption, cyanide distributes to a volume of approximately 40% total body weight. The distribution is rapid and is completed within 5 min after a single intravenous dose (Sylvester et al., 1983). Cyanide is rapidly transported in the body by blood and approximately 60% is bound to plasma proteins, a small amount is present in the RBCs, and the remainder presents as free cyanide (Ryan, 1998). The concentration of cyanide in RBC is estimated to be at a RBC:plasma ratio of 100:1 (Ellenhorn et al., 1997). After acute exposures, the plasma elimination half-life of cyanide was observed to be 14.1 min (Egekeze and Oehme, 1979). After oral poisoning, a significant amount of cyanide was traced in the brain, blood, kidney, stomach wall, liver, and urine (Ansell and Lewis, 1970). This indicates that after absorption, cyanide is widely distributed in all the tissues.

Elimination

Cyanide is rapidly detoxified in mammals. The detoxification rates of cyanide after intravenous administration in humans, dogs, guinea pigs, and rabbits are 0.017, 0.020, 0.04, and 0.008 mg/kg body weight per minute, respectively (Hinwich and Saunders, 1948; NIOSH, 1975). The major pathway of cyanide detoxification (approximately 80%) is through enzymatic transulfuration to thiocyanate (SCN^-), which is nontoxic and excreted in the urine (Lang, 1933). This reaction is catalyzed by an enzyme, rhodanese (thiosulfate cyanide sulfur transferase; EC.2.8.1.1). Rhodanese uses a precursor like thiosulfate, which is a source of sulfane sulfur (divalent ionized sulfur bound to another sulfur atom). The endogenous supply of this substance is very limited. Therefore, detoxification of cyanide largely depends on an exogenous supply of thiosulfate (Westley et al., 1983). However, cyanide detoxification by this pathway is often debated because rhodanese is located principally in the mitochondria and penetration of the cell wall and mitochondrial membrane by thiosulfates is very slow (Bhat and Linnell, 1983). It is presumed that the sulfane sulfur binds first to the serum albumin to yield a sulfane sulfur albumin complex that eventually reacts with cyanide to form thiocyanate (Westley et al., 1983; Way, 1984).

In normal metabolism of cyanide, the serum albumin–sulfane complex may be the primary detoxification mechanism (Sylvester et al., 1983). Unlike other chemical warfare agents, cyanide appears biologically in blood, urine, and expired breath (Lundquist et al., 1988). Cyanide is eliminated from the body by several mechanisms. After 3h, approximately 90% of injected cyanide has been shown to be eliminated in the dog model (Sylvester et al., 1983). A small amount of cyanide is excreted in the urine and via the lungs after being incorporated into cyanocobalamin (vitamin B_{12}), oxidated to formate and carbon dioxide, and incorporated with cystine (Ballantyne, 1987). Other minor pathways of detoxification include enzymes like mercaptopyruvate sulfur transferase, thiosulfate reductase, and cystathionase γ-lyase or disulfide cystine, and 2-iminothiazolidine-4-carboxylic acid (2-ICA) or its tautomer, 2-aminothiazolidine-4-carboxylic acid (2-ACA) (Wood and Cooley, 1956; Baskin et al., 2004). Cyanide reacts with cystine to produce β-thiocyanoalanine, which spontaneously undergoes ring closure to form 2-ICA and 2-ACA, depending on the pH in the cells (Borowitz et al., 2001). To determine the effect of species on cyanide metabolism, toxicokinetics of cyanide was studied in rats, pigs, and goats after oral dosing of KCN (Sousa et al., 2003). The study showed that metabolism of cyanide and its main metabolite, thiocyanate, is species-linked, with goats being the most sensitive to the toxic effects of cyanide.

MECHANISM OF ACTION

The toxic effect of cyanide is attributed predominantly to the production of anoxia after inhibition of the metal-containing enzymes. The critical interaction appears to be the inhibition of the terminal respiratory chain enzyme, cytochrome oxidase a_3 (containing iron), within the mitochondria. The enzyme is essential for the production of adenosine triphosphate (ATP). As a result, aerobic oxidative metabolism and phosphorylation are impaired, leading to cellular hypoxia. The pyruvate that is produced can no longer be used and is now reduced to lactate. The shunt from aerobic to anaerobic metabolism leads to profound lactic acidosis (Solomonson, 1981). Cyanide toxicity may not be ascribed solely to a single biochemical lesion but rather to a complex phenomenon. Cyanide reacts with several metalloenzymes, carbonyl groups of different enzymes, coenzymes, and substrates, resulting in inhibition of normal activity. Cyanide also interacts with sulfhydryl compounds like cystine, mercaptopyruvate, reduced glutathione, and oxidized glutathione to form different complexes (Way, 1984; Borowitz et al., 1992). Cyanide also strongly interacts with iron in protein molecules, inhibiting enzymes including carbonic anhydrase and succinic dehydrogenase (Ballantyne, 1987). Formation of cyanhemoglobin by interaction of cyanide with ferric iron abolishes the ability of hemoglobin to carry oxygen (Way, 1984). The mechanism of cyanide toxicity is summarized in Figure 23.1.

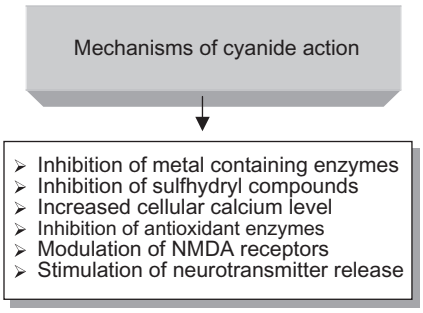

FIGURE 23.1 Mechanisms for the toxic manifestations of cyanide exposure.

Cyanide is regarded as a selective neurotoxin and its toxicity has frequently been associated with elevated levels of cellular calcium (Johnson et al., 1986), inhibition of antioxidant defense enzymes in the brain (Ardelt et al., 1989), and generation of reactive oxygen species (ROS), leading to lipid peroxidation (Ardelt et al., 1994; Kanthasamy et al., 1997). Cyanide increases intracellular calcium through activation of voltage-sensitive calcium channels (Johnson et al., 1987), direct redox modulation and enhancement of *N*-methyl-D-aspartate (NMDA) receptor function (Patel et al., 1992; Sun et al., 1997), and mobilization of intracellular calcium stores (Yang et al., 1997). It was proposed that elevated cytosolic calcium activates proteases, which in turn converts xanthine dehydrogenase to xanthine oxidase. In the presence of oxygen, xanthine oxidase can catalyze the formation of superoxide radicals that initiate lipid peroxidation (Figure 23.2) (Chan and Fishman, 1985). Modulation of NMDA receptor has been widely implicated in cyanide-induced neurotoxicity. Nitric oxide and ROS generation after NMDA receptor activation was found to mediate cyanide-induced neurotoxicity (Gunasekar et al., 1996). Several other studies also showed activation of NMDA receptors during cyanide toxicity (Patel et al., 1992; Sun et al., 1997). Cyanide can stimulate release of glutamate from intracellular stores, resulting in elevation of cytosolic Ca^{2+} through NMDA receptor activation. There is also evidence that cyanide may interact directly with NMDA receptor to enhance NMDA receptor–mediated Ca^{2+} influx (Patel et al., 1994; Sun et al., 1995). It was further shown that cyanide selectively interacted with NMDA subunits, possibly by formation of thiocyanate adduct with a cysteine residue located in NR1 receptor subtype (Arden et al., 1998). Cyanide is also known to interact with cystine to produce 2-ICA and 2-ACA, and the former is responsible for memory loss, convulsions, and loss of consciousness (Bitner et al., 1991). Cyanide

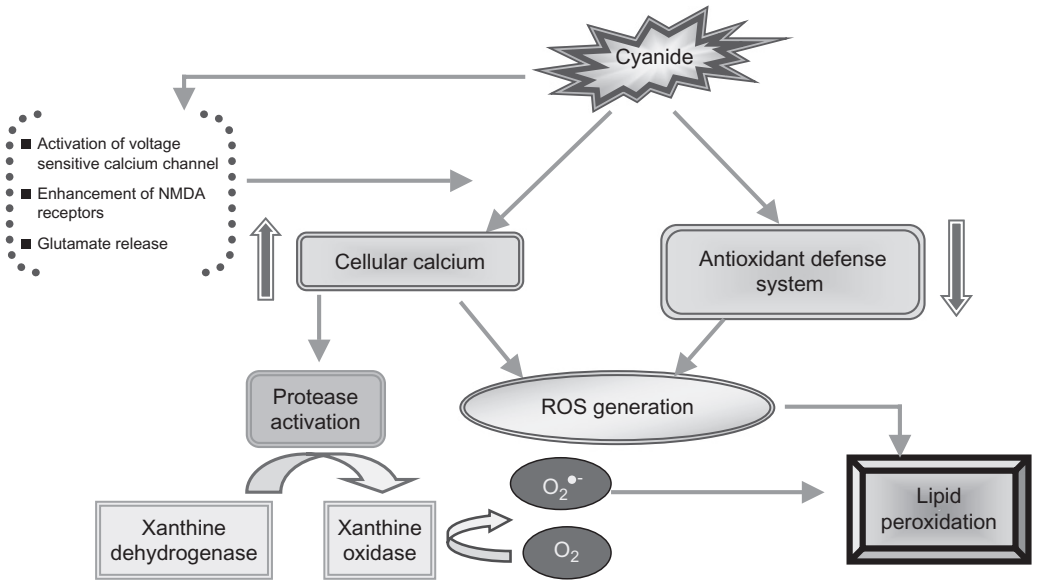

FIGURE 23.2 Pathway of cyanide-induced oxidative stress. Abbreviations used: ROS, reactive oxygen species; O_2, oxygen; $O_2^{\bullet-}$, superoxide anion.

can produce dopaminergic toxicity characterized by loss of dopaminergic neurons in the basal ganglia that is accompanied by impaired motor function (Kanthasamy et al., 1994). Cyanide is also known to stimulate neurotransmitter release in both the central and peripheral nervous systems (Kanthasamy et al., 1991). It is suggested that the convulsive effects of cyanide are due to changes in the levels of dopamine (Cassel, 1995). It has also been opined that protein kinase C, calmodulin, and nitric oxide (NO)-dependent cyclic guanosine monophosphate-dependent enzymes may contribute to the induction of convulsions. ATP depletion may also contribute, in part, to the development of cyanide-induced convulsions (Yamamoto, 1995). Other biochemical processes that may mediate or at least influence cyanide toxicity include lactic acidosis, mitochondrial ADP ribosylation, and hyperammonemia (Borowitz et al., 1992). It is possible that some of these mechanisms are independent of cellular energy deficit but produce severe effects on the cells. Overall, it appears that oxidative stress plays a crucial role in cyanide-induced neurotoxicity (Ardelt et al., 1989; Kanthasamy et al., 1994). Another important aspect of cyanide toxicity is its cardiotoxic manifestations. However, it has been excluded from the present chapter because it has been adequately discussed by others (Baskin, 1991; Borowitz et al., 1992).

DIAGNOSIS AND CLINICAL FEATURES OF CYANIDE POISONING

Signs and symptoms of acute cyanide poisoning are often nonspecific and vary in both time and intensity depending on the scale of exposure (Ballantyne, 1987; Ellenhorn et al., 1997; Nicholson, 2012). Low concentrations may produce delayed and various nonspecific symptoms like headache, dizziness, nausea, vomiting, confusion, coma, and incontinence of feces and urine, but exposure to high concentrations may lead to a series of events like dyspnea, incoordination of movements, convulsive seizures, coma, and cardiac and/or respiratory failure, culminating in death (Ballantyne, 1974; Way, 1984). Pathologically, no particular lesions can define cyanide toxicity, although animal experiments indicate that the lesions are principally in the central nervous system (CNS), particularly necrosis in the white matter (Ballantyne, 1974). Probably the most widespread pathologic condition attributed to chronic cyanide poisoning is tropic ataxic neuropathy after cassava consumption (Rosling, 1989; Kamalu, 1995). Other effects associated with cassava consumption include tropical pancreatitis (Gee Varghese, 1985), tropical neuropathies (Banea et al., 1997), and endemic goiter (Abuye et al., 1998). Repeated or prolonged skin contact to low concentrations of KCN dust may lead to dermatitic effects (cyanide rash) characterized by itching and skin eruptions (NOHSC, 1993).

Although the signs and symptoms of cyanide poisoning are well-documented, more than 55 signs and six biochemical idiosyncrasies are known to possibly occur during cyanide poisoning (Hall et al., 1987). Signs and symptoms of acute cyanide poisoning reflect cellular hypoxia and are often nonspecific. Onset of symptoms depends on dose, route, and duration of exposure. In general, the signs and symptoms correlate with the whole blood cyanide level and are usually tachycardia, flushing, headache, hyperpnea, and dizziness (Table 23.4). Laboratory tests suggestive of cyanide intoxication include arterial blood gases (metabolic acidosis with normal PO_2), serum

TABLE 23.4 Whole Blood Cyanide Levels and Clinical Signs and Symptoms

Whole Blood Cyanide		
μg/mL	**μmol/L**	**Signs and Symptoms**
0–0.5	8–20	No symptoms
0.5–1.0	20–38	Tachycardia, flushing, headache, hyperpnea, dizziness
1.0–2.5	48–95	CNS depression including giddiness, tachypnea, nausea, vomiting feeling, suffocation, confusion
2.5–3.0	95–114	Respiratory depression, convulsion, coma, cyanosis, apnea, circulatory collapse, fixed dilated pupils
≥3.0	114	Death

electrolytes (elevated anion gap), central venous percent O_2 saturation (elevated), calculated arterial percent O_2 saturation (normal), and measured arterial percent O_2 saturation (decreased). Quantitative determination of cyanide in whole blood, urine, gastric contents and tissues, and plasma thiocyanate levels are also important (Ballantyne, 1983). Unfortunately, these tests take several hours and the results may not be available to the clinician during the acute phase of poison management. Usually, the "Lee-Jones test" is a quick bedside test that can qualitatively detect cyanide in gastric aspirate but may give false-positive results with many drugs (Hall et al., 1987).

TREATMENT OF CYANIDE POISONING

The onset of toxicity after cyanide poisoning is very fast. The prognosis of the victim depends on termination of further exposure, supportive care, and institution of immediate and aggressive specific treatment. Early diagnosis and clinical information would enhance the chances of recovery. The first principle of therapy is termination of further exposure, which can be facilitated by the following: (i) remove the victim from the contaminated atmosphere; (ii) apply a protective mask to the patient as soon as possible to prevent further inhalation; (iii) remove any liquid on skin or clothing as soon as possible; (iv) remove all contaminated clothing, rinse skin with soap and copious amounts of water or water alone if there is liquid on the skin; and (v) gavage and administer activated charcoal if cyanide was ingested (Baskin and Brewer, 1997).

Supportive Therapy

The details of supportive therapy are presented in Table 23.5. Briefly, before specific therapy, the patient is administered supportive aid. This includes mechanical airway support, artificial ventilation with 100% oxygen possibly delivered via an Ambu bag containing the contents of two ampoules of amyl nitrite (0.6 mL), and cardiac monitoring (Van Heijst et al., 1987). Anecdotal evidence suggests that hyperbaric oxygen augments the protective efficacy of nitrite–thiosulfate therapy (Goodhart, 1994). Lactic acidosis resulting from anaerobic metabolism and convulsions should be treated with intravenous administration of sodium bicarbonate and diazepam, respectively (Van Heijst and Meredith, 1990; Baskin and Brewer, 1997). The use of antidotes should be restricted to patients in deep coma with respiratory insufficiency.

TABLE 23.5 Supportive Therapy and Specific Antidotal Therapy for Acute Cyanide Poisoning

	Agents	**Drug Category and Action**
Supportive therapy	100% oxygen or hyperbaric oxygen	Oxygen: Potentiates the efficacy of nitrite–thiosulfate therapy
	Sodium bicarbonate	Alkalizing agent: Corrects lactic acidosis
	Diazepam	Anticonvulsant: Depresses CNS activities
	Epinephrine	Sympathomimetic: Improves coronary and cerebral blood flow, corrects anaphylactoid reactions
Specific antidotal therapy	Amyl nitrite	Methemoglobin inducers: Converts hemoglobin to methemoglobin which binds with cyanide to form cyanmethemoglobin. In the presence of sodium thiosulfate cyanide is excreted as thiocyanate
	Sodium nitrite	
	4-Dimethylaminophenol	
	Sodium thiosulfate	Sulfur donors: Facilitates enzymatic conversion of cyanide to thiocyanate
	Dicobalt edentate	Cobalt compounds: Forms stable metal complexes with cyanide
	Hydroxocobalamin	

Specific Antidotal Therapy

Patients who are in critical condition and do not satisfactorily respond to supportive therapy should be administered specific cyanide antidotes as outlined in Table 23.5. Cyanide antidotes have been classified into

three main groups based on their mechanism of action: methemoglobin inducers; sulfur donors; and cobalt compounds. The definitive treatment of cyanide poisoning differs in various countries because of different medical practices and guidelines. The safety and efficacy of all the antidotes are still being debated. There is no worldwide consensus for treatment of cyanide intoxication.

Methemoglobin Inducers

The basic aim of rapid detoxification of cyanide is prevention or reversal of inhibition of cytochrome oxidase by cyanide. This is usually facilitated by providing a large pool of ferric iron in the form of methemoglobin to complex cyanide. Cyanide preferentially competes with the Fe^{3+} of methemoglobin as compared with that of cytochrome oxidase and eventually binds with the former to form cyanmethemoglobin. Thereby, the activity of inhibited cytochrome oxidase is restored (Baskin et al., 1992). The various methemoglobin inducers used as cyanide antidotes are discussed.

Amyl Nitrite

Although inhalation of amyl nitrite as a first aid measure for cyanide poisoning has been known for many years (Pedigo, 1888), its efficacy as a methemoglobin inducer is often disputed because of its inability to generate methemoglobin more than 6% (Jandorf and Bodansky, 1946). Approximately 15% of methemoglobin is required to challenge one LD_{50} of cyanide (Van Heijst et al., 1987). The protective effect of amyl nitrite is attributed to its vasodilatory effect, which can reverse the early cyanide-induced vasoconstriction (Van Heijst and Meredith, 1990). Artificial ventilation with amyl nitrite broken into Ambu bags has been reported as a life-saving therapy in cyanide-poisoned dogs before induction of significant levels of methemoglobinemia (Vick and Froehlich, 1985).

Sodium Nitrite

Sodium nitrite is the most prevalent drug for cyanide poisoning. It takes approximately 12 min to generate approximately 40% of methemoglobin after intravenous administration of the recommended dose (Van Heijst et al., 1987). Despite this delay in inducing a significant level of methemoglobinemia, reasonable protection offered by sodium nitrite can be attributed to its vasodilatory effects (Van Heijst and Meredith, 1990). A major drawback with sodium nitrite is that it causes serious cardiovascular embarrassment, particularly in children (Berlin, 1970). Because methemoglobinemia impairs oxygen transport, sodium nitrite cannot be recommended for fire victims when concomitant exposure of HCN and carbon monoxide usually occurs. Because carbon monoxide also reduces the oxygen-carrying capacity of blood, administration of sodium nitrite would further aggravate the underlying hypoxic condition. Sodium nitrite is also contraindicated for individuals with glucose-6-phosphate dehydrogenase-deficient red cells because of the possibility of serious hemolytic reactions (Way, 1984). Excessive levels of methemoglobin are known to be reverted by intravenous administration of 30 mL of 1% methylene blue solution (Van Heijst et al., 1987). In the United States, the Lilly Cyanide Antidote Kit (manufactured by Eli Lilly and Company, Indianapolis, IN) includes 10 mL ampoules of 3% sodium nitrite solution and 50 mL of 25% sodium thiosulfate solution. The kit also contains amyl nitrite encased in glass "pearls," which are meant to be broken so the drug could be inhaled. In India, a similar kit is manufactured by Troikaa Pharmaceuticals Ltd (Thiol).

4-Dimethylaminophenol

The relatively slow rate of methemoglobin formation by sodium nitrite prompted the development of rapid methemoglobin formers like aminophenols. 4-Dimethylaminophenol (DMAP) is the treatment of choice for cyanide poisoning in Germany. Administered intravenously, a dose of 3.25 mg/kg DMAP was reported to produce methemoglobin levels of 30% within 10 min and 15% methemoglobinemia was attained within 1 min without any immediate effect on the cardiovascular system (Kiese and Weger, 1969). However, there are differences in individual susceptibility to DMAP that may result in undesirable levels of methemoglobin even after normal therapeutic doses (Van Dijk et al., 1987). Intramuscular injection of DMAP results in local abscess and fever. Its clinical utility remains limited because of its other toxicological implications like nephrotoxicity (Weger, 1983).

Sulfur Donors

After the initial therapy of methemoglobin inducers, cyanide has to be converted to thiocyanate, which is eliminated in urine. This enzymatic detoxification of cyanide is facilitated by a sulfur donor like sodium thiosulfate. The mechanism of this reaction was discussed previously under elimination of cyanide. High tissue oxygen markedly potentiates the effects of this reaction. In cases in which methemoglobin formation is not desirable, sodium thiosulfate together with oxygen alone is sufficient. The utility of thiosulfate alone is limited because of its short biological half-life and its small volume of distribution (Sylvester et al., 1983). Also, thiosulfate is contraindicated in patients with renal insufficiency because the thiocyanate formed may cause toxicity (Van Heijst and Meredith, 1990).

Cobalt Compounds

The cobalt ion, which forms a stable metal complex with cyanide, is an effective therapeutic agent against

cyanide poisoning (Evans, 1964; Hillman et al., 1974; Linnell, 1987). Various cobalt-containing compounds known to antagonize cyanide poisoning are discussed.

Dicobalt Edetate (Kelocyanor)

Dicobalt edetate chelates cyanide as cobalticyanide. This drug is known to antagonize cyanide more quickly than the nitrites, but its clear superiority has not been established. Intravenous administration of 300 mg of dicobalt edetate in glucose solution is the current treatment of choice in France and the United Kingdom. Serious side effects like vomiting, urticaria, anaphylactoid shock, hypotension, and ventricular arrythmias have been reported in patients receiving Kelocyanor (Van Heijst and Meredith, 1990).

Hydroxocobalamin (Cyanokit)

With the exchange of the hydroxy group of hydroxocobalamin (vitamin B_{12a}) for cyanide, nontoxic cyanocobalamin (vitamin B_{12}) is formed (Hall and Rumack, 1987). An injectable solution of hydroxocobalamin (5 g in water) has been used in France and Germany. In France, a 4 g hydroxocobalamin solution in 80 mL of sodium thiosulfate has also been used (Van Heijst and Meredith, 1990). EMD Pharmaceutical Company has produced a lyophilized packaging of 2.5 g of hydroxocobalamin that can be readily reconstituted in a 100 mL sodium chloride solution. This product is pending FDA approval. It has been in use in Europe since 1996 as the Cyanokit™. Hydroxocobalamin is also used in other countries, including Sweden, Denmark, Spain, Japan, and Hong Kong. Now, sodium thiosulfate or hydroxocobalamin has also been recommended for empiric treatment of cyanide poisoning (Hall et al., 2007). There are several disadvantages in the clinical use of this drug. It has a relatively short half-life because it decomposes in light, and the dose required to counter cyanide poisoning is quite large. Also, recorded side effects include anaphylactoid reactions and acne (Van Heijst and Meredith, 1990). However, in a case study, clinical laboratory data did not show any evidence of toxicity (Borron et al., 2007).

Investigational Drugs

Numerous agents analogous to the specific antidotes or compounds belonging to different pharmacological classes have also been evaluated against experimental cyanide poisoning. The cardiovascular implications and poor pharmacokinetics of nitrite led to evaluation of yet another group of methemoglobin formers like aminophenones and derivatives. Out of all these compounds, *p*-aminopropiophenone was considered to be the most suitable agent for prophylaxis (Marrs and Bright, 1986; Bright, 1987). Hydroxylamine was examined as a rapid methemoglobin former with anticonvulsive properties, which conferred significant protection against acute cyanide poisoning (Kruszyna, et al., 1982). Combinations of sodium nitrite with DMAP or hydroxylamine were found to provide sustained prophylaxis against acute cyanide poisoning in rats by virtue of rapid, protracted, and desired levels of methemoglobinemia (Bhattacharya et al., 1991, 1993). However, toxicity of these regimens prevented their further use (Bhattacharya et al., 1995). A number of 8-aminoqunoline analogs of primaquine (e.g., WR242511) were also studied as potential prophylactic drugs because they induced elevated levels of methemoglobin for a longer duration (Steinhaus et al., 1990). Stroma-free methemoglobin solution (SFMS) was yet another alternative treatment proposed for cyanide poisoning (Ten Eyck et al., 1985). SFMS was formed by oxidizing outdated human blood and intravenous administration of this solution did not impair the oxygen-carrying capacity of blood as caused by most other methemoglobin formers. It was shown to directly sequester cyanide to protect against a 4x LD_{90} dose of sodium cyanide in rats. Among sulfur donors, several compounds containing sulfane sulfur, like polythionates, thiosulfonates, and persulfides, were examined as cyanide antidotes (Isom and Johnson, 1987). Compounds containing more lipophilic sulfane sulfur (e.g., ICD1021) or those that can be actively transported into the cells to mediate enzymatic detoxification of cyanide were synthesized as new anti-cyanide drugs (Baskin et al., 1999). Antagonism of cyanide intoxication with murine carrier erythrocytes containing bovine rhodanese and sodium thiosulfate has been demonstrated (Cannon et al., 1994; Petrikovics et al., 1995). Exogenous supplementation of the enzyme has been reported to accelerate the transulfuration of cyanide to thiocyanate. However, stability and sensitivity of the enzyme remain to be addressed. Several cobalt compounds were also evaluated against experimental cyanide poisoning, but none of them except cobalt edetate and hydroxocobalamin could be put to human use (Way, 1984; Linnell, 1987; McGuinn et al., 1994).

Cyanide is a nucleophile known to react with various carbonyl moieties like ketones and aldehydes to yield cyanohydrin derivatives (Morrison and Boyd, 1976). Sodium pyruvate (Schwartz et al., 1979), α-ketoglutarate (Moore et al., 1986), pyridoxal-5′-phosphate (Keniston et al., 1987), and many other carbonyl compounds and their metabolites or nutrients that interact with cyanide to form cyanohydrin complexes (Way, 1984; Niknahad et al., 1994; Bhattacharya and Tulsawani, 2008) have been reported to afford significant protection against acute cyanide poisoning *in vitro* or *in vivo*. Out of all these agents, α-ketoglutarate, either alone or with sodium thiosulfate, has been considered to be a promising antidote for cyanide (Borowitz et al., 1992). In a recent study, α-ketoglutarate and *N*-acetyl cysteine were found to protect rat pheochromocytoma (PC12) cells from cyanide-induced cytotoxicity and altered energy metabolism (Satpute et al., 2008). *N*-acetyl cysteine, a free

radical scavenger, was previously found to attenuate cyanide-induced cytotoxicity and DNA damage in isolated rat thymocytes (Bhattacharya and Lakshmana Rao, 1997). Also, in several other acute (Tulsawani and Bhattacharya, 2007) and subacute studies (Tulsawani et al., 2005), α-ketoglutarate was found to antagonize cyanide poisoning in experimental animals. This agent is being actively pursued as an oral treatment for cyanide poisoning, particularly in instances when nitrites are not recommended (Bhattacharya, 2004). At present, phase I clinical trials with α-ketoglutarate are being conducted in India.

There are several other compounds that are regarded as nonspecific cyanide antidotes, and their mechanisms have been only tentatively explained. These compounds are usually not very effective *per se* but as adjuncts significantly augment the efficacy of conventional antidotes. Chlorpromazine (phenothiazine), phenoxybenzamine (α-adrenergic blocker), centrophenoxine (neuroregulator), etomidate (nonbarbiturate hypnotic and anticonvulsant), naloxone (morphine antagonist), and flunarizine, verapamil, and diltiazem (calcium-channel blockers) are some of the compounds (Way, 1984; Marrs, 1988; Bhattacharya, 2000). Additionally, a few more compounds have been found to antagonize cyanide poisoning in the recent past. They include protein kinase C inhibitor (Maduh et al., 1995), nitric oxide generator (Sun et al., 1995), endothelium-derived relaxing factor/nitric oxide releasers or calcium ionophore A23187 (Baskin et al., 1996), local anesthetic, procaine hydrochloride (Jiang et al., 1998), and antioxidants like melatonin, a pineal hormone (Yamamoto and Tang, 1996), and its metabolite, 6-hydroxymelatonin (Maharaj et al., 2003). In general, antioxidants are found to be good adjuncts to nitrite–thiosulfate therapy. Another study has shown that ATP, encapsulated in unilamellar fusogenic lipid vesicle when injected intraperitoneally in mice before cyanide, significantly increased the survival time and the fatal dose of cyanide (Chiang et al., 2004). Most importantly, extracorporeal filtering or hemodialysis has also been found to supplement specific cyanide antidotes. This would hasten the elimination of cyanide and its metabolites. Hemodialysis achieved dramatic response in patients with severe acidosis (Wesson et al., 1985). This is only an anecdotal case report and has not been substantiated further.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Currently, cyanide is considered to be neither an important CW agent nor an agent of choice for suicidal or homicidal purposes. However, the threat of its possible use in local terrorism cannot be overlooked. Also, the occupational hazard of cyanide is likely to increase with its escalating industrial use. Serious epidemiological studies and reporting in poison control centers can be observed in many developed nations. However, many cases of fatal cyanide poisoning from fire smoke, drug side effects, dietary sources, and industrial exposures may go undetected because of poor surveillance in many countries. Cyanide toxicity is mainly ascribed to its ability to inhibit cytochrome oxidase, an end-chain enzyme of cellular respiration. Numerous new mechanisms of action of cyanide unfold the complex toxic phenomena occurring at cellular and molecular levels. Many of these mechanisms have been the target for pharmacological interventions. Although very primitive, the combination of sodium nitrite and sodium thiosulfate still continues to be the most favored treatment for cyanide poisoning. Hydroxocobalamin has also been used as a cyanide antidote with considerable success in the recent past. If we could circumvent the side effects of this drug, then it would possibly be the most successful second-generation antidote for cyanide. Cyanide antagonism by scavengers like carbonyl compounds, particularly α-ketoglutarate, has also been very encouraging in animal models. Further clinical trials would determine its safety for human use. Also, the stability of this molecule needs to be addressed. Perhaps these kinds of molecules could be of use for occupational exposures, chronic dietary cyanide poisoning, or as prophylaxis for fire-fighters/personnel engaged in evacuation operations in cyanide-contaminated areas. Another area of interest would be the development of field-based detection systems for rapid diagnosis of cyanide poisoning or for retrospective detection of cyanide in biological samples. This is a prerequisite for effective therapeutic measures.

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CHAPTER

24

Chlorine

Sylvia Milanez

INTRODUCTION

Diatomic chlorine (Cl_2) is a gas under ambient conditions, with a pungent, suffocating odor. Because its vapor density is twice that of air, chlorine forms a greenish-yellow cloud near the ground upon release into the environment (Haynes et al., 2013; O'Neil et al., 2013). The Cl_2 odor detection threshold is approximately 0.2–0.4 ppm, with considerable variation among humans, although odor perception tends to decrease over time (NIOSH, 1976; ATSDR, 2010). Chlorine gas is slightly water-soluble (~4.4 g/L; O'Neil et al., 2013), but it reacts quickly with water to form hydrochloric acid (HCl) and hypochlorous acid (HOCl). Selected chemical and physical properties of Cl_2 are listed in Table 24.1.

Very little chlorine gas is found naturally in the environment due to its high reactivity with other compounds and its degradation by sunlight; it has a half-life of <10 min (Hov, 1985). Chlorine and sodium hydroxide are co-produced by the electrolysis of brines (the chlor-alkali process) using mercury, membrane, or diaphragm cells; the ion exchange membrane cell has become the predominant process, for economic and environmental reasons (Burridge, 2005). The major uses of Cl_2 are in the manufacture of polyvinyl chloride and other plastics, chlorinated solvents, and pharmaceuticals; as a bleaching agent and biocide in pulp and paper production; and in water purification and waste treatment systems (Evans, 2005; CEH, 2011). Worldwide, there are over 500 chlor-alkali producers at over 650 sites, with an annual chlorine production capacity of 58 million tons (World Chlorine Council, 2012). Chlorine is one of the top 10 production volume chemicals in the United States, with over 10 million tons produced in 2010 (US Census Bureau, 2011).

Chlorine is a contact irritant in both humans and animals, and the respiratory tract is the primary, and most sensitive, target organ. Toxicity resulting from short- and long-term chlorine inhalation has been studied in humans and laboratory animals, and exposures associated with various endpoints have been defined.

HISTORY OF USE

Chlorine's utility as a bleaching agent was discovered in the late 1700s, and its use as a water disinfectant in the United States began in the early 1900s, dramatically cutting the death rate from typhoid fever (Toren and Blanc, 1997; Evans, 2005). Chlorine gas was used as a chemical warfare agent during World War I, most notably when the German army buried 5,730 chlorine-containing cylinders in trenches along a 6-mile front near Ypres, Belgium. On April 22, 1915, over 150 tons of chlorine gas was released from the cylinders in 5 min and was blown by wind toward the Allied forces. It was estimated that 800 soldiers were killed and 2,500–3,000 more were incapacitated from lack of adequate protective gear (Joy, 1997). Because chlorine gas has significant warning properties (i.e., color and odor) and its toxicity is relatively easy to avoid, it is less than ideal as a warfare agent, and its use declined after World War I. Recently, however, chlorine has been used by terrorists in the Iraq war, with bombs being rigged to chlorine tanker trucks or cylinders intended for use in water treatment or for other industrial purposes (Wetz et al., 2007).

A number of accidents have occurred during chlorine production and transport, which occurs primarily by rail. In a recent fatal accident in January 2005, 9 people died and nearly 600 required medical attention when a chlorine tank ruptured from a train collision in Graniteville, South Carolina (Van Sickle et al., 2009; Jones et al., 2010). However, due to its great utility as a water disinfectant, bleaching agent, and industrial chemical, chlorine continues to be produced, transported, and used in great quantities worldwide, making it a persistently hazardous material (HAZMAT) threat (Jones et al., 2010).

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TABLE 24.1 Chemical and Physical Data for Chlorine

Parameter	Value	Reference
Molecular formula	Cl_2	Haynes et al. (2013)
Molecular weight	70.90	Haynes et al. (2013)
CAS registry number	7782-50-5	Haynes et al. (2013)
Synonyms	Bertholite, molecular chlorine, chlorine mol, dichlorine	ATSDR (2010)
Physical state	Greenish-yellow gas	Haynes et al. (2013)
Odor threshold	0.2–0.4 ppm	ATSDR (2010)
Conversion factors in air	1 ppm = 2.90 mg/m^3; 1 mg/m^3 = 0.34 ppm	NIOSH (2010)
Boiling point	−34.04°C	Haynes et al. (2013)
Melting point	−101.5°C	Haynes et al. (2013)
Vapor pressure (mmHg)	5,830 mmHg at 25°C	ATSDR (2010)
Vapor density (air = 1)	2.48 at 20°C	O'Neil et al. (2013)
Liquid density (water = 1)	2.898 g/L at 25°C	Haynes et al. (2013)
Flash point/flammability	Not flammable	O'Neil et al. (2013)
Water solubility (g/L)	4.4 g/L at 25°C	O'Neil et al. (2013)

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Almost all inhaled chlorine (>90%) is absorbed via the respiratory tract, in both humans and animals (Abdel-Rahman et al., 1983; Nodelman and Ultman, 1999a,b; Morris et al., 2005). Humans absorbed >95% of an inhaled bolus of 0.5–3 ppm chlorine in the upper airway and <5% in the lower airway, regardless of the mode of breathing or respiratory flow rate (Nodelman and Ultman, 1999a,b). Chlorine that is absorbed is not subject to metabolic biotransformation and is distributed widely throughout the body and joins the pool of chloride ions. Rats that were orally administered HO^{36}Cl in distilled water excreted the majority of ^{36}Cl in the urine (36.43%) and feces (14.80%) over a 96 h post-exposure period (Abdel-Rahman et al., 1983).

MECHANISM OF TOXICITY

Chlorine is a strong oxidizing agent that hydrolyzes in water, and in the fluid lining of the respiratory tract surface (pH ~7), where it exists predominantly as HOCl and the hypochlorite ion (OCl^-) (ATSDR, 2010; Squadrito et al., 2010; O'Neil et al., 2013). In both humans and animals, the respiratory tract is the primary target organ for inhaled chlorine, which reacts with the epithelial lining of the airways and lungs. Depending on the chlorine exposure concentration and duration, respiratory toxicity can be manifested as sensory irritation, increased airway resistance, and respiratory tract lesions, and can lead to death (Withers and Lees, 1985; Bitron and Aharonson, 1978). In rats and mice, chlorine sensory irritation decreases the respiratory rate by stimulating the trigeminal nerve endings in the respiratory mucosa (Alarie, 1981).

The precise mechanism by which chlorine or its reaction products cause cellular toxicity is unknown, but it is widely believed that chlorine toxicity is due to its oxidant properties, and is mediated largely by the hypochlorite ion (Morris et al., 2005; ATSDR, 2010). An alternative model has proposed that Cl_2 itself can react with biomolecules in the epithelial lining fluid in the respiratory tract, and may be able to penetrate the lung surface (Squadrito et al., 2010). This hypothesis was based on a kinetic analysis that found that the reaction rate of chlorine with water was several orders of magnitude lower than its reaction rate with low molecular weight antioxidants and N-terminal functions of peptides and proteins in lung epithelial fluid (Squadrito et al., 2010). In addition to causing epithelial injury, the presence of HCl and HOCl in the airway causes an inflammatory response. The recruited neutrophils and macrophages increase the local concentration of nitric oxide (NO) and H_2O_2, which can react with HOCl and HCl to produce hydroxyl radicals and reactive intermediates able to nitrate, chlorinate, and dimerize aromatic amino acids (Olin et al., 1999; Evans, 2005; Jones et al., 2010).

In vitro mechanistic studies with bacteria and mammalian cells have led to various theories for the chlorine mechanism of action, including direct interaction of

chlorine or hypochlorite with enzymes, other proteins, and nucleotide bases, and disruption or degradation of cell membrane structure and function (US EPA, 1994). Chlorine was proposed to cause systemic endothelial dysfunction by inhibiting endothelial nitric oxide synthase (eNOS)–dependent signaling, based on its inhibition of eNOS-dependent vasodilation in isolated rat aortas (Honavar et al., 2011).

TOXICITY

Chlorine is a direct-contact irritant, of which the primary and most serious target is the respiratory tract, although it can also damage the skin, eyes, and internal organs at high exposure levels (Evans, 2005). The acute, repeat-exposure, and chronic inhalation toxicity of chlorine has been studied in humans and in laboratory animals, which showed many similarities in their response.

Human Studies

The effects on humans of a single chlorine gas exposure lasting up to 8 h have been described anecdotally, as well as in controlled experiments with volunteers. In a summary of various anecdotal reports, Winder (2001) noted that exposures of up to 3 ppm caused mild irritation; 5–15 ppm caused moderate irritation; >15 ppm caused severe irritation with pulmonary involvement, and death occurred from exposure to ≥430 ppm after 30 min or less.

Controlled human studies (Table 24.2) generally found that exposure to 0.5–2 ppm Cl_2 for a period of a few minutes to 8 h caused slight to moderate irritation of the eyes, nose, and throat, and small decrements in pulmonary function (such as the forced expiratory volume in 1 s (FEV_1) and airway resistance (R_{aw})) (Anglen, 1981; Rotman et al., 1983; D'Alessandro et al., 1996; Shusterman et al., 1998; Schins et al., 2000). The severity of irritation increased with exposure duration and concentration, and effects were greater in subjects with preexisting respiratory conditions (such as asthma and airway hyperreactivity) and in smokers. A number of cases of reactive airways dysfunction syndrome have been reported, and all were seen in individuals with preexisting respiratory conditions and current or former smokers (Evans, 2005). Withers and Lees (1985) applied probit analysis to human and animal lethality data to estimate that chlorine concentrations of 250 and 100 ppm were lethal to 50% of a regular and vulnerable population (LC_{50}), respectively, that was exposed for 30 min with an inhalation rate of 12 L/min.

Few repeat-exposure human studies have been conducted with chlorine gas (Table 24.3). Subjects exposed to 0.1, 0.3, or 0.5 ppm for 6 h/day for 3 days had no pulmonary function decrements, but did report eye and respiratory tract irritation (Schins et al., 2000). Workers exposed chronically to <0.1 ppm experienced no chlorine-related toxicity; exposure to ~0.2–0.3 ppm had little or no effect on pulmonary function but did result in fatigue, anxiety, wheezing, and chest tightness; and exposure to 1.7 ppm was associated with numerous respiratory symptoms (including sore throat, shortness of breath, chest tightness, wheezing, and rhinitis), chest X-ray abnormalities, and pulmonary function decrements (Capodaglio et al., 1970; Patil et al., 1970; Chan-Yeung et al., 1980; Enarson et al., 1984; Shi, 1990). The severity of effects increased with the number of working years and was greater in smokers.

Laboratory Animal Studies

Animal studies with chlorine gas have been conducted using monkeys, dogs, rabbits, guinea pigs, rats, and mice, with the majority of the data being for rats and mice. Single-exposure durations ranged from 2 min to 24 h (Table 24.4), and repeat-exposure durations were 3 days to 2 years (1–8 h/day) (Table 24.5). Early studies tested concentrations causing severe toxicity, often extending into the lethal range (Weedon et al., 1940; Schlagbauer and Henschler, 1967; Faure et al., 1970; MacEwen and Vernot, 1972; Barrow and Smith, 1975; Barrow et al., 1979; Jiang et al., 1983; Klonne et al., 1987; Zwart and Woutersen, 1988). Early studies also examined the sensory irritation of chlorine gas in rodents, determining the concentration that caused a 50% decrease in the inhalation rate (i.e., the RD_{50}) (Barrow et al., 1977; Barrow and Steinhagen, 1982; Buckley et al., 1984; Chang and Barrow, 1984; Gagnaire et al., 1994). Several more recent studies, as well as a few earlier studies, were designed to explore aspects of the mechanism of chlorine toxicity, including its progression over time and post-exposure tissue repair (Dodd et al., 1980; Demnati et al., 1995; Wolf et al., 1995; Martin et al., 2003; Yildirim et al., 2004; Morris et al., 2005; Hoyle et al., 2010; Jarabek et al., 2010; Leikauf et al., 2010, 2012; Peay et al., 2010; Song et al., 2010; Musah et al., 2012; Mo et al., 2013). Some of the more recent mechanistic studies evaluated the ability of various classes of compounds to mitigate chlorine toxicity (see the next section of this chapter, "Risk Assessment").

The respiratory tract was consistently the primary target of toxicity, and similar effects were seen following single and repeated inhalation exposures. The upper respiratory tract was primarily affected at lower chlorine concentrations, with symptoms such as sensory irritation and nasal lesions. Sensory irritation was manifested as a decreased respiration rate, sneezing, flared nostrils, reddened eyes, lacrimation, salivation, and foamy nasal secretions. Nasal symptoms included inflammation, epithelial necrosis, deciliation,

TABLE 24.2 Human Single-Exposure Chlorine Inhalation Studies

Exposure Time	Concentration (ppm)	Effects	Reference
15 min	0.5	At 0 and 15 min post-exposure, NAR was ↑ in rhinitics but not normal subjects; rhinitics had greater nasal irritation and congestion; no effect on pulmonary peak flow, rhinorrhea, or headache in either group.	Shusterman et al. (1998)
15 min	1.0	At 0 and 15 min post-exposure, NAR was ↑ in rhinitics but not normal subjects; effect ↑ with age; very slight nasal irritation and odor perception.	Shusterman et al. (2003b)
15 min	1.0	At 0 and 15 min post-exposure, NAR ↑ in rhinitics but not normal subjects; no effect on nasal lavage fluid levels of mast cell tryptase, or plasma neuropeptides, albumin, urea, lysozyme, or 7F10-mucin.	Shusterman et al. (2003a, 2004)
1 h	0.4	No pulmonary function changes in those with airway hyperreactivity or asthma.	D'Alessandro et al. (1996)
1 h	1.0	Modest changes in pulmonary function measurements, greater in hyperreactive subjects (± asthma), of which 2/7 had "respiratory symptoms."	
15 min – 2 h (left room every 15′)	0.5, 1.0	Barely perceptible irritation of eyes, nose, and/or throat.	Joosting and Verberk (1974)
	2.0	Distinctly perceptible irritation of eyes, nose, and throat; some coughing.	
	4.0	Nuisance level irritation of eyes, nose, and throat; some coughing.	
4 h + exercise 15 min/h	0.5	Moderate or mild itching or burning of throat and nose; severity generally ↑ with exposure time.	Anglen (1981)
	1.0	Moderate or mild irritation of throat, eyes, and nose; runny nose; severity ↑ over time; few had pharynx injection, ↑ mucous secretion, and inspiratory rales.	
	2.0	Moderate or mild irritation of throat, eyes, and nose; runny nose; severity ↑ over time; few had pharynx injection or palpebral fissures, ↑ mucous secretion, inspiratory rales, altered pulmonary function.	
8 h + exercise 15 min/h; had 30 or 60 min break after 4 h	0.5	Moderate or mild itching or burning of throat and nose; severity generally ↑ with exposure time.	Anglen (1981)
	1.0	Moderate or mild itching or burning of throat, eyes, and nose; runny nose; urge to cough; severity ↑ with exposure time; few had pharynx injection or palpebral fissures, ↑ mucous secretion, and inspiratory rales after 4 and 8 h; after 8 h had slight changes in pulmonary function.	
8 h + exercise 15 min/h	0.5	Slight changes in pulmonary function measurements after 4 and 8 h; changes were greater in an atopic subject.	Rotman et al. (1983)
	1	Itchy eyes, runny nose, mild burning in throat, and slight changes in pulmonary function after 4 and 8 h; changes were sufficient in an atopic subject to terminate exposure after 4 h due to dyspnea and wheezing.	

↑, increase(d); ↓, decrease(d); NAR, nasal airway resistance.

hyperplasia, and metaplasia. At higher chlorine concentrations, the lower respiratory tract was also affected. Lower respiratory tract effects included dyspnea, emphysema, bronchopneumonia, pulmonary edema, and hemorrhage, and increased protein content and IgM in the bronchoalveolar (BAL) fluid. In many cases, the irritation and pulmonary toxicity were reversible, but at sufficiently high doses, lung toxicity led to death. Exposure to near-lethal concentrations caused lesions in organs outside the respiratory tract, including the

TABLE 24.3 Human Repeat-Exposure Chlorine Inhalation Studies

Exposure Time	Concentration (ppm)	Effects	Reference
6 h/day for 3 day	0.1, 0.3, and 0.5	At unspecified concentration(s) had eye, nose and throat irritation, wheeze, nasal congestion and mucous production; pulmonary function normal.	Schins et al. (2000)
10.9 ± 2.8 years	0.146 ± 0.287	Male workers had fatigue, anxiety, dizziness, and white blood cell count; ↓ hematocrit. No effect on chest X-ray or pulmonary function.	Patil et al. (1970)
>10 years	0.298 ± 0.181	Workers had ↓CO lung diffusing capacity but no effects on pulmonary function measurements or ↑ in pulmonary emphysema.	Capodaglio et al. (1970)
13.9 ± 9.3 years	<0.05 (mean)	Male workers had no ↑ in respiratory symptoms or abnormalities in pulmonary function or chest radiographs. Possible co-exposure to low levels of H_2S, methyl mercaptan, SO_2 and particulates.	Chan-Yeung et al. (1980)
8.9 ± 8.6 years	0.18 (mean)	Male workers had ↑ frequency of wheeze, chest tightness, chest illness, and decrements in pulmonary function. No ↑ in cough, phlegm, or dyspnea. Possible co-exposure to low levels of SO_2, CH_3SH, and particulates.	Enarson et al. (1984)
<10–25 years	1.7 ± 0.12	Workers had sore or dry throat, shortness of breath, chest tightness or pain, headache, wheezing, chronic rhinitis and tonsillitis, olfactory deficiency, chest X-ray abnormalities, and impaired pulmonary function.	Shi (1990)

↑, increase(d); ↓, decrease(d).

TABLE 24.4 Animal Single-Exposure Chlorine Inhalation Studies

Species	Exposure Time	Concentration (ppm)	Effect	Reference
Rabbit	30 min	50	Temporary ↓ in lung compliance.	Barrow and Smith (1975)
		100–250	Lung edema, hemorrhage, emphysema, inflammation, impaired function.	
		500–1,000	LC_{100}	
Guinea pig	15–30 min	200	Severe lesions of upper respiratory tract mucous membranes, emphysema, inflammation and exudate in bronchioles.	Faure et al. (1970)
Rat	2, 10 min	1,500	Lung edema with bronchial epithelial sloughing and airway wall leucocyte infiltration; some epithelial regeneration after 72 h.	Demnati et al. (1995)
	15 min	1,330	Lung eosinophilic accumulation, edema, and bleeding, which became interstitial fibrosis and thickening of alveolar septa after 45 days.	Yildirim et al. (2004)
	30 min	184, 400	Labored breathing, expiratory grunting; 1 h after exposure, had ↓ arterial oxygen pressure and↑CO_2 levels, and respiratory acidosis. Had lung necrosis and epithelial sloughing, neutrophil accumulation, and mild alveolitis; BAL fluid had ↑ albumin, IgG, IgM, and phospholipid levels (400 ppm), and ↓ levels of ascorbate and reduced glutathione.	Leustik et al. (2008)
	30 min	400	Transient hypoxemia, severe epithelial pathology in airways; increased BAL protein, neutrophils, epithelia; lung edema; effects lessened by sodium nitrite given (i.p.) 2–6 h post-exposure.	Yadav et al. (2011)
	10 min	579–2,248	LC_{50} = 1,931, 690, 448 ppm for 10, 30, 60 min; majority died in 1 week; were restless, had eye and nasal irritation, dyspnea, and lesions in nose, larynx, trachea, and lung; some were reversible.	Zwart and Woutersen (1988)
	30 min	547–645		
	60 min	322–595		
	60 min	213, 268, 338, 427	LC_{50} = 293 (260–329) ppm; had eye and nose irritation, and after 60 min had lacrimation, rhinorrhea, and gasping; survivors had lower weight gain. Liver tissue mottling was most common finding at necropsy.	MacEwen and Vernot (1972)

(*Continued*)

TABLE 24.4 (Continued)

Species	Exposure Time	Concentration (ppm)	Effect	Reference
	60 min	6, 30, 60	Nasal lesions (necrosis, inflammation, hyperplasia, degeneration), severity increasing with concentration and greatest in more anterior regions.	Peay et al. (2010)
	6 h	0.1, 1.0, 5.0, 10	Nasal epithelial inflammation and hyperplasia, olfactory necrosis and degeneration; severity increased with concentration and was greatest in anterior regions; lesions seen at ≥1.0 ppm persisted at least 5 days.	Peay et al. (2010) and Jarabek et al. (2010)
	6 h	9.1 ± 1.02	Nasal cavity lesions (epithelial degeneration, ulceration, and necrosis); most severe in olfactory mucosa.	Jiang et al. (1983)
	16 h	63	All survived 5 months post-exposure; had gross changes in brain, lungs, heart, stomach, intestines, liver, kidneys, and lung adhesions.	Weedon et al. (1940)
		250, 1,000	Lacrimation, dyspnea; all died by 16 h at 250 ppm and 1.7 h at 1,000 ppm; had lesions in brain, lungs, heart, liver, stomach, intestine, and kidneys.	
	24 h	0.25, 1.25, 2.5	Nasal necrosis/degeneration followed by inflammation and hyperplasia; severity increased with concentration and greatest in anterior regions.	Peay et al. (2010)
Mouse	5 min	100	Airway hyperresponsiveness, increased cells and glutathione in BAL, dimethylurea given (i.p.) 1 h before/after exposure decreased BAL protein and glutathione levels and prevented lung lipid peroxidation.	McGovern et al. (2010)
		200, 400	As at 100 but more severe; had microscopic pathological changes in airway epithelium (BAL fluid and effect of dimethylurea not evaluated).	
	5 min	100, 200, 400, 800	Dose-related increases in airway responsiveness, airway epithelial loss, alveolar damage and inflammation, leukocytes and nitrate in BAL fluid, nitric oxide synthase expression and oxidation of lung proteins.	Martin et al. (2003)
	10 min	0.7–38.4	RD_{50} = 9.3 ppm; respiratory rate ↓ was dose-related and was attained at each concentration within 5–7 min.	Barrow et al. (1977)
	10 min	1.0–760	RD_{50} = 25 ppm; appearance of animals was not addressed.	Barrow and Steinhagen (1982)
	10 min	~2.5–1,000	RD_{50} = 10.9 ppm; animal appearance was not addressed.	Chang and Barrow (1984)
	10 min 30 min	579–1,654 458–645	LC_{50} = 1,034 and 517 ppm for 10 and 30 min, respectively. Animals had signs of restlessness, eye and nasal irritation, dyspnea, and ↑ lung weight.	Zwart and Woutersen (1988)
	15 min	0.8, 2.0, 3.1, 3.8	RD_{50} = 2.3 ppm; dose-related ↓ in respiratory rate (30–80% of baseline) and ↑ in airway resistance (64–186%) and length of the expiratory pause (20–522 ms).	Morris et al. (2005)
	15 min	200	Anorexia, lethargy, hypothermia; acute lung edema, collagen deposition (fibrosis), and inflammation; dexamethasone (i.p.) reduced severity if given within 1–6 h after exposure.	Jonasson et al. (2013)
	7.5 min 15 min 30 min 60 min	800 400 200 100	After 6 h: mortality was 8/8; 6/41; 0/40, 0/40; had increased lung weights (pulmonary edema), BAL protein and IgM levels, neutrophil infiltration, and airway epithelial denudation – more severe for the shorter exposures. After 24 h: only lung weight returned to sham control levels.	Hoyle et al. (2010)
	30 min	400	Lung deciliation, epithelial sloughing, alveolar inflammation, ↑ airway resistance, ↑ protein and inflammatory cells in BAL; arformoterol (β_2-agonist) given nasally starting 10 min post-exposure reduced airway resistance and improved alveolar clearance.	Song et al. (2010, 2011)

(*Continued*)

TABLE 24.4 (Continued)

Species	Exposure Time	Concentration (ppm)	Effect	Reference
	30 min	55–179	The 30 min LC_{50} = 127 (106–152) ppm; ↑ lung weight; tracheal, bronchiolar, and alveolar lesions; regenerative changes seen after 9–10 days.	Schlagbauer and Henschler (1967)
	3 h	10, 22	Pathology as 30 min exposure; 8/10 died at 10 ppm and 10/10 at 22 ppm.	
	6 h	10	Similar pathology as after 30 min exposures, 9/10 died.	
	45 min	600	Most (14/18) died within 72 h of exposure; had labored breathing, lung inflammation, epithelial sloughing; lipid peroxidation; had less mortality (4/18) and toxicity if given ascorbate and deferoxamine (i.m., inhalation) starting 1 h post-exposure.	Zarogiannis et al. (2011)
	60 min	122–193	LC_{50} = 137 (119–159) ppm; had eye and nose irritation, and gasping; survivors had weight loss; liver tissue mottling was seen at necropsy.	MacEwen and Vernot (1972)
	60 min	228–270	Lung edema and ↑ protein and IgM in BAL; airway hyperreactivity and impaired pulmonary function; rolipram given post-exposure (i.p. or intranasally) inhibited pulmonary edema and airway hyperreactivity.	Chang et al. (2012)
	60 min	240	Sloughing of Clara and ciliated cells from tracheal epithelium, after 2–4 days had cell proliferation in K5- and K14-expressing basal cells; at d 7–10 fibrosis was seen primarily in distal trachea.	Musah et al. (2012)
	60 min	240	Airway epithelial damage and hyperreactivity, neutrophil inflammation, lung edema and dysfunction; mometasone and budesonide (i.p., given 1 h post-exposure) decreased lung neutrophil levels and lung edema.	Chen et al. (2013)
	60 min	240	FVB/NJ mice and A/J mice had fibroproliferative lesions in large airways 4 days post-exposure, which by day 7 was repaired in A/J mice but fibrosis in FVB/NJ mice, latter had less keratin 5 in basal cells of large airways.	Mo et al. (2013)
	60 min	1.7–8.8	RD_{50} = 3.5 ppm; appearance of animals was not addressed.	Gagnaire et al. (1994)
	120 min	2.2–6.6	Respiratory rate ↓ 31–65% after 60 min, and ~38–72% after 120 min.	
	67 min	179–263	Several mice died within 24 h after exposure; had edema, airway epithelial sloughing, and ↑ protein and neutrophils in BAL.	Tian et al. (2008)
	6 h	9.1 ± 1.02	Marked nasal cavity epithelial degeneration, ulceration, and necrosis, most severe in olfactory mucosa of anterior dorsal meatus.	Jiang et al. (1983)
	16 h	63	All survived; showed less distress than at higher concentrations.	Weedon et al. (1940)
		250, 1,000	Lacrimation, dyspnea, convulsions; all died after 8.4 h (250 ppm) and 50 min (1,000 ppm); had lesions in lungs and numerous internal organs.	
	24 h	45	Most died; mean survival time was 7.6–38.1 h for 40 different strains; had lung hemorrhage, alveolar wall thickening, ↑ BAL total protein and altered metabolite content.	Leikauf et al. (2010, 2012)

↑, increase(d); ↓, decrease(d); BAL, bronchoalveolar lavage (fluid); BW, body weight(s); LC_{50}, the concentration that results in 50% mortality; OE, olfactory epithelium (nasal); RD_{50}, concentration causing a 50% decrease in the respiration rate.

spleen, thymus, brain, lungs, heart, stomach, intestines, liver, and kidneys. In the single-exposure studies, toxicity was generally influenced more by exposure concentration than exposure duration; this relationship was less clear in the repeat-exposure studies, which were complicated by intermittent periods of nontreatment (e.g., 5 days/week), allowing partial recovery of the chlorine-induced lesions.

No animal studies have linked chlorine exposure with developmental or reproductive toxicity or cancer; genotoxicity studies with chlorine gas yielded negative results, but mixed results were obtained with the chlorine hydrolysis product hypochlorite (US EPA, 1999; ATSDR, 2010).

RISK ASSESSMENT

There are a number of factors to consider in defining the risk of chlorine inhalation on a diverse human population and in defining levels that are safe for short- and long-term exposures. For example, how do age, gender, and preexisting respiratory conditions such as asthma and airway hyperreactivity affect susceptibility

TABLE 24.5 Animal Repeat-Exposure Chlorine Inhalation Studies

Species	Exposure Scenario	Concentration (ppm)	Effect	Reference
Monkey	6 h/day, 5 days/week × 12 months	0.10 ± 0.03, 0.50 ± 0.10, 2.3 ± 0.4	Eye tearing and reddening during exposure to 2.3 ppm seen after ~6 week; after 1 year had conjunctival irritation but no gross eye lesions. Had trace or mild nasal epithelial hyperplasia, loss of cilia and goblet cells (dose-related), with tracheal involvement at 2.3 ppm.	Klonne et al. (1987)
Rat	6 h/day × 3 days 6 h/day × 5 days	9.1 ± 1.02 (TWA)	BW ↓; marked nasal epithelial degeneration, cell exfoliation, ulceration, and necrosis; neutrophil infiltration of epithelium and squamous metaplasia after 5 days; milder lesions in pharynx, trachea, and lungs.	Jiang et al. (1983)
	6 h/day × 5 days	0.1, 0.5, 1.0 2.5	Nasal inflammation and hyperplasia in squamous and respiratory epithelium (concentration-dependent) but not OE; extended to the nasopharyngeal duct at 2.5 ppm.	George et al. (2010); Jarabek et al. (2010)
	With 7 days recovery	1.0, 2.5	Hyperplasia (concentration-dependent) in squamous and respiratory epithelium of anterior nasal sections.	
	6 h/day, 5 days/week × 2 weeks	12	Looked unkempt, wheezed, had swelling around eyes and nares, ↓ BW, and reversible ↑ in lung sulfhydryl level after 3–6 days.	Dodd et al. (1980)
	6 h/day, 5 days/week × 6 weeks	1	Signs of irritation, mild nasal, tracheal, bronchial, alveolar inflammation.	Barrow et al. (1979)
		3	As at 1 ppm; also, lacrimation, conjunctivitis, nasal discharge, liver lesions.	
		9	As at 3 ppm; also, gasping, lung rales, emaciation; 3/20 died by 30th exposure; lesions in lungs, liver, kidney, stomach, spleen, and thymus.	
	6 h/day × 104 weeks; 5 days/week (M) or 3 days/week (F)	0.4, 1.0, 2.5	BW ↓ in all M and at 1.0 and 2.5 ppm in F after > 3 months; closed eyes at 2.5 ppm in F. All had nasal lesions, most severe in anterior and generally concentration-related (inflammation; OE degeneration; squamous metaplasia; goblet cell epithelial hypertrophy and hyperplasia).	Wolf et al. (1995)
Mouse	8 h/day × 3 days	2.5	BW was 93.2% of initial weight.	Schlagbauer and Henschler (1967)
		5	BW was 87.5% of initial weight; lesions in tracheal epithelium and mucosa, bronchioles, and alveoli.	
	6 h/day × 3 days 6 h/day × 5 days	9.1 ± 1.02	BW ↓; marked nasal lesions (epithelial degeneration, ulceration, necrosis); neutrophil infiltration of epithelium and squamous metaplasia after 5 days; milder lesions in pharynx, larynx, trachea, lungs.	Jiang et al. (1983)
	6 h/day × 5 days	9.7	Nasal, tracheal, and lung lesions, with anterior to posterior severity gradient (exfoliation, necrosis, inflammation, squamous metaplasia, hyperplasia, bronchiolitis).	Buckley et al. (1984)
	6 h/day, 5 days/week × 104 weeks	0.4, 1.0, 2.5	BW ↓ for M at 1.0 and 2.5 ppm and F at 2.5 ppm after >3 months. All had nasal lesions, most severe in anterior and generally concentration-dependent (septal fenestration; proteinaceous accumulation; epithelial hyperplasia; squamous metaplasia; OE atrophy).	Wolf et al. (1995)

↑, increase(d); ↓, decrease(d); BW, body weight(s); F, female; M, male; OE, olfactory epithelium (nasal).

to chlorine toxicity? How do chlorine exposure concentration and duration contribute to chlorine toxicity?

A review of human chlorine inhalation studies led White and Martin (2010) to conclude that there was no basis for considering young subjects more susceptible than adults to chlorine toxicity. Although it was speculated that children might be more susceptible than adults to chlorine toxicity, based on reports of illness following several accidental chlorine releases, the actual air chlorine levels and exposure durations of the subjects were unknown (ATSDR, 2010).

Among healthy non-asthmatic adults aged 18–69 years who were exposed to 1.0 ppm chlorine for 15 min, nasal airway resistance, congestion, and irritation increased with age and preexisting allergic rhinitis, independent of gender (Shusterman et al., 2003a,b, 2004). Adults with nonspecific airway hyperreactivity or asthma, or those who smoke, were more susceptible to chlorine respiratory symptoms and pulmonary dysfunction and recovered more slowly after chlorine exposure (Rotman et al., 1983; D'Alessandro et al., 1996; ATSDR, 2010).

The degree to which endpoints such as respiratory tract irritation and mortality are influenced by chlorine exposure concentration or duration have been evaluated using studies that varied both parameters. The concentration-time relationship for many irritants and systemically acting vapors and gases can be described by the equation $C^n \times t = k$ (ten Berge et al., 1986), which is equivalent to Haber's rule ($C \times t = k$) when $n = 1$. A value of $n = 1.9$ was determined for chlorine gas nuisance irritation by ten Berge and Vis van Heemst (1986) using the Anglen (1981) human data. This value of n indicates that exposure concentration was a greater determinant of toxicity than duration; a similar conclusion was drawn by Shusterman et al. (2004) in their analysis of chlorine sensory irritation in a number of controlled human studies. Haber's rule did not adequately capture the type, incidence, and severity of nasal lesions in female rats exposed once to 0.1–10 ppm chlorine for 1–24 h, such that the product of the test concentration and duration ($C \times t$) was constant (Peay et al., 2010). Mice exposed to 100–800 ppm chlorine at a constant $C \times t$ of 100 ppm h had much greater mortality and lung injury at 800 ppm (0.125 h exposure) than at 100 ppm (1 h exposure) (Hoyle et al., 2010).

Current US workplace standards limit chlorine air concentrations to 0.5 ppm (NIOSH REL-TWA Ceiling (NIOSH, 2010); ACGIH TLV-TWA (ACGIH, 2010)) or 1 ppm (OSHA Ceiling (OSHA, 2006); ACGIH TLV-STEL (ACGIH, 2010)). These standards reflect human data showing that inhalation of 0.5–1 ppm chlorine, whether once or repeatedly, causes mild upper respiratory tract irritation, which is more severe in individuals with preexisting conditions such as asthma, rhinitis, and airway hyperreactivity (Joosting and Verberk, 1974; Anglen, 1981; Rotman et al., 1983; D'Alessandro et al., 1996; Shusterman et al., 1998, 2003a,b, 2004). The human studies also served as the basis for guidelines to prevent adverse but nondisabling effects from a single 10 min to 24 h inhalation exposure. The Acute Exposure Guideline Levels (AEGLs) limit chlorine air concentration to 0.5 ppm for a 10–480 min exposure (Talmage, 2004). The provisional advisory levels (PALs) limit chlorine levels to 0.096 ppm for a 24 h exposure (Milanez et al., 2014). Additional AEGL and PAL values were developed for chlorine that are associated with serious irreversible effects or lethality (Talmage, 2004; Milanez et al., 2014).

TREATMENT

Currently, treatment for chlorine inhalation is largely nonspecific and geared to alleviating the patient's symptoms, and a chlorine-specific antidote is unavailable (Jones et al., 2010). All persons exposed to Cl_2 should be given humidified oxygen, and inhaled beta-adrenergic agents should be administered if there is evidence of airway obstruction (White and Martin, 2010). If ocular irritation is present, the eyes should be copiously irrigated. Respiratory peak flow should be measured in patients with mild or greater symptoms, and patients with moderate or severe effects should have a chest X-ray (IPCS, 2008). All symptomatic patients should be observed for 8–24 h for delayed pulmonary edema, which can be treated with positive airway pressure (Traub, 2006; IPCS, 2008). Endotracheal intubation and mechanical ventilation may be used in the most extreme cases (IPCS, 2008). However, the majority of individuals exposed to mild to moderate chlorine levels see resolution of their symptoms in 3–5 days and recover normal pulmonary function within several months (Jones et al., 2010).

The most commonly prescribed medications for over 60 people who were hospitalized following the release of chlorine gas in the 2005 train accident in Graniteville, SC, were inhaled β-agonists, ipratropium bromide (an anticholinergic drug used to treat chronic obstructive pulmonary disease), and inhaled and oral corticosteroids (Van Sickle et al., 2009). Less common treatments included antibiotics and intravenous or nebulized sodium bicarbonate. Sodium bicarbonate can theoretically neutralize the HCl and HOCl formed in the respiratory tract from Cl_2 hydrolysis, although its efficacy and optimal treatment have not been clearly established (Jones et al., 2010). Corticosteroids are used to reduce the inflammatory response and lung scarring resulting from acute exposure to respiratory irritants, although the ideal steroid and treatment regimen for Cl_2 exposure is undefined (Jones et al., 2010; Chen et al., 2013).

Various compounds have been tested in animal studies in the quest to develop an effective chlorine-specific treatment based on its mechanism of action (Table 24.4). The evaluated compounds include the antioxidants dimethylthiourea, ascorbic acid, *N*-acetyl-L-cysteine, and deferoxamine mesylate (Leustik et al., 2008; McGovern et al., 2010; Zarogiannis et al., 2011); sodium nitrite (Yadav et al., 2011); the corticosteroids mometasone, budesonide, and dexamethasone (Chen et al., 2013; Jonasson et al., 2013), the type 4 phosphodiesterase inhibitor rolipram (Chang et al., 2012), and the β2-agonist arformoterol (Song et al., 2011).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Chlorine studies have been conducted with humans and laboratory animals since before its use as a chemical weapon in World War I. Chlorine gas is a direct-acting irritant that primarily affects the respiratory tract, causing irritation and tissue damage that can lead to death at sufficiently high doses. Chlorine toxicity is believed to be due to its oxidant properties and to be mediated by the hypochlorite ion, although the precise mechanism of cellular damage is unknown. An alternative hypothesis suggests that chlorine itself may play a substantial role in toxicity. The chlorine exposure concentrations and durations associated with various degrees of toxicity in humans have been defined, taking into consideration the impact of human variability such as preexisting respiratory conditions, age, and gender.

Despite its toxicity and potential for nefarious use, chlorine continues to be widely used in manufacturing and as a disinfectant. Currently, treatment for chlorine inhalation is largely supportive and nonspecific. Research continues into elucidating chlorine's mechanism of action and into finding methods to mitigate its toxic effects.

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CHAPTER

25

Phosgene

Cheryl B. Bast and Dana F. Glass-Mattie

INTRODUCTION

Phosgene is a colorless gas at ambient temperature and pressure. Its odor has been described as similar to newly mown hay (Leonardos et al., 1968). This mild odor and the weak acute irritant properties, however, provide little warning of its presence (Lipsett et al., 1994).

Phosgene is manufactured from a reaction of carbon monoxide and chlorine gas in the presence of activated charcoal. Phosgene is used in the manufacture of isocyanates, polycarbonates, pesticides, dyes, and pharmaceuticals. Manufacture of phosgene in the United States is almost entirely captive in that more than 99% is used in the manufacture of other chemicals within a plant boundary (US EPA, 2003). The odor threshold is between 0.5 and 1.5 ppm (NIOSH, 1976); unfortunately, the odor threshold is inadequate to protect against toxic inhalant exposure because damage to the deep respiratory tract can take place at lower concentrations (Sidell et al., 1997). Phosgene has an odor safety classification rating of "E," which indicates that fewer than 10% of attentive persons can detect the Threshold Limit Value (TLV) (Amoore and Hautala, 1983).

Inhalation is the most important route of exposure for phosgene. Because of its mild upper respiratory, eye, and skin irritancy, and a mildly pleasant odor, an exposed victim may not actively seek an avenue of escape before lower respiratory damage has occurred (Currie et al., 1987a; Lipsett et al., 1994). Small amounts of phosgene can be irritating to the eyes (lacrimation) and throat (coughing) as phosgene undergoes hydrolysis to create hydrochloric acid, which acts as an irritant. Far more dangerous, however, is the pulmonary edema that can develop after a latent period of 1–24 h after exposure. Exercise can increase the extent of pulmonary edema (Marrs et al., 1996). If severe clinical signs are not present after 48 h, then chances for full recovery are great. Physical and chemical properties of phosgene are summarized in Table 25.1.

BACKGROUND

Phosgene was first made in 1812 and was eventually used as a chemical warfare agent in WWI. Because of its higher density compared with air, phosgene gas can accumulate in low-lying areas; thus, concentrated pockets within war trenches caused significant exposure during WWI. German troops in WWI used phosgene gas as a chemical warfare agent against the British troops. Possibly up to 85% of the deaths in WWI were a result of phosgene gas exposure (Ministry of Defense, 1987). Sandall (1922) examined 83 British soldiers 3 years after phosgene exposure. Shortness of breath on exertion (70%), cough with expectoration (54%), tight feeling in the chest (25%), sporadic giddiness (14%), and nausea (12%) were the most frequently reported symptoms. No physical lung abnormalities were noted in 53% of the men.

The concept of a "death product" was introduced by Haber to explain the relationship between the extent of exposure to phosgene and death (Haber, 1924). According to "Haber's law," the biological effect of phosgene is directly proportional to the exposure expressed as the product of the atmospheric concentration (C) and the time of exposure (T) or $CT = k$, where k can be death, pulmonary edema, or other biological effects of phosgene exposure (US EPA, 1986). Haber's law has subsequently been shown by other investigators to be valid for both nonlethal and lethal effects within certain limits. There appears to be little species variability with regard to lethality between rats, mice, and guinea pigs, and the $CT = k$ relationship appears to be generally

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TABLE 25.1 Physical and Chemical Data

Parameter	Data	Reference
Synonyms	Carbonyl chloride, carbon oxychloride, carbonic dichloride, chloroformyl chloride	US EPA (1986) and Lipsett et al. (1994)
Chemical formula	$COCL_2$	Lipsett et al. (1994)
Molecular weight	98.92	Lipsett et al. (1994)
CAS registry No.	75-44-5	Lipsett et al. (1994)
Physical state	Colorless gas at room temperature	Budavari et al. (1989)
Odor threshold	0.5–1.5 ppm	NIOSH (1976)
Odor description	Pleasant, like newly mown hay	Dunlap (2001)
Vapor pressure	1215 mmHg at 20°C	Budavari et al. (1989)
Vapor density	3.5 (air = 1)	NIOSH (2010)
Specific gravity	1.92 (water = 1)	CRC Handbook (1988)
Melting/boiling/flash point	−118°C/8.2°C/non-flammable	NIOSH (2010)
Water solubility	Slightly soluble in water, decomposes rapidly ($t_{½}$ = 0.26 s)	US EPA (2003); NIOSH (2010)
Reactivity	Reacts with alcohols, alkalis, ammonia, and copper	NIOSH (2010)
Conversion factors in air	1 ppm = 4.1 mg/m^3 1 mg/m^3 = 0.25 ppm	NIOSH (2010)

valid (although at very high or very low concentrations or exposure times so short that the animal can hold its breath, the $CT = k$ relationship may not be relevant).

TOXICOKINETICS

After inhalation exposure, a small portion of phosgene hydrolyzes to hydrochloric acid (HCl) and carbon dioxide (CO_2) in the mucous coating of the upper respiratory tract (Diller, 1985), but in the moist atmosphere of the terminal spaces of the lungs more extensive hydrolysis is thought to occur (Beard, 1982). Although phosgene is only slightly soluble in water, once in solution it rapidly hydrolyzes to HCl and CO_2. However, phosgene reacts even faster with other functional groups such as amino, hydroxyl, and sulfhydryl groups (Diller, 1985; Jaskot et al., 1991). Because of the affinity for lung tissue and the hydrolysis and acylation that take place in the pulmonary system, very little, if any, phosgene is dispersed to other locations in the body.

MECHANISM OF ACTION

The toxicity of phosgene is attributable to both hydrolysis and acylation, with the latter being most important.

Diller (1985) accumulated data and described the clinical signs associated with phosgene exposure. Phosgene inhaled at concentrations of more than 1 ppm triggers a transient vagal reflex and causes shallow, rapid respiration with a decrease in respiratory volume and capacity, a decrease in arterial oxygen partial pressure, and bradycardia. The intensity varies greatly between individuals. Phosgene at more than 3 ppm becomes moderately irritating to the eyes and upper airways (cough) as some of the phosgene undergoes hydrolysis, producing HCl.

The acylation reaction of phosgene with nucleophiles, such as amino, hydroxyl, and sulfhydryl groups, also occurs rapidly, causing lipid and protein denaturation, irreversible membrane changes, and disruption of enzymatic function. These acylation effects observed at exposures more than 30 ppm·min produce pulmonary edema as the blood–air barrier becomes more permeable to blood plasma after a clinical latent period (1–24 h). This is called the clinical edema phase, which is characterized by increasing inefficiency of gas exchange as more defects in the blood–air barrier occur, allowing more accumulation of a protein-rich fluid. Clinical signs in this phase are labored breathing and a frothy expectorant. Progression usually results in death from paralysis of the respiratory center due to anoxemia with secondary cessation of heart function. If anoxemia is controlled, then circulatory shock may still occur. At very high concentrations (>200 ppm), phosgene may cause death within a few minutes from "acute cor pulmonale" (acute overdistention of the right heart), often before pulmonary edema can develop.

Phosgene depletes lung glutathione, whereas glutathione reductase and superoxide dismutase increase as a result of the lung's response to injury. On exposure to

TABLE 25.2 Summary of Major Signs and Symptoms of Phosgene Inhalation Exposure in Humans

Exposure					
Concentration	**Duration**	**$C \times T$ product (ppm min)**	**Acute Effect**	**Time to Onset of Pulmonary Edema**	**Time to Death**
3 ppm	"Acute"	–	Throat irritation	–	–
4 ppm	"Acute"	–	Ocular irritation	–	–
4.8 ppm	"Acute"	–	Cough	–	–
1 ppm	20 min	20	–	–	–
5 ppm	5 min	25	–	–	–
1 ppm	150 min	150	–	10 h	–
50 ppm	5 min	250	–	5 h	–
100 ppm	5 min	500	–	3 h	24 h
1.3 ppm	400 min	520	–	7 h	30 h
300 ppm	2 min	600	–	–	Minutes

Source: Diller and Zante (1982) and Borak and Diller (2001).

phosgene, cellular glycolysis, oxygen uptake, intracellular ATP, and cyclic AMP are decreased and associated with increased permeability of pulmonary vessels, leading to pulmonary edema. Phosgene exposure also causes increased lipid peroxidation and leukotriene synthesis, with no change in cyclooxygenase metabolism (Borak and Diller, 2001).

TOXICITY

Human

Noncancer

Reports of human phosgene poisoning present a relatively consistent set of clinical effects and sequelae (Delephine, 1922; Hegler, 1928; Galdston et al., 1947a,b; Herzog and Pletscher, 1955; Everett and Overholt, 1968; Henschler, 1971; Stavrakis, 1971; Diller et al., 1979; Bradley and Unger, 1982; Misra et al., 1985; Regan, 1985; Wells, 1985; Cordasco et al., 1986; Kaerkes, 1992; Hardison et al., 2014). After acute phosgene exposure, brief (20 min) ocular and throat irritation, cough, nausea and vomiting, and dizziness are experienced, followed by a period (24 h) of apparent well-being. After this clinical latent phase, cough accompanied by expectoration, a sensation of pain or tightness of the chest, shortness of breath, and a choking sensation are experienced. Clinical findings may include hemoconcentration, leukocytosis, rales, and pulmonary edema. After recovery, rapid and shallow breathing, shortness of breath on exertion, and a sense of decreased physical fitness may persist for months. Pulmonary emphysema may occur with repeated exposure to phosgene.

Diller and Zante (1982) identified ocular irritation, throat irritation, and cough as acute irritating effects of phosgene. In a follow-up analysis, Borak and Diller (2001) also performed an extensive literature review concerning human phosgene exposure and concluded the following: the smell has no warning property; immediate irritation is not prognostic; and pulmonary edema can appear several hours after exposure and the length of clinical latency can be used as a prognostic indicator (i.e., the shorter the time to effects, the worse the prognosis). The data also show that although concentration is the primary driver for the onset and severity of symptoms, duration of exposure also plays a role. Information synthesized from these reviews is presented in Table 25.2.

Cancer

Epidemiology studies have shown no increase in cancer in workers exposed to phosgene compared with controls. Polednak (1980) and Polednak and Hollis (1985) examined a cohort of chemical workers exposed to chronic low levels of phosgene as well as daily exposures more than 1 ppm. Approximately 35 years after exposure to phosgene, no increase in overall mortality or mortality from cancer or respiratory disease was noted.

Animal

Noncancer

Animal studies with phosgene show a steep concentration–response curve for pulmonary edema and mortality. Acute animal studies also indicate little species variability because rats, mice, sheep, pigs, and dogs

exposed had development of similar clinical signs (dyspnea, labored breathing, and pulmonary edema after a latent period) and histopathological lesions in the lungs. Although there are no chronic animal data, subchronic studies indicate little accumulation of phosgene or increased severity of lesions with continuous exposure.

Bronchoalveolar lavage (BAL) fluid analysis may be used to assess pulmonary edema/lung injury after acute inhalation exposure to phosgene (Pauluhn et al., 2007). Maximum protein concentrations in BAL fluid typically occur within 1 day after exposure, followed by a latency period up to 15 h. For acute exposures, the *CT* relationship is constant over a wide range of concentrations. However, after noncontinuous and repeated exposure, increased tolerance to subsequent exposures is observed. Although limited, the longer-term data indicate that effects do not increase in severity over time but do increase with increased concentration, suggesting that chronic toxicity is dependent on an acute pulmonary threshold dose. A comparison of BAL fluid constituents from acute inhalation studies in rats and dogs suggests that dogs are three- to four-times less sensitive to phosgene than rats (Pauluhn et al., 2007).

A summary of selected lethal and nonlethal animal toxicity studies is presented in Tables 25.3 and 25.4, respectively.

Animal Cancer

A study by Selgrade et al. (1989) showed that exposure to very low levels of phosgene enhances the susceptibility of mice to lung tumor formation. Female C57BL/6 mice were exposed for 4 h to 0.01 ($N = 13$), 0.025 ($N = 28$), or 0.05 ppm phosgene ($N = 35$) and injected intravenously with syngeneic B16 melanoma cells the next day. Controls were injected with tumor cells and exposed to air. The

TABLE 25.3 Summary of Lethal Animal Inhalation Studies with Phosgene

Concentration (ppm)	Time	Species	Effect	Reference
Various	30 min	Dog	LC_{50} = 66 ppm	Boyland et al. (1946)
Various	30 min	Dog	LC_{50} = 61–70 ppm	Underhill (1920)
Various	60 min	Dog	LC_{50} = 42 ppm	Boyland et al. (1946)
Various	8 min	Rat	LC_{50} = 92 ppm	Boyland et al. (1946)
12, 37, 75, 80, 88, 93, or 106	10 min	Rat	LC_{50} = 82 ppm	Zwart et al. (1990)
41, 44, 52, or 61	10 min	Rat	LC_{50} = 62 ppm	Pauluhn (2006a)
12, 15, 16, 17, or 25	30 min	Rat	LC_{50} = 21 ppm	Zwart et al. (1990)
12, 13, 17, or 22	30 min	Rat	LC_{50} = 13.5 ppm	Pauluhn (2006a)
Various	32 min	Rat	LC_{50} = 17 ppm	Boyland et al. (1946)
6.4, 8.8, 9.0, or 12	60 min	Rat	LC_{50} = 12 ppm	Zwart et al. (1990)
7.3, 9.6, or 12	60 min	Rat	LC_{50} = 7.7 ppm	Pauluhn (2006a)
Various	64 min	Rat	LC_{50} = 11 ppm	Boyland et al. (1946)
2.2 or 2.7	240 min	Rat	LC_{50} = 2.1 ppm	Pauluhn (2006a)
10, 15, 25, 35, 50, 70, or 90	5 min	Mouse	LC_{50} = 33 ppm	Kawai (1973)
Various	8 min	Mouse	LC_{50} = 77 ppm	Boyland et al. (1946)
12, 37, 75, 80, 88, 93, or 106	10 min	Mouse	LC_{50} = 79 (m) and 60 (f) ppm	Zwart et al. (1990)
12, 15, 16, 17, or 25	30 min	Mouse	LC_{50} = 19 (m) and 11.5 (f) ppm	Zwart et al. (1990)
1.0, 2.0, 3.0, 6.0, 9.0, or 13.5	30 min	Mouse	LC_{50} = 5.1 ppm	Kawai (1973)
Various	32 min	Mouse	LC_{50} = 15 ppm	Boyland et al. (1946)
6.4, 8.8, 9.0, or 12	60 min	Mouse	LC_{50} = 9.5 (m) and 5.0 (f) ppm	Zwart et al. (1990)
Various	64 min	Mouse	LC_{50} = 7 ppm	Boyland et al. (1946)
Various	8 min	Guinea pig	LC_{50} = 43 ppm	Boyland et al. (1946)
Various	32 min	Guinea pig	LC_{50} = 13 ppm	Boyland et al. (1946)
Various	64 min	Guinea pig	LC_{50} = 11 ppm	Boyland et al. (1946)

TABLE 25.4 Summary of Non-Lethal Animal Inhalation Studies with Phosgene

Concentration (ppm)	Time	Species	Effect	Reference
0 or 60	10 min	Pig	LOAEL ≥ 60 ppm, based on increased lung wet weight, mortality	Brown et al. (2002)
137, 244, 435, or 773	10 min	Sheep	LOAEL ≥ 137 ppm based on pulmonary edema, shallow breathing	Keeler et al. (1990a)
0 or 490 to 611	10 min	Sheep	LOAEL ≥ 490 ppm based on lung edema	Keeler et al. (1990b)
0 or 8	20 min	Mouse	LOAEL ≥ 8 ppm, based on acidosis, clinical signs and ↓body wt, ↑lung ww/dw	Sciuto et al. (2001)
0 or 22	20 min	Mouse, rat, and guinea pig	LOAEL ≥ 22 ppm, based on ↑LFP[a]	Sciuto (1998)
0, 2.1, 4.3, or 8.8	30 min	Dog	LOAEL = 4.3 ppm, based on increased PMNs in BAL[a] fluid NOAEL = 2.1 ppm	Pauluhn (2006c)
0.2, 0.5, 1.0, 2.0, or 4.0	30 min	Rat	LOAEL = 2.0 ppm, based on clinical signs and ↓body wt, ↑LFP NOAEL = 1.0 ppm	Pauluhn (2006b)
0, 0.05, 0.1, 0.2, 0.4, or 1.0	240 min	Rat	LOAEL = 0.2 ppm, based on based on clinical signs and ↓body wt, ↑LFP NOAEL = 0.1 ppm	Pauluhn (2006b)
0, 0.1, 0.5, or 1.0	240 min	Rat	LOAEL = 0.5 ppm based on decrease in NK cell activity NOAEL = 0.1 ppm	Burleson and Keyes (1989)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↓body wt, ↑lung wts	Ehrlich et al. (1989)
0 or 0.5	240 min	Rat	LOAEL ≥ 0.5 ppm based on ↑LFP and lung wts	Jaskot et al. (1989)
0, 0.25, or 0.5	240 min	Guinea pig	LOAEL ≥ 0.25 ppm based on ↑LFP	Slade et al. (1989)
0.1 to 0.5	240 min	Mouse	LOAEL = 0.15 ppm based on ↑phenobarbital induced sleeping times NOAEL = 0.10	Illing et al. (1988)
0, 0.125, 0.25, 0.5, or 1.0	240 min	Rat	LOAEL = 0.25 ppm based on ↑PMNs in lavage fluid NOAEL = 0.125 ppm	Currie et al. (1987a)
0, 0.05, 0.125, 0.25, 0.5, or 1.0	240 min	Rat	LOAEL ≥ 0.05 ppm based on ↓ATP in lungs	Currie et al. (1987b)
0, 0.1, 0.2, 0.5, or 1.0	240 min	Rat, mouse, and hamster	LOAEL = 0.2 ppm based on ↑LFP NOAEL = 0.1 ppm	Hatch et al. (1986)
0, 0.1, 0.2, 0.5, or 1.0	240 min	Rabbit and guinea pig	LOAEL = 0.5 ppm based on ↑LFP NOAEL = 0.2 ppm	Hatch et al. (1986)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↓body wt, ↑lung wts	Franch and Hatch (1986)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↑pulmonary edema	Frosolono and Currie (1985)
0 or 1.0	420 min	Rat	LOAEL ≥ 1.0 ppm based on ↑lung wts	Franch and Hatch (1986)
0.125 or 0.25	4 h/day, 5 days/week for 17 days	Rat	LOAEL = 0.25 ppm based on ↑lung wts and ↑NPSH and G6PD activity NOAEL = 0.125 ppm	Franch and Hatch (1986)

(*Continued*)

TABLE 25.4 (Continued)

Concentration (ppm)	Time	Species	Effect	Reference
0, 0.1, 0.2, 0.5, or 1.0	0.1 ppm for 5 days/week; 0.2 ppm for 5 days/week; 0.5 ppm for 2 days/week; 1.0 ppm for 1 day/weekAll exposed 6 h/day for up to 12 weeks	Rat	LOAEL = 0.1 ppm based on reversible lung histopathology; ↑lung displacement volume NOAEL = none	Kodavanti et al. (1997)
0, 0.1, 0.2, or 0.5	0.1 and 0.2 ppm for 5 days/week; 0.5 ppm for 2 days/week All exposed 6 h/day for up to 12 weeks	Rat	LOAEL = 0.1 ppm based on decreased bacterial clearance after infection with *Streptococcus zooepidemicus* NOAEL = none	Selgrade et al. (1995)

[a]*LFP, lavage fluid protein; BAL, bronchiolar lavage.*

lungs were removed 2–3 weeks after tumor cell injection and the tumors were counted. Compared with controls, there was a statistically significant ($P < 0.05$) increase in the number of B16 melanoma tumors in the lungs of mice treated with 0.025 or 0.05 ppm phosgene. Exposure to 0.025 ppm was considered the lowest observed effect level. Extending the exposure time from 4 to 8 h did not alter the susceptibility to B16 tumors at 0.01 ppm.

RISK ASSESSMENT

Many inhalation regulatory and guideline levels have been derived for phosgene. These values are summarized in Table 25.5, and the definitions and basis for the values are described in the footnotes to Table 25.5.

TREATMENT

Treatments that have been proposed to prevent pulmonary edema in exposed persons include sedation, steroids, ibuprofen, *N*-acetylcysteine, B2-andregenic agonists, aminophylline/theophylline, leukotriene agonists, and positive pressure airway ventilation. However, there is no known antidote for phosgene poisoning, and although animal studies suggest that these treatments may be effective, no clinical data are available to verify efficacy in humans (Borak and Diller, 2001; Grainge and Rice, 2010; ACC, 2013). Asymptomatic individuals exposed to phosgene should be observed and evaluated to determine if symptoms develop. Vital signs and lung auscultation should be evaluated every 30 min and serial chest X-rays should be performed starting 2 h after exposure. If no clinical signs occur and no signs of pulmonary abnormalities are detected on the X-ray after 8 h, then patients may be discharged. If no X-ray is available, then patients should be observed for 24 h after exposure. If signs develop, then patients should be treated. Therapy for the cardiogenic pulmonary edema may include positive airway pressure and monitoring (endotracheal intubation and mechanical ventilation with high oxygen concentrations), steroids, theophylline, diuretics, and antibiotics (in patients who develop bacterial pulmonary infections) (Borak and Diller, 2001).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Phosgene is a colorless gas at ambient temperature and pressure, and inhalation is the most important route of exposure for phosgene. The odor of phosgene has been described as similar to newly mown hay. Because of its mild upper respiratory, eye, and skin irritancy, and a mildly pleasant odor, an exposed victim may not actively seek escape before lower respiratory damage has occurred (Currie et al., 1987a; Lipsett et al., 1994). Pulmonary edema is the cause of death after a clinical latent period of 24 h (Franch and Hatch, 1986). Phosgene exhibits a steep concentration–response curve and little species variability with regard to lethality. Data (Pauluhn, 2006a–c; Pauluhn et al., 2007) suggest that with regard to physiology of the respiratory tract and acinar structure of the lung, dogs are more similar to humans than rodents. Thus, it may be most appropriate to base future phosgene risk assessments on data extrapolation from dogs to humans when dog data are available. Recent studies appear to focus on acquiring an additional in-depth understanding of the mechanism of action of phosgene through use of animal models (Chen et al., 2009; Li et al., 2013; Wang et al., 2013).

TABLE 25.5 Inhalation Standards and Guidelines for Phosgene

	Exposure Duration				
Guideline	**10 min**	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	NA	NA	NA	NA	NA
AEGL-2[a]	0.60 ppm	0.60 ppm	0.30 ppm	0.08 ppm	0.04 ppm
AEGL-3[a]	3.6 ppm	1.5 ppm	0.75 ppm	0.20 ppm	0.09 ppm
ERPG-1[b]			NA		
ERPG-2[b]			0.5 ppm		
ERPG-3[b]			1.5 ppm		
EEGL (NRC)[c]			0.2 ppm		0.02 ppm (24 h)
NIOSH IDLH[d]	2 ppm				
NIOSH STEL[e]	0.2 ppm (15 min ceiling)				
NIOSH REL[e]					0.1 ppm (10 h)
OSHA PEL-TWA[f]					0.1 ppm
ACGIH TLV[g]					0.1 ppm
MAK (Germany)[h]					0.1 ppm
MAC (Netherlands)[i]					0.1 ppm
RfC[j]					7.33×10^{-5} ppm

	Exposure Duration			
	24 h	**30 days**	**90 days**	**2 years**
PAL 1[k]	0.0017 ppm	0.0006 ppm	0.0006 ppm	0.0006 ppm
PAL 2[k]	0.0033	0.0012 ppm	0.0012 ppm	0.0012 ppm
PAL 3[k]	0.022	NA	NA	NA

[a]*AEGL (Acute Exposure Guideline Levels) (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels (AEGL-1, AEGL-2, and AEGL-3) are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects. The three AEGLs tiers are defined as follows.*

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, non-sensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. The AEGL-1 is not recommended for phosgene because the odor threshold is at the concentration approaching AEGL-2 values, and odor cannot be used as a warning.

AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. The AEGL-2 for phosgene is based on chemical pneumonia in rats (Gross et al., 1965).

AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 is based on concentrations causing no death in rats or mice (Zwart et al., 1990).

[b]*ERPG (Emergency Response Planning Guidelines, American Industrial Hygiene Association (AIHA, 2013). The ERPG-1 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing other than mild, transient adverse health effects or without perceiving a clearly defined objectionable odor. The ERPG-1 for phosgene is not derived. The ERPG-2 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing irreversible or other serious health effects or symptoms that could impair an individual's ability to take protection action. The ERPG-3 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing life-threatening health effects.*

[c]*EEGL (Emergency Exposure Guidance Levels, National Research Council (NRC, 1985). The EEGL is the concentration of contaminants that can cause discomfort or other evidence of irritation or intoxication in or around the workplace, but avoids death, other severe acute effects and long-term or chronic injury. The EEGL for Phosgene is based on the "most relevant animal exposure studies (Rinehart and Hatch, 1964; Gross et al., 1965)" and studies suggesting that animals do not tolerate phosgene at 0.2 ppm administered 5 h/day for 5 days (Cameron and Foss, 1941; Cameron et al., 1942).*

[d]*IDLH (Immediately Dangerous to Life and Health, National Institute of Occupational Safety and Health) (NIOSH, 2010) represents the maximum concentration from which one could escape within 30 min without any escape-impairing symptoms, or any irreversible health effects. The IDLH for phosgene is based on acute inhalation toxicity data in humans (Diller, 1978).*

[e]*NIOSH REL-STEL (Recommended Exposure Limits—Short Term Exposure Limit) (NIOSH, 2010) is defined analogous to the ACGIH TLV-TWA.*

[f]*OSHA PEL-TWA (Occupational Health and Safety Administration, Permissible Exposure Limits—Time Weighted Average) (NIOSH, 2010) is defined analogous to the ACGIH-TLV-TWA, but is for exposures of no more than 10 h/day, 40 h/week.*

[g]*ACGIH TLV-TWA (American Conference of Governmental Industrial Hygienists, Threshold Limit Value—Time Weighted Average) (ACGIH, 2013) is the time-weighted average concentration for a normal 8 h workday and a 40 h workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect. The ACGIH TLV-TWA was derived from the marked potential for pulmonary irritation after exposure to phosgene at concentrations greater than 0.1 ppm; this conclusion was based on two studies. Gross et al. (1965) found that phosgene at 0.5 ppm for 2 h caused some pathologic changes in the lungs of rats and Cameron et al. (1942) found that 0.2 ppm, 5 days/week for 5 consecutive days caused pulmonary edema in 41% of animals exposed (goats, cats, rabbits, guinea pigs, rats, and mice).*

[h]*MAK (Maximale Argeitsplatzkonzentration (Maximum Workplace Concentration)) Deutsche Forschungsgemeinschaft (German Research Association) 2012 is defined analogous to the ACGIH-TLV-TWA.*

[i]*MAC (Maximaal Aanvaaarde Concentratie (Maximal Accepted Concentration)). SDU Uitgevers (under the auspices of the Ministry of Social Affairs and Employment), The Hague, The Netherlands 2012 is defined analogous to the ACGIH-TLV-TWA.*

[j]*US EPA (US Environmental Protection Agency Reference Concentration). (US EPA, 2005). The RfC is an estimate of a continuous inhalation exposure concentration to people (including sensitive subgroups) that is likely to be without risk of deleterious effects during a lifetime. The RfC was developed by using incidence of the lung histopathological findings from Kodavanti et al. (1997) in a bench-mark dose analysis.*

[k]*PAL (Provisional Advisory Levels). (Glass et al., 2008). Provisional Advisory Levels represent exposure limits for the general public applicable to emergency situations. Three levels (PAL 1, PAL 2, and PAL 3), distinguished by the degree of severity of toxic effects, are developed for up to 24 h, 30-day, 90-day, and 2-year durations of potential drinking water and inhalation exposures. The PALs have not been promulgated nor have they been formally issued as regulatory guidance. They are intended to be used at the discretion of risk managers in emergency situations when site specific risk assessments are not available.PAL 1 represents the assumed continuous exposure concentration of a chemical in air or drinking water above which changes from baseline of specific biomarkers or physiological responses could have adverse health effects in the general population. Concentrations at or below PAL 1 are not expected to be associated with adverse health effects. Increasingly greater concentrations above the PAL 1 value could cause progressively harmful effects in the general population, including all ages and sensitive subpopulations.PAL 2 represents the assumed continuous exposure concentration of a chemical in air or drinking water above which serious, irreversible, or escape-impairing effects could result. Increasingly greater concentrations above the PAL 2 value could cause progressively harmful effects in the general population, including all ages and sensitive subpopulations.PAL 3 represents the assumed continuous exposure concentration of a chemical in air or drinking water above which lethality in the general population, including all ages and sensitive subpopulations could occur.*

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CHAPTER

26

Other Toxic Chemicals as Potential Chemical Warfare Agents

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INTRODUCTION

For the development of new chemical weapons (CWs), a number of criteria are necessary: a research base including scientists and equipment, access to information, chemical and arms industries, and, of course, financial support. It is noteworthy that the development of CWs is possible not only for states, but also for terrorists. It is necessary to stress that the intention of this chapter is not to describe new CWs or chemical warfare agents (CWAs), but to comment on a number of trends in toxicology with the aim that these chemicals may be proposed for inclusion in the Chemical Weapons Convention (CWC) verification mechanisms. However, the text of the CWC is comprehensive and covers practically all chemicals that may be misused as CWs.

The objective of this chapter is to briefly describe a number of chemicals that could be used as toxic compounds or CWAs against humans.

GENERAL

Chemical Weapons Convention: Article II, Definitions and Criteria

1. "CWs" means the following, together or separately:

- **a.** Toxic chemicals and their precursors, except where intended for purposes not prohibited under this Convention, as long as the types and quantities are consistent with such purposes.
- **b.** Munitions and devices, specifically designed to cause death or other harm through the toxic properties of those toxic chemicals specified in subparagraph (a), which would be released as a result of the employment of such munitions and devices.
- **c.** Any equipment specifically designed for use directly in connection with the employment of munitions and devices specified in subparagraph (b).

2. "Toxic chemical" means:

Any chemical which, through its chemical action on life processes, can cause death, temporary incapacitation or permanent harm to humans or animals. This includes all such chemicals, regardless of their origin or their method of production, and regardless of whether they are produced in facilities, in munitions, or elsewhere.

Therefore, CWAs can be characterized as toxic chemicals. Initially, it is difficult to differentiate between the research aimed at protection against CWAs (defense) and development of new CWAs (offense). Both actions deal with the synthesis of chemicals, based on either information or ideas, or incidentally synthesized toxic compounds. A typical example is the synthesis of organophosphates (OPs) by G. Schrader, originally dedicated to the development of new pesticides. Another example of the development of a new CW would be the synthesis of OP compounds of the V series. After synthesis, the compound in question will be characterized chemically and biologically, and sometimes modified to increase its military properties (toxicity and physicochemical properties, such as stability and volatility). Then the compound can be studied in detail for its pharmacological and toxicological characteristics by using more convenient species and routes of administration. At this stage, it is practically impossible to decide if the research is offensive or defensive though some indications would lead to the opinion that the direction is offensive; for example, when attention is given to its efficacy following percutaneous

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or inhalation administration. Studied methods of dispersion under field conditions are an indication of an offensive approach; testing for protective qualities under real conditions can be regarded as a defensive approach. However, further steps, like production of large quantities and weaponization, are clearly offensive. It is useful to compare the time from synthesis to production or use of certain CWs. After the synthesis of phosgene and diphosgene (1812 and 1887), their use in 1916 was observed; a similar situation was observed for mustard (1866–1917); for CS, this period was shortened (synthesis in 1928 and use in 1950); and VX was synthesized in the early 1960s and weaponized in 1968. The big question is: What is meant by large quantities? This can be addressed using the approach contained in the CWC. Quantities would also be compared with the contamination density prescribed for different CWAs. For instance, for yperite, it is 19 tons/km^2 for percutaneous administration and 4 tons/km^2; for *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX) (percutaneous) it is 2 tons/km^2; for sarin and 3-quinuclidinyl benzilate (BZ) (by inhalation), this value is about 0.5–0.6 tons/km^2 (Robinson, 1985). Of course, it does not apply to the synthesis or production of these substances for terroristic purposes.

Apart from this "classic" approach, it would be possible to "improve" the properties of known CWs (e.g., microencapsulation so that less stable or highly volatile substances) can be used. Nanotechnology offers new possibilities, as described recently by Price and Peterson (2008). The other option is to improve penetration using known enhancers like dimethyl sulfoxide (DMSO). While the percutaneous toxicity (expressed as LD_{50} in rats) of one of the toxic OPs (namely, *O*-isopropyl *S*-2-diisopropylaminoethyl methyl phosphnothiolate) is 59.1 μg/kg, when it is mixed with DMSO, this value decreases to 10.1 μg/kg (Bajgar, 1989).

Binary technologies are also acknowledged; however, the development of other methods of synthesis is not excluded (the more steps involved in synthesis, the more difficult it is to control the process). An alternative is to search for compounds either used or synthesized already. From the groups of highly toxic chemicals, these could be fluorophosphorylcholines (unstable) or toxic silatrans. In the group of medications, there are also highly toxic chemicals like cardiac glycosides (digoxin), sympathomimetics (noradrenalin), and myorelaxans (succinylcholine, curare derivatives). The other compounds to be included are insulin, cantharidin, aconitin, galantamine, pancuronium, pipecuronium, some derivatives of vitamin D (cholecalcipherol), some antibiotics, cytostatics, etc. It is necessary to point out that their use is limited (e.g., parenteral administration of insulin or delayed acute effect of cytostatics). A possible candidate would be centrally acting alpha 2-adrenergics with antihypertensive and sedative properties. All bioregulators are of great interest, especially in connection with the increased possibility of obtaining significantly sufficient quantities for military and terroristic purposes. All these examples are more or less hypothetical and require further testing. There exist some groups of compounds whose misuse is more probable and some of these chemicals are under suspicion (not proved) of being introduced into military arsenals. An illustrative but not exhaustive list of warfare agents is given next.

SPECIFIC AGENTS

Carbamates

Compounds in the carbamate group have a broad spectrum of toxicities—from relatively slightly toxic (carbaryl) to highly toxic compounds comparable with nerve agents (T-1123) (Figure 26.1). As described by Robinson (1971), carbamates including T-1123 had been studied by British and Canadians as CW agents since 1940. Other carbamates were described in detail by Badawi and Hassan (1995). They are well absorbed by the lungs, gastrointestinal tract, and the skin. The clinical representation of

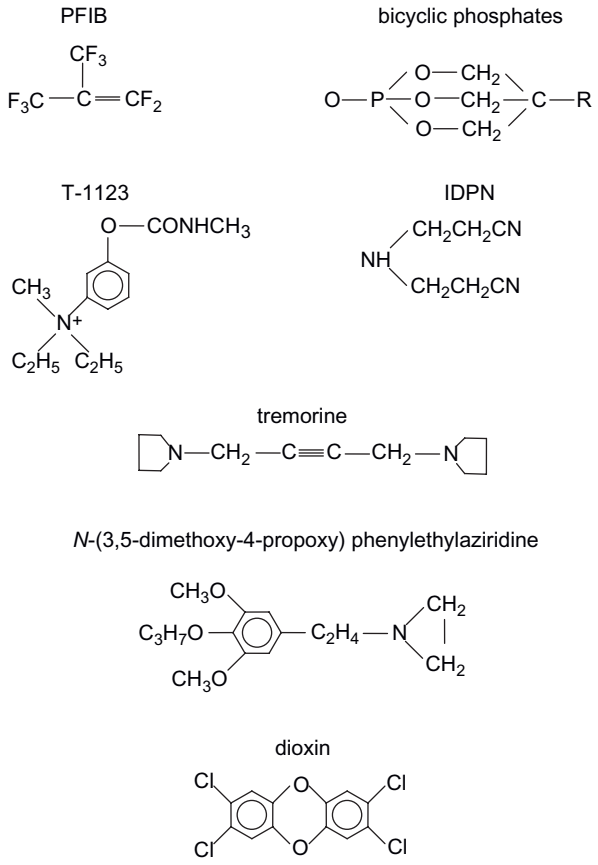


FIGURE 26.1 Chemical formulas of some toxic chemicals.

poisoning is similar to that for nerve agents, though perhaps with more expressed peripheral signs because of quaternary nitrogen in the molecule (T-1123); therefore, penetration through the blood–brain barrier is difficult (Bajgar and Patocka, 1976; Fusek et al., 1996a). The basic mechanism of action is reversible inhibition of cholinesterases. However, the inhibition of carbamates is based on carbamylation of the active center of the acetylcholinesterase (AChE). Spontaneous decarbamylation is a relatively quick process (taking approximately 24 h) and carbamylated cholinesterases are resistant to the effect of reactivators. Therefore, the treatment is symptomatic, preferably using only atropine. These difficulties would be reasons for military use (Patocka, 1990).

Dioxin

Dioxin is one of the most toxic low-molecular-weight compounds (Figure 26.1). Its oral LD_{50} for guinea pigs is 2–20 μg/kg; for monkeys, it is 2 μg/kg; for rats, it is 18–60 μg/kg; and for humans (subcutaneous administration), it is about 107 μg/kg (Bajgar, 2006; Patocka and Fusek, 2004). Dioxin interferes with the metabolism of porphyrins (and the main symptom of poisoning is derived from an increase of porphyrins in the organism—i.e., porphyria cutanea tarda) by induction of delta aminolevulate synthetase. Dioxin also has carcinogenic, hepatotoxic, nephrotoxic, teratogenic, and embryotoxic effects and causes dermal changes (except porphyria cutanea tarda), including chloracne, followed by development of cachexia. There is no specific antidote, making treatment very difficult and symptomatic. Effects of dioxin following acute administration are relatively delayed. This is a limiting factor for its use as a CW. Dioxin in the organism is bound to lipids and concentration of dioxin in plasma fat in persons exposed to dioxin was 100–1,000× higher than that of normal population (Neuberger et al., 1999; Pelclová et al., 2011; Klement et al., 2013). It should be mentioned that dioxin is one of the polychlorinated biphenyls and dibezofurans that appears to be problematic for the environment (Sofronov et al., 2001; Bajgar, 2006). Dioxin was used to poison Ukraine's President Viktor A. Yushchenko (Sorg et al., 2009).

Bicyclic Phosphates

Bicyclic phosphates have been used as flame retardants, antioxidants, stabilizers, and for spectroscopic studies. At present, however, they are being replaced by other compounds that are not so highly toxic. In a chemical structure (Figure 26.1), when R is substituted by isopropyl, the toxicity is very close to that of sarin (LD_{50} = 0.18 mg/kg, i.m. in rats). Bicyclic phosphates act rapidly—within minutes following parenteral administration. Clinical symptoms include behavioral perturbation, muscle weakness, hyperactivity, muscle tremors, and convulsions leading to paralysis. Intoxication is slightly similar to poisoning with nerve agents, but with a different mechanism of action; i.e., probably connected with gamma-aminobutyric acid receptors. Specific antidotal therapy does not exist, but a relatively good effect was observed following administration of benzodiazepines (Patocka and Fusek, 2004; Bajgar, 2006).

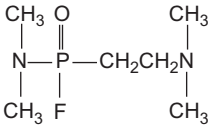
Perfluoroisobutene

Perfluoroisobutene (PFIB) is designated a chemical 2 by the CWC and therefore is contained in Schedule 2A. Its chemical structure is shown in Figure 26.1. PFIB is produced by thermal decomposition of Teflon and has high inhalation toxicity characterized by pulmonary edema. PFIB has been characterized in more detail in an *ASA Newsletter* (Patocka and Bajgar, 1998). Therapy is symptomatic.

Organophosphates

There are other known OPs having relatively high toxicity, like amiton (Tetram®), Armin®, dimefox (Hanane, Terrasytam®), paraoxon (E 600®), and TEPP (Tetron®). These compounds could be used for military and terrorist purposes; however, it would be uneconomic for the military to replace these substances. However, a new group of OP compounds has been described and characterized. This class of OPs can be described in general as 2-dialkylaminoalkyl-(dialkylamido)-fluorophosphates. In their chemical formulas, structural similarities with the group of so-called G-compounds (i.e., sarin, soman, and tabun) and V-compounds (i.e., VX and others) are found. These chemicals were designated as *GP* or *GV compounds* (Bajgar, 1992; Bajgar et al., 1992; Halámek et al., 1995; Fusek et al., 1996b). The toxicities of the most toxic derivatives are shown in Table 26.1. Intoxication

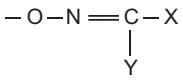
TABLE 26.1 LD_{50} Values of GV in Mice and Rats Following Various Routes of Administration



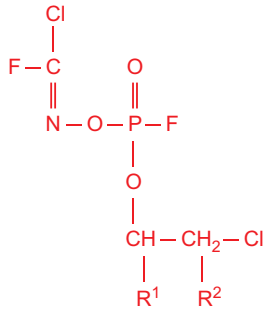
LD_{50} (μg/kg) with their 95 % Confidence Limits		
Route of Administration	**Mice**	**Rats**
i.v.	27.6 (25.6–29.4)	11 (8.5–17.6)
i.m.	30.5 (28–55)	17 (15.5–23.6)
s.c.	32 (29–53)	21 (18–26)
p.o.	222 (194–255)	190 (881–272)
p.c.	Not tested	1366 (881–3138)

with this compound has practically the same syndromes as observed with nerve agents. Treatment with atropine and reactivators is difficult because of the absence of the ability to reactivate inhibited cholinesterases by common oximes (Kassa, 1995; Fusek et al., 1996b; Kassa et al., 2006; Kuca et al., 2006). The lack of reactivation is different from that observed for soman (i.e., aging and dealkylation) and it is probably caused by steric hindrance in the cavity of cholinesterase. The volatility of GV compounds is between VX and sarin and therefore these agents are effective when penetrating through uniforms. This is an example of an intermediate volatility agent.

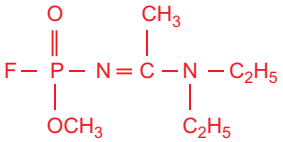
There are sources of information that suggest a new nerve agent known as Novichok or Novichok 5. In 1982, the Soviets began a secret CW development program that was code-named Foliant. The program had the apparent goal of developing new binary nerve agent weapons. Novichok has been described as a new toxic agent, and it is very difficult to treat poisoning by it (practically impossible; the toxicity was about 10 times greater than VX agents). Its exact chemical structure is unknown. The Novichok class of chemicals almost certainly belongs to the organophosphorus compounds containing the dihaloformamide group (Bajgar, 2006):



where X and Y are Cl, F, Br, or even a stable pseudohalogen like–CN. An example of Novichok (Kuca et al., 2013) could be as follows:



Another possibility could be compound A232:



There are other analogs of A232, including A230 and A234. Because these chemicals are relatively unstable, binary recepture was probably developed (Halamek and Kobliha, 2011).

Toxins

Toxins are prohibited by the Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction (known for short as the Biological Weapons Convention), signed in London, Moscow, and Washington on April 10, 1972. Their isolation from natural sources is sometimes difficult, but some toxins are possible to synthesize using biotechnology. Their toxicity is very high (e.g., the inhalation LD_{50} of botulinum toxin and tetanotoxin is measured in tens of $mg/kg/m^3$). Some other known toxins are saxitoxin, tetrodotoxin, and batrachotoxin. They are highly effective by other routes of administration as well. Effects are observed within 10–20 min, beginning with muscle weakness, insensitivity of tongue, fingers, and mouth, followed by muscle paralysis, including respiratory muscles. Specific therapy is unknown, so it is necessary to save basic life functions (using artificial ventilation, etc.). When death is not observed within the first 24 h after exposure, the prognosis is relatively good.

Other types of toxins are mycotoxins, mostly trichothecenes. Their production is straightforward by fermentation, and they are highly stable and therefore may be stored for a long time. They are effective by all routes of administration, including inhalation and percutaneous absorption. Their toxicity is not very high, and the effect is prolonged. Symptoms of intoxication vary greatly, including fever, hemorrhagic eruption, bleeding, necrotic angina, decrease of leukocytes, and sepsis. Some toxins are carcinogenic, teratogenic, mutagenic, and hepatotoxic, while some of them also have a neurotoxic effect. There is no specific therapy; treatment is focused only on relieving symptoms (Patocka, 2004). They caused an epidemic (alimentary toxic aleukia) in the former Soviet Union and were the subject of discussion for possible use in Asia (yellow rain).

In August 1981, based on limited physical evidence, the United States announced that trichothecene mycotoxins had been used—but the findings were less than convincing to the scientific community and the issue became extremely contentious. This controversy was never totally resolved, and no definitive evidence was found (Bajgar, 2006).

The question of neurotoxins and neurotoxicity mechanisms was extensively described by Segura-Aguilar and Kostrzewa (2006). Some of them are described next.

Aziridines

Aziridines are 2-(trisubstituted phenyl) ethyl aziridines that induce changes in behavior and motor influencing neurotransmission. Their toxicity is not very high, and they play a role in modeling a number of diseases. The effect is long-lasting, and mostly irreversible without

specific antidotal treatment. Some aziridines, e.g., *N*-(3,5-dimethoxy-4-propoxy) phenylethylaziridinium (Figure 26.1), have convulsive properties. Convulsions are treatable with benzodiazepines (Herink, 1977, 1995).

Tremorine

A relatively simple compound (Figure 26.1), tremorine is known to induce symptoms similar to Parkinson's disease in mice and monkeys. The onset of symptoms, such as salivation, miosis, lacrimation, muscle weakness, and bradycardia, was evident within 15–30 min after administration. Typical symptoms are muscle twitch or fine tremor of the head and extremities, decrease in body temperature, and analgesia. This stage usually lasts for a few hours. Therapy is symptomatic only and not very effective (Bajgar, 2006; Patocka, 2004).

Imino-β,β-Dipropionitrile

Imino-β,β-dipropionitrile (IDPN) is one of the compounds (Figure 26.1) isolated from *Lathyrus sativus*, also called *lathyrogenic substances.* The toxicity of IDPN (and also of aziridines and tremorine) expressed as LD_{50}, is not very high, falling in the range of tens of mg/kg. Following administration of lower doses of IDPN, a condition called *waltzing syndrome* is characterized by a circling movement in both directions, and sometimes movement of the head similar to chorea (and hyperkinetic syndrome). High doses of IDPN are known to produce conjunctivitis and edema of the eyelids. In severe cases, it causes hemorrhages in the retina with the possibility of blindness. Hyperkinetic syndrome is irreversible and does not react to therapy (Bajgar, 2006).

Bioregulators

A variety of agents have the potential to be used as weapons of bioterrorism. These weapons have been used in wars from the start of recorded history (Metcalfe, 2002). The development of technologies on a modern militarily significant scale was initiated in several countries during the period between the two world wars (Roffey et al., 2002). However, as a result of modern technology, the risks are greater now, and the outcomes more serious (Henderson, 1999). Today, agents include not only toxins, but also a new group of compounds and bioregulators. Bioregulators are naturally occurring organic compounds that regulate diverse cellular processes in all organisms. There are substances normally found in the body that regulate normal and critical biological processes, such as blood pressure, heart rate, breathing, muscle contraction, temperature, mood control, consciousness, sleep, emotions, and immune responses. Their characteristics include activity in extremely low doses and they frequently have rapid effects. Unlike traditional disease-causing biowarfare agents that take hours and days to act, bioregulators can act within minutes after administration. There is comprehensive knowledge available on these compounds because all these compounds work as regulators and modulators of all vital biochemical pathways, linked with physiological functions of living organisms. If bioregulators were exploited for the purpose of terrorism, they could potentially cause profound pathophysiological effects. The main group of bioregulators under discussion includes different biochemicals such as neurotransmitters, hormones, and proteolytic enzymes. The common property of all bioregulators is their ability to induce biological effects and a consequential rapid fall in their concentrations in tissues. The problem is their route of administration (Patocka and Merka, 2004; Patocka et al., 2013).

A brief description of some bioregulators from a military viewpoint is given next. These peptides have been chosen based on the criteria of bioregulators intended for terrorism and warfare agents (Bokan et al., 2002).

Angiotensins

Angiotensins regulate blood pressure and contribute to sustaining hypertension (Mazzolai et al., 1998). The principal effect of angiotensin is to stimulate the synthesis of aldosterone and elevate blood pressure via vasoconstriction of the smooth muscle in arterioles.

Bombesin

Bombesin is a tetradecapeptide isolated from the skin of the amphibian frog *Bombina bombina* (Anastasi et al., 1971). It has been proposed that bombesin-related peptides may be released from the gastrointestinal tract in response to ingested food, and that they bridge the gut and brain via neurocrine means to inhibit further food intake (Merali et al., 1999).

Bradykinin

Bradykinin is a vasoactive nonapeptide, which is the most important mediator generated by the kinin system and it is involved in inflammation processes (Calixto et al., 2000). Kinins identified thus far include bradykinin and kallidin. They cause local increases in the permeability of small blood vessels. Bradykinin is a potent stimulator of pain receptors in the skin and has a powerful influence on stimulating smooth muscle contraction, inducing hypotension, and increasing blood flow and permeability of capillaries (Cyr et al., 2001).

Endorphins

Endorphins are peptides that bind to the neuroreceptors in the brain to give relief from pain (Terenius, 1992). Beta-endorphin is the most active, and it is about 20 times more potent than morphine.

Endothelins

Endothelins constitute a family of peptides (Hart and Hart, 1992). They are very potent endogenous vasoconstrictors and vasopressors and are secreted by various cells and tissues in the human body. Of the three isoforms, endothelin-1 (ET-1) is one of the most potent contractors of vascular smooth muscles (Miller et al., 1993). Endothelins have very similar structure and biological properties to sarafotoxins (Kloog and Sokolovsky, 1989), and the toxic peptides are obtained from the venom of mole vipers (Atractaspidae).

Enkephalins

Enkephalins are endogenous pentapeptides. Two enkephalins have been identified: Met-enkephalin and Leu-enkephalin. Both enkephalins are relatively weak analgesics, which activate all opioid receptors but appear to have the highest affinity for the delta-receptors. In the central nervous system, enkephalins have been found in many areas, but predominantly those associated with nociception (Przewlocki and Przewlocka, 2001).

Histamine Releasing Factor

Histamine releasing factor (HRF) is one of the many immune system protein molecules called *cytokines*, which trigger allergic reactions. Unlike other cytokines, HRF stimulates basophils to release histamine (MacDonald, 1996).

Neuropeptide Y

Neuropeptide Y (NPY) is the most abundant neuropeptide in the brain. Its concentration is many times higher than other neuropeptides. It is a member of a family of proteins that include pancreatic polypeptide, peptide YY, and seminalplasmin. In addition to its function of stimulating feeding behavior, several other physiologic roles have been assigned to NPY, including involvement in circadian rhythms, sexual function, anxiety responses, and vascular resistance (DiBona, 2002; Halford and Blundell, 2000).

Neurotensin

Neurotensin is an endogenous peptide neurotransmitter inducing a variety of effects, including analgesia, hypothermia, and increased locomotor activity. It is also involved in regulation of dopamine pathways. Neurotensin is found in endocrine cells of the small intestine, where it leads to secretion and smooth muscle contraction (Moore and Black, 1991).

Oxytocin

Oxytocin is a nine amino acid peptide that is synthesized in hypothalamic neurons and transported down through axons of the posterior pituitary for secretion into blood. Oxytocin has three major physiological effects: stimulation of milk ejection, stimulation of uterine smooth muscle contraction at birth, and establishment of maternal behavior.

Somatostatin

Somatostatin is a cyclic tetradecapeptide hormone, characterized as the major physiological inhibitor of growth hormone released from the pituitary, but inhibits the release of many other physiologically important compounds, including insulin, glucagon, gastrin, and secretin (Wolkowitz, 1994).

Substance P

Substance P is an 11–amino acid polypeptide, and a physiologically significant (and the best-known) member of a family of three related peptides known as *neurokinins.* The specific receptor subtypes corresponding to these three neurokinins are known (Sandberg and Iversen, 1982). These neurotransmitters appear to play a key role in the regulation of emotions, and antagonists of their receptors may be the novel psychotropic drugs of the future. Koch et al. (1999) demonstrated that substance P, in combination with thiorphan, administered as an aerosol, is highly potent and extremely toxic. Exposure to the substance at extremely low air concentrations may result in incapacitation of humans.

Vasopressin

Vasopressin, also called *antidiuretic hormone (ADH)*, is a cyclic nonapeptide hormone released from the posterior pituitary. Its primary function in the body is to regulate extracellular fluid volume by affecting renal handling of water. Specific actions include inhibition of diuresis, contraction of smooth muscles, stimulation of liver glycogenesis, and modulation of adrenocorticotropic hormone release from the pituitary gland. ADH belongs to the family of vasoactive peptides involved in normal and pathological cell growth and differentiation.

Thyroid-Stimulating Hormone

Thyrotropin (also known as *thyroliberin* and *thyroid-stimulating hormone (TSH)*) is a peptide released by the anterior pituitary gland that stimulates the thyroid gland to release thyroxine (Ladram et al., 1994). The release of TSH is triggered by the action of thyrotropin-releasing factor, a peptidic substance found in the hypothalamus of the brain and influencing the secretion of glandula thyroidea.

Not long ago, most bioregulators had been unavailable in the amount needed for terroristic attacks or military operations. However, by the end of the twentieth century and beginning of the twenty-first century, there had been intensive developments in biomedical sciences,

biotechnology, and chemical engineering in the pharmaceutical industry, and because of the revolution in the science and technology of drug discovery, the control of bioregulators will be significantly complicated.

In the near future, genomic and proteomic methods will stimulate increasing use of computer modeling techniques to identify new biologically active compounds and then determine their mode of action. Currently, new compounds are being generated in large numbers by combinatorial methods and assayed for potential activity, and it seems likely that genomic and proteomic methods will make these compounds accessible in amounts necessary for terroristic use. This is a very disagreeable situation and a new problem for the control of chemical and biological weapons.

NONLETHAL WEAPONS

Nonlethal weapons can be of a chemical, physical, or pharmacological character (Hess et al., 2005). Such weapons are designed and primarily employed for the purpose of incapacitating personnel or material while minimizing fatalities, permanent injury to personnel, and undesired damage to property and the environment (Pearson, 2006). Weapons used for physically immobilizing personnel include lasers, microwave impulses, ultrasound, electric current, nets, solidifying foams, and sliding gels. For CWs, immobilizing gases and irritants and smelly bombs can be considered. For pharmacological immobilization, ketamine, benzodiazepines, and onset accelerators are the compounds of real interest. Basic requirements for the use of immobilizing drugs are necessary, such as minimal cardiovascular and respiratory side effects; easy administration, primarily by inhalation; rapid onset; high biological accessibility and well-controlled effects; and specific antagonists that can be used within a large therapeutic range. These drugs are used for animal immobilization, using narcotizing blowpipes, darts, or guns. This group is also called *calmatives.* The compounds considered include ketamine and phencyclidine, alpha-2-agonist, opioids (including etorphine, fentanyl, and carfentanyl) and muscle relaxants (Patočka and Cabal, 2001; Dejmek, 2004; Patočka and Fusek, 2004; Streda and Patočka, 2004; Hess et al., 2005; Halamek and Kobliha, 2011; Klement et al., 2013).

As for fentanyl derivatives, there are more effective drugs than fentanyl. These include carfentanil (probably used in Moscow in 2002 against terrorists), whose effectiveness in 500–3,000 times greater than morphine, and remifentanil, which is 5,500–11,000 times more effective (Halamek and Kobliha, 2011). Methods of determination of fentanyl derivatives were described, too (Jelinkova et al., 2013).

Genetic and Ethnic Weapons

Another possible threat is modification of the effects of commonly used chemicals or biological agents. The first administration produces no toxic effect, but the second administration of the same or another compound causes damage.

The present genetic material of a human population could be misused. Recent advances in biological research could eventually lead to the creation of new types of CWs targeting a specific group of human beings with common genetic characteristics, as is perhaps the case with certain ethnic groups. Biologically, there are more similarities than differences between human beings. However, differences do exist, and if the data on ethnic differences are known, the selective effect of different chemicals to the groups cannot be excluded. Glucose-6-phosphate dehydrogenase, the enzyme that catalyzes the dehydrogenation of glucose-6-phosphate to 6-phosphogluconate, has been genetically determined and found to be low or nonexistent in some groups (though it appears more frequently in Africans and Scandinavians). It is connected with the male chromosome, and in men where it appears, hyperbilirubinemia is observed. Following administration of some normal medications, like acetylsalicylic acid, sulfadimidine, chinine, and chloramphenicol, a hemolytic syndrome is induced. Individuals with other pathologic states (e.g., chronic methemoglobinemia) will be more sensitive to drugs that are able to increase the level of methemoglobin, including analgesics, antipyretics, and nitrates. Deficiency of alpha-1-antitrypsine can cause increased sensitivity to asphyxiation agents. Plasma cholinesterase activity is also genetically determined, and individuals with decreased cholinesterase activity are more sensitive to myorelaxants. It is very probable that these people will be more sensitive to nerve agents (Bajgar, 2006).

Comparison of some criterias of the use of new chemicals for military or terroristic purposes is shown in Table 26.2.

For military purposes, using new nerve agents is the most likely possibility; however, their use is generally prohibited by the CWC. For terroristic purposes, there is a wide range of toxic chemicals and their choice is very dependent on the capabilities of potential users and their aims—namely, to achieve immediate effects or the liquidation or incapacitation of people over longer periods.

In the end, let us consider (very hypothetically!) terroristic use of one of the most obvious drugs: insulin. It is known that some other routes (e.g., inhalation) of administration for medical purposes have been developed (Mastrandrea, 2010). Therefore, this does not rule out the development of effective administration by inhalation. If suitable stability and aerosolization is achieved, could we realistically conclude that this drug would *not*

TABLE 26.2 Different Criteria for the Suitability of Different Chemicals to be CW

Criterium	Carbamates	New OP	Dioxin	Bicyclic Phosphates	Toxins	Incapacitants
Occurrence of first symptoms	Minutes	Minutes	Hours–days	Minutes	Different	Minutes
Death observed within	Hours	Hours	Weeks–months	Hours	Hours–weeks	Not in incapacitant doses
Availability of antidotes	Symptomatic	Yes	no	Symptomatic	Symptomatic	Yes
Easy to synthesize	+	+	+	++	+++	++
Toxicity	High	High	Very high	High	Varied	Low
Stability	+	+	+++	+	Different	++
Cost	+	+	+	++	+++	+++

be misused by terrorists? The effects are quick and antidotes to protect potential users would not be a problem. Military use of this drug for states or groups that are not CWC members is not ruled out either.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This chapter explains that the misuse of knowledge of pharmacology and toxicology is possible and can be applied to all human activities. The task is to be well informed in cases where it is important to know the best protection and therapeutic means, and to control all activities connected with the synthesis and the possible availability of CWs/CWAs to both nonqualified and qualified people.

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CHAPTER

27

Ricin

Russell Dorsey, George Emmett and Harry Salem

INTRODUCTION

Biological toxins are metabolic products from living organisms. They are produced by a vast number of different life forms ranging from the simplest to the most complex, and each has a distinctive mode of action in conjunction with a characteristic molecular structure and biochemistry. A common feature to all toxins is that minute quantities will exert a pronounced effect on their intended targets. Algal toxins constitute a very diverse group of compounds ranging from simple ammonia to complex polypeptides and polysaccharides. Dinoflagellates are a source of some potent, nonprotein toxins, such as saxitoxin and tetrodotoxin; one such toxin causes the deleterious effects during a red tide. A red tide is the common name for an estuarial algal bloom when the photosynthetic pigments of certain species of phytoplankton can alter the hue of water; colors vary from purple to almost pink, but normally they are red or green. The mycotoxins are secondary metabolites, which are nonprotein substances produced by molds and fungi. Many molds produce more than one toxin and, in several cases, combinations of mycotoxins synergize to enhance toxicity (Weber et al., 2005). Leaves, roots, or seeds from some plants can be poisonous. Examples include the protein toxins ricin, derived from castor beans, and abrin, from the jequirity pea. Finally, there are a number of toxins produced by animals, including the nonprotein batrachotoxin found in certain species of frogs (poison dart frog), Melyridae beetles, and birds (*Pitohui*, *Ifrita kowaldi*), and a wide variety of peptide and proteins from marine snails, scorpion venoms, and snake venoms.

In the wake of recent terrorist-related incidents across the globe, biological agents have become well-known for the threat they pose to the US and Allied militaries and the civilian populations they guard. The agents are attractive to both foreign states and terrorists because they are relatively inexpensive to produce and require minimal technical infrastructure.

Ricin is defined as a biological category B agent that has the potential to pose a severe threat to public health and safety. There are currently 41 agents and toxins, including ricin, listed in the Center for Disease Control and Prevention (CDC) regulation found in Part 73 of Title 42, Code of Federal Regulations (Possession, Use, and Transfer of Select Agents and Toxins).

Both the ricin toxin and castor bean plant have a long history of human use. The earliest human records contain references to the castor bean plant, which was known and cultivated by the ancient Egyptians. Oil pressed from the beans was used as a lubricant, and the beans were sometimes ingested to treat constipation. Even today, castor oil is still commercially produced globally. Castor oil is presently used in bath oil products, detergents, lubricants, dying agents, and most recently in the production of biodiesel fuel. The ricin toxin is easily produced from the castor bean mash, remaining after the beans are pressed to extract castor oil (Wannemacher et al., 1992). Peter Hermann Stillmark first isolated and named the toxin ricin in 1888. Initially, the ricin protein was not known for its toxicity, but rather for causing the agglutination of erythrocytes. Later, the field of immunology was founded based on Paul Ehrlich's research with ricin. By introducing small amounts of the toxin to mice, Ehrlich was able to induce immunity specifically to ricin. This seminal work proved that in response to a challenge, certain serum proteins (antibodies) are produced that afford the host protection.

During the era of the biological weapons offensive program in the United States, ricin, by virtue of its toxicity, availability, and ease of production, was one of the first agents to be weaponized (Cookson and Nottingham, 1969). Today, ricin is one of the few biological agents utilized with known success. The most prominent of these was the attack leading to the death of the Bulgarian exile,

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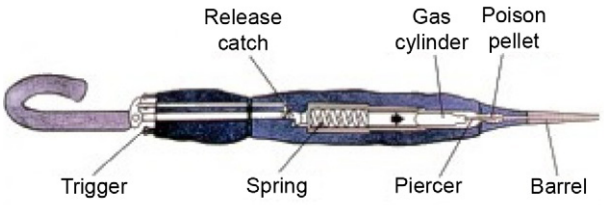

FIGURE 27.1 An artist's conception of the "umbrella gun." *Source: Figure from James (2014).*

Georgi Markov. On September 7, 1978, Markov was jabbed in the leg with an umbrella while waiting at a bus stop. A man holding the umbrella apologized to Markov before casually walking away from the scene (Crompton and Gall, 1980). Markov would later tell doctors that the man had spoken with a foreign accent. He recalled feeling a stinging pain from the site where he had been jabbed with the umbrella tip and noticed that a small, red, raised wound had formed at the site. That evening, he developed a high fever and was admitted to a hospital, where he died 3 days later. An autopsy revealed a 1.52-mm-wide metal pellet in the wound, speculated to be a watch bearing. Two 0.34-mm-diameter holes had been drilled at right angles to form a well inside the bearing. The pellet contained no trace of the poison that killed Markov, and investigators relied on standard differential diagnosis techniques and intel (military discipline that exploits a number of information collection and analysis approaches to provide guidance and direction to commanders in support of their decisions) to identify the causative agent. A host of toxins were considered, including tetanus, diphtheria, dioxin, and several nerve agents. Ricin seemed most likely because intelligence agents knew that it had been under research for decades in the Soviet Union. Working together, scientists and investigators surmised that an "umbrella gun" shot a ricin-laced pellet. The modified umbrella may have contained a cylinder of compressed air that propelled the pellet after pulling a trigger mounted in the umbrella's handle. An artist's conception of the "umbrella gun" is shown in Figure 27.1.

Two years later, another assassination attempt with ricin occurred at a shopping mall parking lot in Tyson's Corner, Virginia. Boris Korczak, a Soviet citizen who had been a CIA double agent, felt a slight sting, "something like a mosquito bite" he reported, while walking across the parking lot. He developed a fever of 106°F a few hours later, followed by a sharp decline in body temperature. Internal bleeding and an irregular heartbeat ensued. After his recovery, he extracted a tiny metal ball from his "mosquito bite" (Figure 27.2) (Douglas and Livingston, 1987).

More recently, ricin was discovered in a Las Vegas motel room. Roger Von Bergendorff awoke from a

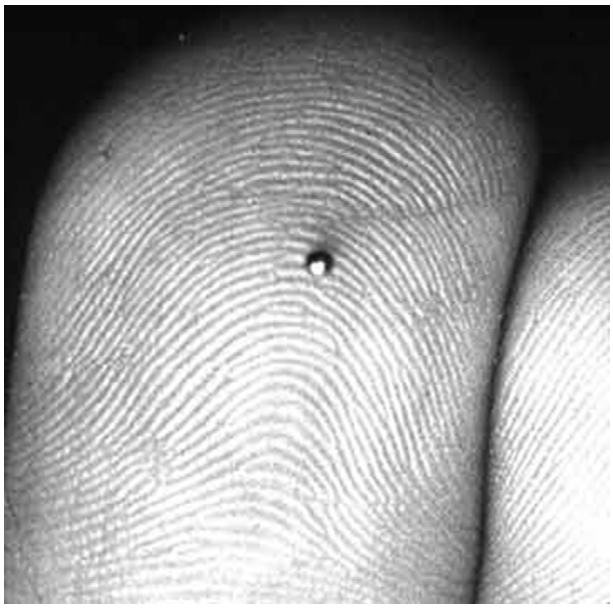

FIGURE 27.2 Ricin-laced pellet extracted from Boris Korzak.

comatose condition on March 14, 2008, at which time he was questioned by police as to why he had such a large quantity of ricin. Subsequently, he was arrested on April 16, 2008, and charged with possession of a biological toxin and two weapons offenses. Several other related cases involving ricin on US soil occurred in November 2003. Ricin was found in the mailroom that served Senate Majority Leader Bill Frist's office. No injuries were reported. The White House mail-processing facility and a mail-sorting facility in Greenville, South Carolina discovered ricin. The envelope discovered in Greenville was addressed to the US Department of Transportation and labeled "Caution Ricin Poison." The enclosed letter threatened to "turn DC into a ghost town." There are more than a dozen other events that have occurred in the past (CNS, 2008).

HISTORY OF BIOLOGICAL WEAPONS

The known use of biological weapons dates back to the beginning of written record, with many examples of use on the battlefield. In 190 BC, Hannibal hurled venomous snakes onto enemy ships on Pergamus at Eurymedon. Scythian archers used arrows dipped in the blood of decomposing bodies in 400 BC. Biological weapons have also seen use in more recent times on the North American continent. In 1763, during the Pontiacs Rebellion in New England, the British officer Colonel Henry Bouquet gave blankets infected with smallpox to Native Americans at Fort Pitt, Pennsylvania, an act that devastated the Native American population. In 1863, Confederate soldiers retreating through Mississippi left dead animals in

wells and ponds to deny drinking water to Union troops. Another US Civil War use of biological weapons was executed by Dr. Luke Blackburn, who attempted to infect clothing with smallpox and yellow fever for sale to unsuspecting Union troops (Diamond, 1997).

Biological weapons research and development in the United States was very active during World War I, with the impetus provided by the American Anton Dilger. After joining the German Army in 1914, Dilger experienced a nervous breakdown and returned home to the United States, which was neutral at that point. At the request of the German Government, Dilger brought strains of anthrax and glanders with him for inoculating horses awaiting shipment to Europe (Erhard, 1999). After becoming aware of Germany's involvement with biological weapons, the US Government initiated its own biological weapons program, using ricin for retaliatory use in 1918 (DA, 1977; Carter, 2000). Two methods of dispersal were examined: the adherence of ricin to pellets contained in an artillery shell and the production of a ricin dust cloud. Because of limited amounts of purified ricin on hand and its less efficient delivery via the respiratory tract, the latter approach was considered less promising. Although both approaches were laboratory-tested to some degree, neither was perfected for battlefield use before the end of the war. During the early 1920s, the US military leadership determined that conducting further research into the use of biological weapons was not an efficient use of resources. Germany, France, Great Britain, Canada, and Japan, however, aggressively pursued the development of offensive biological weapons (Carter, 2000). The reported use of cholera, dysentery, typhoid, plague, and anthrax by the Japanese against the Chinese motivated the United States to reinitiate a biological offensive development program. The US Army's biological warfare program began at the Edgewood Arsenal (now the Edgewood Area of the Aberdeen Proving Ground, MD) and was later moved to Camp Detrick (now Fort Detrick, Frederick, MD). The first agents produced at Camp Detrick were botulinum toxin, anthrax, and brucella, but none was weaponized before the conclusion of World War II. After the war, research continued into the weaponization of biological agents until President Nixon's decision in 1969 to redirect the research toward a defensive posture, where the United States stands today. Because biological warfare agents are typically etiological agents of naturally occurring disease, the majority of military defensive efforts are focused on vaccine development and diagnostic testing. Although research continued, the program was only a skeleton of the former offensive effort. Despite a 1979 anthrax outbreak in Sverdlovsk, Russia, which exposed Russia's ongoing biological weapons research, the United States did not place much weight on the possibility of its forces facing biological weapons. This position, however, changed after the invasion of Kuwait by Iraqi troops and the deployment of US troops to the Persian Gulf. Iraq had a large biological agent production facility and a history of using biological and chemical agents during military offensives (Warrick, 2006). To counter this, intensive research focused on measures for protection, detection and destruction; these efforts are still ongoing today. In addition to state-sponsored entities, the revelation that terrorist organizations, such as the Aum Shinrikyo in Japan, were actively developing biological agents has brought attention to domestic defense against biological weapons. Defending against these agents requires not only an understanding of the agent but also an understanding of how an adversary might utilize it.

THE WEAPONIZATION OF BIOLOGICAL AGENTS

Although many pathogens and toxins cause disease or toxicity, relatively few of the naturally occurring agents can be adapted for use as a biological weapon. Paramount is its ability to survive the mechanical rigors of large-scale aerosol dissemination. Other important characteristics include an agent's availability, ease of production and storage, and its lethality, or its ability to incapacitate through morbidity (NATO, 1992). While some pursue agents of death, the philosophy of others is that the most attractive agents are those that incapacitate an adversary. During the era of the US biological offensive program, agents such as *Staphylococcus aureus* enterotoxin B (SEB) and Venezuelan equine encephalitis were selected for their ability to incapacitate rather than kill. It was estimated that an afflicted soldier would require the care from a minimum of three personnel, whereas a dead soldier placed no burden on personnel. Providing care to sickened forces can quickly render entire military units mission-ineffective by overloading field-forward medical facilities intended to treat the wounded. In addition, these same agents could also be turned on civilians, concentrating the population at care centers, which in turn would facilitate attack and invasion by our forces. A final mark of an ideal agent is one that renders the adversary defenseless while the disseminating force is unaffected.

Terrorist organizations have historically lacked technical expertise; this often leaves insurmountable challenges for large-scale production and agent delivery. Thus, these organizations require an agent that can be easily disseminated in open air using common items such as crop dusters or liquid spray devices. Agents that can survive drying have high inhalational efficiency and can effectively cause infection or toxicity, which makes them ideal biological threat agents. The options

for indoor dissemination or individual targets are much broader and essentially only limited to the creativity of the perpetrator. Use of the postal service, for instance, before the anthrax attacks on the United States, was believed to be impossible. During decontamination efforts after the anthrax attack, it was also discovered that reaerosolization was a threat. The optimal agent particle size is dependent on the delivery mode. For aerosol dissemination, the smaller the particle size, the longer it will stay airborne and the more likely it can be reaerosolized after settling. Bacterial agents have a minimum size that is limited by their cellular dimensions; this is typically 1–5 μm. Alternatively, viral agents can be as small as 0.1 μm, and toxins are limited only by their molecular weight. It has been shown that particles of less than 10 μm can stay airborne for extended periods of time (Winters and Chenoweth, 2002). Particle size is inconsequential for attack via injection, ingestion, or transdermal transmission.

Agent stability after production is another important factor to consider when selecting an agent for weaponization. Environmental factors, including temperature, relative humidity, atmospheric pollution, and sunlight, can all have an effect on agent viability. Feasible options for storage of agents are as liquid samples at room temperature, as liquid samples under refrigeration, as dry samples at room temperature, and as dry samples under refrigeration. The most desirable option is dry sample storage at room temperature, because this eliminates the need for refrigeration, allows for particle sizing before dissemination, and minimizes the environmental effects on dissemination.

Considering all factors, toxins are probably the most suitable biological agents for use in any attack. In contrast to replicating agents, using toxins as biological agents requires that a lethal amount must be injected, ingested, or inhaled. Given this caveat, a sizeable amount of toxin must be available for dispersion in large-scale attacks, severely limiting the effective toxins to those that are the most lethal and easily produced. Ricin is one of only a few toxins that meet these requirements, along with botulinum toxin, SEB and the trichothecene mycotoxins. Principally because of its suitability as a biological warfare agent, the ricin toxin has been one of the biological weapons of choice for state-sponsored organizations (Kortepeter and Parker, 1999).

THE FAMILY OF RIBOSOME-INACTIVATING PROTEINS

Ribosome-inactivating proteins (RIPs) are toxins that act intracellularly. They consist of two different subunits: a subunit A that is responsible for the enzymatic activity of the toxin and a subunit B that binds to a specific receptor on the host cell membrane, thus allowing the A subunit, the enzyme, to cross the cell membrane. The enzymatic component is not active until it is released from the native (A + B) toxin. Isolated A subunits are enzymatically active but lack binding and cell entry capability. Isolated B subunits may bind to target cells (and even block the binding of the native toxin), but they are nontoxic.

There are a variety of ways that toxin subunits may be synthesized and arranged: A + B indicates that the toxin is synthesized and secreted as two separate protein subunits that interact at the target cell surface; A-B or A-5B indicates that the A and B subunits are synthesized separately, but associated by noncovalent bonds during secretion and binding to their target; 5B indicates that the binding domain of the protein is composed of five identical subunits. A/B denotes a toxin synthesized as a single polypeptide, divided into A and B domains that may be separated by proteolytic cleavage (Todar, 2014).

Ricin is a ribosome inactivating lectin isolated from the beans of *Ricinus*. Ricin and other plant lectins, e.g., abrin and modeccin, consist of two peptide chains, A, the toxin, and B, a lectin, linked together by a disulfide bond. Lectins are a class of carbohydrate-binding proteins that interact specifically with glycoconjugates present in other organisms. Specific carbohydrate-binding activity distinguishes lectins from other plant proteins (Zhu-Salzman et al., 1998). This interaction is as specific as enzyme–substrate or antigen–antibody interactions.

Another characteristic property of lectins is that they agglutinate cells and precipitate polysaccharides and glycoproteins. That is because lectins are, as a rule, divalent or oligovalent, i.e., each lectin molecule has at least two carbohydrate binding sites that allow cross-linking between cells (by combining with sugars on their surfaces) or between sugar-containing macromolecules. Lectins have accordingly been defined as sugar-binding proteins of nonimmune origin that agglutinate cells and precipitate polysaccharides or glycoproteins (Goldstein et al., 1980).

Lectins are present in numerous edible plants. For many years it has been known that they occur in major food sources for humans and animals such as soybeans, kidney beans, lima beans, mung beans, lentils, garden peas, and peanuts. One family is composed of proteins that contain one or more 30- to 43-amino acid cysteine-rich chitin-binding domains. Another family, mainly legume lectins, binds carbohydrate substrates by way of interactions involving specific amino acid residues that are located spatially throughout the peptide. The exact role of lectins in plants is unclear, although they can serve as potent insecticides. Castor beans contain so much lectin that they are toxic to most organisms.

The binding ability and specificity of the lectin is preserved when bound to the toxic A chain. It is the lectin that directs the toxin to its target within the cell. There are a number of toxins that are bound to lectins. These

are all classified as type III toxins. This classification is based on the mode of entry into the cell. There are two categories of type III toxins: those that are bound to a single lectin, referred to as an AB toxin, and those that are bound to four lectins, known as AB5 toxins. The most commonly known type III toxins are:

AB toxins (usually plant toxins):

1. Ricin
2. Abrin
3. Mistletoe (viscumin)
4. Modeccin (Gasperi-Campani et al., 1978)
5. Diphtheria

AB5 toxins (usually bacterial toxins):

1. Campylobacter jejuni enterotoxin (from *Campylobacter jejuni*)
2. Cholera toxin (*Vibrio cholerae*)
3. Heat-labile enterotoxins (LT and LT-II) (*Escherichia coli*)
4. Pertussis toxin (*Bordetella pertussis*)
5. Shiga toxin (*Shigella dysenteriae*)
6. Shiga-like toxin (or verotoxin) (enterohemorrhagic varieties of *E. coli* including O157:H7)

Cholera toxin, Shiga toxin, ricin, and abrin are protein toxins that damage mammalian cells by a mechanism that includes four essential events: receptor-mediated endocytosis; retrograde transport into the lumen of the endoplasmic reticulum (ER); passage through the ER membrane into the cytoplasm; and catalytic inactivation of a target substrate in the cytoplasm. The receptor-binding site of a protein toxin is usually within a subunit or domain that is distinct from the subunit or domain that bears the catalytic activity of the toxin. Both cholera toxin and Shiga toxin belong to the AB5 protein toxin family in which the A chain is the enzymatic subunit, and each of the five identical B chains bind cell surface receptors.

Some peptide and protein toxins have been classified into families containing a number of different toxins with the same biological activity. Within any one family, the toxins can differ in amino acid sequence and number, but their molecular architectures and active site conformations are conserved. One of the most intensely studied toxin families is that of the RIPs, which includes ricin. The term "ribosome-inactivating protein" was introduced to designate plant proteins that inactivate animal ribosomes. The toxins were later found in certain bacteria and fungi (Endo et al., 1988). These are cytotoxins that catalytically and specifically inactivate the large subunit of eukaryotic or prokaryotic ribosomes (Stirpe and Barbieri, 1986). Historically, RIPs have been classified into two types based on their quaternary structure.

The type I RIPs are the most numerous and are all synthesized as a single chain enzyme of approximately 30 kD. Type II RIPs are synthesized as larger precursors that accumulate in protein bodies and contain an approximately 30-kD A-chain linked by a disulfide bond to a lectin B-chain of similar size (Stirpe and Barbieri, 1986). Plant RIPs, including all type I toxins and the A-chains of type II toxins, are RNA *N*-glycosidases capable of hydrolyzing the nitrogen–carbon glycosidic bond of a specific adenosine located in the sarcin/ricin domain of the largest ribosomal RNA (Barbieri et al., 2006). The B-chain is able to bind the D-galactose-terminated receptors on the membranes of animal cells and facilitates the internalization of the enzymatic A-chain. To date, more than 50 type I RIPs and approximately 15 type II RIPs have been identified (Stirpe and Barbieri, 1986). Although there is some variation at the N- and C-termini of their respective polypeptide chains, the active sites of the type I RIPs and the A-chains of type II RIPs are well-conserved, as are their three-dimensional conformations (Gasperi-Campani et al., 1985).

Originally, type I and type II RIPs were identified based on biological activities. Type II RIPs were discovered more than a century ago, when Stillmark isolated the toxic protein ricin from castor beans. As mentioned, the toxicity of ricin was initially attributed to its agglutination activity for red blood cells and not by ribosome inactivation. Type II RIPs attribute their carbohydrate binding activity to their B-chain, which contains two or possibly three binding sites (Robertus, 1991). The multiple binding sites allow B-chains to aggregate with red blood cells and platelets.

Evidence for type I RIPs was not uncovered until 1925. Duggar and Armstrong observed that the so-called *Phytolacca Americana* antiviral protein (PAP) inhibited the transmission of tobacco mosaic virus in plants. In 1978, PAP was recognized as an inhibitor of protein synthesis. Not all type I RIPs, however, are believed to be antiviral proteins (Lam and Ng, 2001). Unlike their type II counterparts, type I RIPs exhibit low toxicity because they are not able to bind and cross the cell membrane efficiently. Type I RIPs, however, are cytotoxic to certain cells such as macrophages. The cells can absorb type I RIPs by pinocytosis and subsequently succumb to RIP activity. Recently, a type III RIP has been isolated from *Hordeum vulgare*, the common barley plant. The RIP consists of an amino-terminal domain resembling type I RIP and is linked to an unrelated carboxyl-terminal domain with unknown function (Bolognesi et al., 2002). The type III RIP accumulates in the barley kernel as an inactive precursor that, on germination, is processed into a two-chain RIP by removal of an internal peptide from the catalytic domain (Peumans et al., 2001).

RIPs have been identified in more than 50 different plant species and have also been found in fungi, bacteria, and at least one algal species. The highest quantity of RIPs has been isolated from the carnation family Caryophyllaceae, the elder family Sambucaceae, the family of gourd-producing

plants Cucurbitaceae, the Euphorbiaceae family of flowering plants, the pokeweed family Phytolaccaceae, and the family of flowering grasses Poaceae (Reinbothe et al., 1994). Presently, the role of RIPs in plant physiology is not entirely clear. Based on their variable activity toward heterologous and autologous plant ribosomes, several possible roles have been proposed: anti-viral activity, anti-fungal activity, herbivore defense, a role in the arrest of cellular metabolism during periods of senescence, and, finally, a role as storage proteins (Bolognesi et al., 2002). Conclusive evidence has been obtained that RIPs not only deadenylate ribosomal RNA but are also capable of removing adenine residues from DNA and several other polynucleotide substrates. Thus, it has been proposed to rename RIPs as polynucleotide: adenosine: glycosidases (PAGs) (Shakirova and Bezrukova, 2007).

THE RICIN TOXIN STRUCTURE AND BIOSYNTHESIS

Ricin is a toxin originating from *Ricinus communis*, the castor bean plant. A photograph of the plant is shown in Figure 27.3A; a photograph of the beans it produces is presented in Figure 27.3B. As for all type II RIPs, ricin is a protein consisting of two folded peptide chains, designated the A-chain and B-chains. The two are linked by a single disulfide bridge. Ricin has a molecular weight of approximately 64–65 kDa; ricin A-chain (RTA) is a 267-amino acid globular protein domain containing eight α-helices and eight β-sheets (Wright and Robertus, 1987). The catalytically active site occurs as a long cleft on the A-chain surface. Within the active site, Glutamic acid 177 is a key catalytic residue, and conversion to Glutimate reduces activity 180-fold.

Ricin B-chain (RTB) is a 262-amino acid protein domain that conforms to a barbell-shaped architecture. The folded domain has a sugar-binding site at each end, allowing hydrogen bonding to galactose and *N*-acetyl galactosamine typically found on cell surfaces. The B-chain is not toxic and the A-chain alone is not toxic. In fact, many edible plants such as barley synthesize A chain. The B-chain has been expressed in monkey COS-M6 cells with no adverse effects (Weston et al., 1994). A representation of the ricin protein based on X-ray crystallographic data is presented in Figure 27.4.

FIGURE 27.4 Representation of the ricin protein derived from X-ray crystallographic data. The A-chain and B-chain are blue and orange, respectively. Random coil regions are strings, β-sheets are flat ribbons, and α-helices are coiled ribbons.
Source: Illustrations taken from GS (2014).

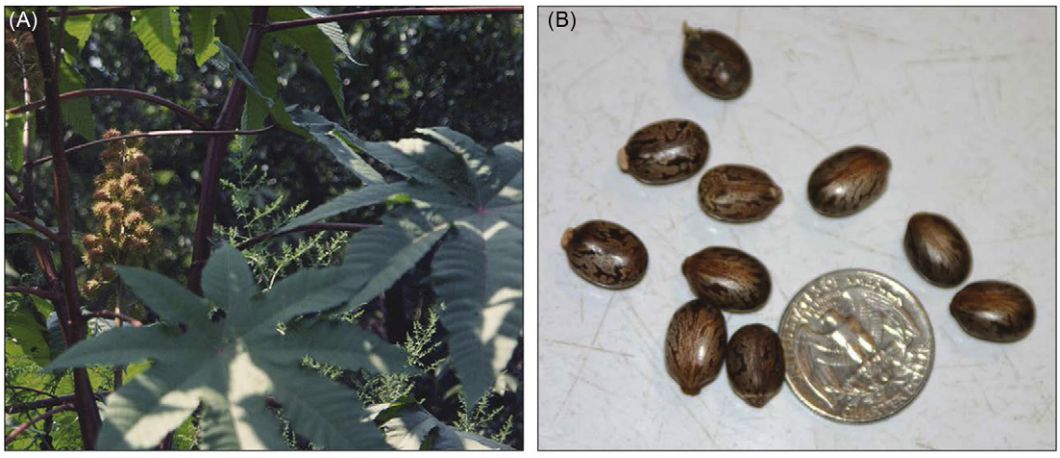

FIGURE 27.3 Photograph of a castor bean plant (A) and castor beans (B). *Source: From Audi et al. (2005).*

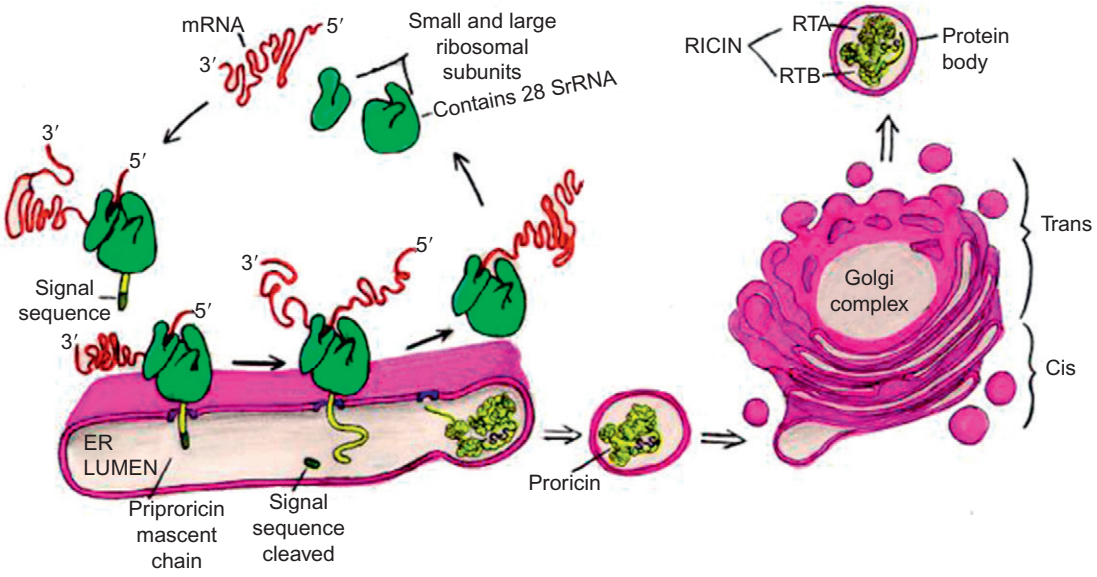

FIGURE 27.5 Biosynthesis of the ricin toxin. *Source: Illustration taken from EHSO (2014).*

The biosynthesis of ricin and RCA occurs in the endosperm cells of maturing *R. communis* seeds for storage in subcellular vacuolar compartments referred to as protein bodies. This entire process is illustrated schematically in Figure 27.5. Biosynthesis begins with the translation of a propolypeptide encompassing both the A-chain and B-chain as well as an amino-terminal signal sequence (Butterworth and Lord, 1983; Lord, 1985). The signal sequence directs the nascent chain to the ER lumen, where it is cleaved and removed from the rest of the long polypeptide chain. The resulting proricin is N-glycosylated in the lumen, and protein disulfide isomerases catalyze disulfide bond formation as the proricin chain folds into two globular protein domains. Proricin undergoes further oligosaccharide modifications within the Golgi complex before it is transported within vesicles to the protein bodies (Hiraiwa et al., 1997). The toxins are stored in the protein bodies until their ultimate destruction by hydrolysis, typically only a few days after germination of the mature (Lord, 1985).

THE CELLULAR INTERNALIZATION OF RICIN

The entry of ricin into a living cell begins with the reversible binding of its B-chain to cell-surface glycolipids and glycoproteins bearing β(1→4)-linked galactose residues (Moya et al., 1985). The toxin has two galactose binding sites for cell surface binding, but ricin may also be bound to certain cells by an entirely different mechanism. Ricin, a glycoprotein, contains mannose-rich N-linked oligosaccharides in both of its constituent subunits. On the surface of cells in the reticuloendothelial system, mannose receptors are present that have been shown to bind these carbohydrates (Simmons et al., 1986). Further, the binding by these mannose receptors results in subsequent cell death (Simmons et al., 1986), and deglycosylation of the toxin abolishes both binding and intoxication by the mannose receptor pathway (Foxwell et al., 1987). It is estimated that 10^6–10^8 toxin molecules may bind to a single cell at one time. The toxin molecules are internalized into cells via endocytosis in coated pits and vesicles, or smooth pits and vesicles (Moya et al., 1985). Once in the cytosol, these vesicles fuse with endosomes that can return some of the toxin molecules to the cell surface by exocytosis or, as shown in Figure 27.5, endosomes can fuse with lysosomes that will destroy the ricin molecules (Sandvig and Olsnes, 1979; Sandvig et al., 2004). Alternatively, the endosomes can carry ricin molecules to the Golgi complex and ER by retrograde transport (Sandvig et al., 2004) (Figure 27.6). Ricin molecules reaching the trans Golgi network can penetrate its membrane and reach the cytosol or, in some cases, return to the cell surface (Sandvig et al., 2004). For ricin molecules reaching the ER lumen, the disulfide bond connecting their A-chain and B-chain will be reduced, facilitating a partial unfolding of the A-chain (Kornfeld et al., 1991). The unraveling toxin protein will be translocated across the ER membrane via the Sec61p translocon, following the same pathway as that for incorrectly folded ER proteins targeted to the ER-associated degradation machinery (Simpson et al., 1999; Lord et al., 2003). Ricin molecules escaping the degradation machinery may find their way into the cytosol. These ricin molecules, and those crossing the trans

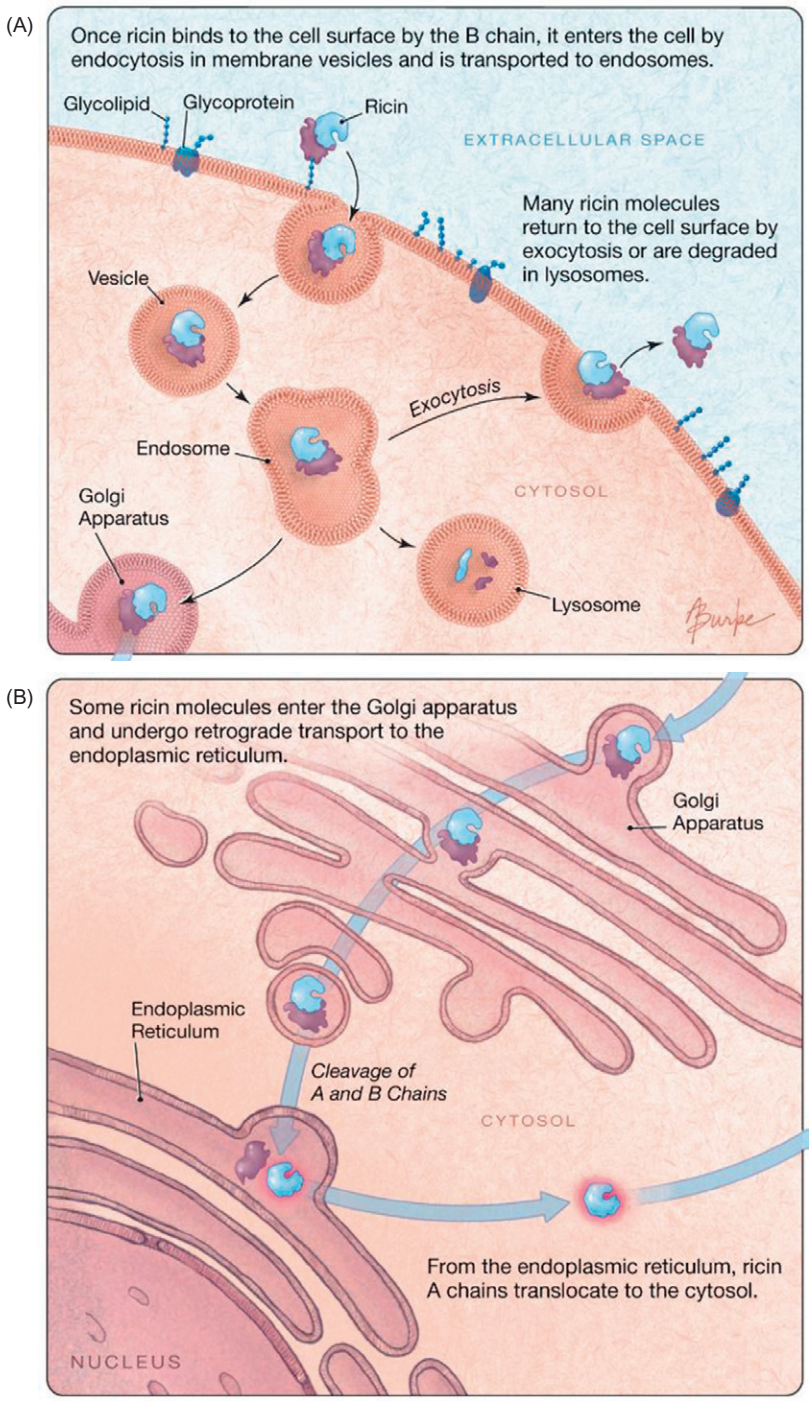

FIGURE 27.6 Example of the cellular internalization of ricin. The process involves endocytosis by coated pits and vesicles (A) or smooth pits and vesicles, followed by vesicle–endosome fusion (B). Ricin molecules can then return to the cell surface by exocytosis, or the vesicles may fuse to lysosomes for toxin destruction. *Source: Illustrations taken from Audi et al. (2005).*

Golgi network membrane into the cytosol, can refold into a protease-resistant, enzymatically active structure (Lord et al., 2003). Just a single ricin molecule entering the cytosol of a living cell with a K_{cat} of 1,500/min can inactivate enough ribosomes to ultimately result in cell death (Endo et al., 1987).

N-GLYCOSIDASE ACTIVITY OF RICIN

The ricin A-chain contains an enzymatically active site that recognizes and binds a highly conserved region in the large 28S rRNA of the intoxicated cell, referred to as the sarcin/ricin loop (Rajamohan et al.,

2001). This loop is a very short stem-loop structure of the overall ribosome and the loop is located in domain VII, approximately 400 nucleotides from the 3′-end of the rRNA. Within the stem-loop, the ring of a single adenine (A_{4324}) becomes sandwiched between two tyrosine rings in the catalytic cleft of the ricin A-chain and is hydrolyzed at the carbon–nitrogen glycosidic bond by the N-glycosidase action of ricin (Endo et al., 1987). This particular site-specific RNA N-glycosidase activity is a common property of all identified type I and type II RIPs (Barbieri et al., 2006). The activity prevents the binding of elongation factors EF-1 and EF-2, resulting in the cessation of mRNA translation.

Although all RIPs exhibit N-glycosidase activity toward ribosomes, they display marked differences in substrate specificity. Most type I toxins exhibit very broad specificities, whereas type II toxins display a preference for animal ribosomes. Ricin, for example, is highly active against mammalian and yeast ribosomes but poorly active, or even inactive, against plant and bacterial ribosomes (Yoshinari et al., 1997). In contrast, pokeweed antiviral protein depurinates ribosomes from plants, bacteria, yeast, and various evolutionally diverse animals (Rajamohan et al., 1999). Both the RIP and ribosome in a RIP–ribosome interaction contribute to the apparent substrate specificity (Kurinov et al., 1999). Because the rRNA sarcin/ricin loop structure is universally conserved, differences in sensitivity to different RIPs may possibly reside within the associated ribosomal proteins that confer tertiary structure to the ribosome (Kurinov et al., 1999). These differences may either allow or prevent access of the RIPs to the sarcin/ricin loop. Vater et al. (1995) identified the rat liver ribosomal proteins L9 and L10e as the binding target of the ricin A-chain, whereas yeast ribosomal protein L3 has been identified as the binding factor for pokeweed antiviral protein. The specific interaction between pokeweed antiviral protein and L3 is probably explained by the broad-spectrum activity of pokeweed antiviral protein toward ribosomes from species of different taxonomic groups, because L3 is highly conserved in ribosomes (Rajamohan et al., 1999). Differences in activity and ribosome substrate specificity are also due to differences in the structure of different RIPs. This was demonstrated by an approach in which pokeweed antiviral protein–ricin A-chain protein hybrids were created and examined for activity on rabbit reticulocyte and *E. coli* ribosomes (Vater et al., 1995). Experimental results demonstrated that the amino-terminal half of the hybrid proteins determine the substrate specificity. Structurally dissimilar surface polypeptide loops apparently do not play a role. In addition to the highly specific action on ribosomes, ricin and related RIPs have a less specific action *in vitro* on super-coiled double-stranded DNA (dsDNA), dsDNA, single-stranded DNA (ssDNA), and RNA substrates releasing multiple adenine residues and, in some instances, guanine residues (Wang and Tumer, 1999). RTA also catalyzes the hydrolysis of synthetic oligonucleotides as short as 6 base pairs, provided a GAGA tetra loop is present (Amukele and Schramm, 2004). Other reported RIP activities are lipase, chitinase, and superoxide dismutase (Xu et al., 2008). To date, activity against synthetic DNA or RNA oligonucleotides for the various RIPs has not been compared.

SIGNS AND SYMPTOMS OF RICIN EXPOSURE

As with most biological warfare agents, ricin remains biologically active through several different modes of entry into a living animal, including ingestion, injection, and inhalation. Symptoms and toxicity, however, can vary dramatically with mode of entry (Olsnes, 2004). Response to cutaneous contact with ricin, however, is not particularly noteworthy. An urticarial, IgE-mediated allergic reaction may occur after handling the intact castor bean plant or exposure to castor bean dust or pomace. Irritation and the development of pseudomembranous conjunctivitis after ocular exposure to very low ricin concentrations are commonly reported. In humans, general signs and symptoms of ricin exposure include fever, fatigue, weakness, muscle pain, and dehydration.

Most of what is known about the effects of ingested ricin derives from animal studies, which reflect a 100-fold variation in lethal toxicity between the various animals studied. Of all tested animals, the chicken is the least sensitive and the horse is the most sensitive. In mice, the lethal dose for 50% of an animal test group (LD_{50}) is 20 mg/kg when ingested (Ishiguro et al., 1992). Absorption occurs within 2 h with uptake by both gastrointestinal lymphatic and blood vessels, and approximately 20–45% of the ingested ricin is excreted unchanged in the feces (Ishiguro et al., 1992). The toxin accumulates mainly in the liver and spleen, with the onset of symptoms usually occurring within 4–6 h (Challoner and McCarron, 1990; Ishiguro et al., 1992). Initial symptoms are nonspecific and may include abdominal pain, vomiting, diarrhea, heartburn, and oropharyngeal pain (Klaim and Jaeger, 1990). Without treatment, fluid losses may lead to electrolyte imbalances, dehydration, hypotension, and circulatory collapse. Blood chemistry changes can lead to leukocytosis, elevated transaminases and creatinine kinase activities, hyperbilirubinemia, renal insufficiency, and anemia. Postmortem findings of diffuse intestinal hemorrhagic lesions are common (Fodstad et al., 1976; Klaim and Jaeger, 1990).

Many of the results for ricin injection derive from rodent studies. The highest concentration of ricin was found to localize to the spleen after either intravenous or intraperitoneal administration of ^{125}I-ricin, whereas

the urine was found to be the major route of elimination. The ricin LD_{50} in mice is approximately 5 μg/kg by injection (Fodstad et al., 1976). In general, for rodents, the majority of ricin excretion occurs in the urine over the course of the first 24 h (Blakey et al., 1988). The onset of nonspecific signs and symptoms, which may be similar to sepsis (fever, headache, dizziness, nausea, anorexia, hypotension, abdominal pain), occurs within 12 h after dosing. Abnormalities include elevated liver transaminases, amylase, and creatinine kinase activities, hyperbilirubinemia, myoglobinuria, and renal insufficiency, and these can progress to multisystem organ failure (Ishiguro et al., 1992; Bradberry et al., 2003). Postmortem findings are consistent in forensic case investigations as well as animal studies and include focal hemorrhage in the intestines, brain, myocardium, and pleura (Fodstad et al., 1976). The lymph nodes, kidneys, and intestines may also show signs of necrosis, hemorrhage, and edema (Flexner, 1897).

In cases of ricin inhalation, particle size can significantly influence lung deposition and lethality. Smaller particles typically deposit deeper into the respiratory tract, resulting in higher mortality. Larger particles deposit higher in the airways and can be swept up into the mouth by the mucociliary system and subsequently swallowed (Roy et al., 2003). The LD_{50} in mice exposed to ricin particles smaller than 5 μm is between 3 and 5 μg/kg (Wilhelmsen and Pitt, 1996). For monkeys, inhalation of 1- to 2-μm particles at 21–42 μg/kg results in progressive dyspnea 20–24 h after dosing (Wilhelmsen and Pitt, 1996). Postmortem findings typically include diffuse pulmonary edema with multifocal areas of necrosis and inflammation, and injury tends to be significantly worse in the distal airways and alveoli (Soler-Rodríguez et al., 1993; Griffiths et al., 1995; Wilhelmsen and Pitt, 1996; Brown and White, 1997). Toxicity results directly from the inhibition of protein synthesis, release of cytokine mediators, and direct injury to the epithelial membrane, with the primary targets of toxicity as the type I and type II pneumocytes (Soler-Rodríguez et al., 1993; Griffiths et al., 1995; Wilhelmsen and Pitt, 1996; Brown and White, 1997; Roy et al., 2003). Significant systemic absorption is usually not observed after inhalation, and toxicity is primarily limited to the respiratory tract (Soler-Rodríguez et al., 1993; Wilhelmsen and Pitt, 1996). Respiratory failure is likely to be the primary cause of morbidity and mortality in humans after ricin inhalation, and symptoms include severe lung inflammation with progressive cough, dyspnea, cyanosis, and pulmonary edema (USAMRIID, 2011).

The CDC recommends taking the following precautions when handling ricin in the laboratory (CDC, 2014):

- Avoid any activity that places persons at risk for exposure, especially activities that might create aerosols or droplet dispersal.
- Follow laboratory safety practices to prevent exposure.
- Decontaminate laboratory benches after each use and dispose of supplies and equipment in proper receptacles.
- Avoid touching mucosal surfaces with gloved or ungloved hands and never eat or drink in the laboratory.
- Remove gloves before leaving the laboratory, dispose of gloves in a biohazard container, wash hands, and remove laboratory coat.

FIELD-FORWARD BIOLOGICAL AGENT DETECTION

Immunoassays

Conventional assays used to detect biological toxins involve traditional biochemical techniques such as immunoassays based on antibodies raised against the toxin target. Immunoassays were first developed in the 1950s; 30 years later, the assays were in common use due to the development of standard reagents and automated assay readers (Ngundi et al., 2006). The immunoassay is a rapid and reliable technique for identifying a molecule that contains at least one antigenic moiety. Immunoassays rely on the inherent ability of an antibody to bind specific chemical groups on conformationally flexible antigens, such as carbohydrates, or specific molecular conformations in the case of large, conformationally rigid molecules, such as proteins. These chemical groups and conformations are referred to as antigenic determinants and epitopes, respectively. Because antibodies are produced against a specific determinant or epitope, they are highly specific and selective and, therefore, are ideal for use in the detection of biological molecules. Today, the gold standard of immunoassays for toxin detection is the enzyme-linked immunosorbent assay (ELISA) (Hammond et al., 2006; Guglielmo-Viret and Thullier, 2007; Garber and O'Brien, 2008). In its simplest terms, the ELISA is a technique used to detect the presence of an antigen in a sample by affixing the sample contents to a solid support and visualizing the immobilized antigens with specific antibodies. Performing an ELISA involves at least one antibody with specificity for the target antigen. The sample is first immobilized to a solid support such as the surface of a polystyrene microtiter plate, either nonspecifically via direct adsorption or specifically via capture by another antibody specific for the target antigen that is affixed to the solid support. After antigen immobilization, a second mixture of antibodies sometimes referred to as detector antibodies are added to the complex with target antigens. Detector antibodies can be covalently linked to an enzyme or a chromophoric

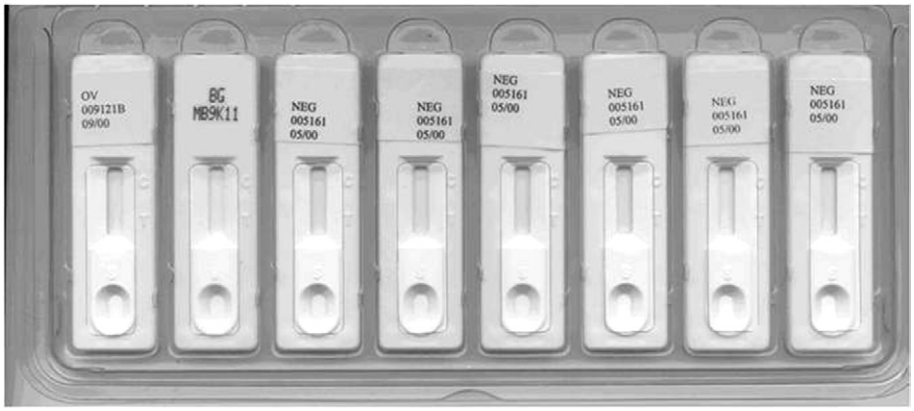

FIGURE 27.7 Photograph of an HHA packet.

reporter group, or itself can be detected by a secondary antibody linked to an enzyme or chromophoric reporter group. The labeling of the detector antibody allows for visualization of the antibody/antigen complex. Washing with a mild detergent solution typically is used between each step to remove nonspecific antibodies or other proteins. After a final wash, the assay is developed to visualize the results, either by addition of an enzymatic substrate to produce a color change or precipitate, or by irradiation of a specific wavelength of light in the case of chromophoric reporter groups. Older ELISAs typically used chromogenic substrates, whereas newer assays use fluorogenic or electrochemiluminescence-based technology to significantly enhance assay specificity.

The most recently developed technique aimed at enhancing immunoassay sensitivity is immune-polymerase chain reaction (I-PCR). The technique is markedly more sensitive than ELISA, primarily because it combines the detection specificity of an antibody with the amplification power of real-time PCR for a nucleic acid. The technique was first described by Sano et al. (1992). Realized limits of detection have surpassed ELISA by 10-fold to 10,000-fold. Typically, lateral flow immunochromatographic assays are used in field-forward situations due to lenient storage and equipment requirements (Pastoor et al., 2008). The problem with PCR-based assays is that they do not detect toxin protein, but they do detect co-purifying DNA, which is not always present. Cell-based bioassays are sometimes used to confirm intact toxin-killing activity of the ricin in environmental samples (Brzezinski and Craft, 2007). Field-forward analysis for biothreat agents is limited to antibody-based tests and PCR. The most commonly used tests are the ELISA and immunochromatographic tests typically referred to as hand-held assays (HHAs), as shown in Figure 27.7.

The ricin toxin has been detected in tissue sections, some tissue specimens, nasal swabs, and fluids by using immunologically based methods to study animals and animal tissues (DOD, 1999; Brzezinski and Craft, 2007). Immunologically based methods applied to human and animal fluid specimens have the potential to measure ricin concentrations as low as 0.1 ng/mL (1.54 pmol/L) (Shyu et al., 2002a,b). However, such applications have not been clinically validated, and concentrations after toxic exposures are unknown. Reference laboratories such as the US Army Medical Research Institute of Infectious Diseases (Aberdeen Proving Ground, MD) and the CDC are currently adapting these and other analytic methods for application to human specimens. At the Edgewood Chemical Biological Center (ECBC, Aberdeen Proving Ground, MD), matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) is used as a definitive method for identification in biological specimens, but this technique is not currently able to provide quantitative results. Technologies used for assessing enzymatic activity are the cell-free luciferase-based assay, the cell-based apoptosis assay, and a recently developed liquid chromatography tandem mass spectrometry (LC-MS) technique (Zamboni et al., 1989). Another technique that has been described measures the liberation of radio-labeled adenosine from RIP substrates resulting from their enzymatic activity. This technique has similar sensitivity to the LC-MS method with a range of 0.01–10 pmol, but it involves the hazards of radio-labeled nucleotides and is not readily transferable to field-forward use (Brigotti et al., 1998).

DNA-Based Assays: The PCR

The PCR is a technique for logarithmically amplifying a DNA sequence to the extent that a sufficient quantity of material may be available to detect through either fluorescence or gel electrophoresis. The technique was developed in 1993 by Kary Mullis, who was awarded the Noble Prize in Chemistry that year for his pioneering work (Bartlett and Stirling, 2003). Because of its simplicity, PCR is a highly popular technique with a

wide range of applications, including direct nucleic acid sequencing, genomic cloning, DNA typing, the detection of infectious organisms, site-directed mutagenesis, parental genetic disease research, and the analysis of allelic sequence variations (Sambrook and Russell, 2001). Typically, when PCR is used as an identification technique, the target sequence to be amplified is a sequence of DNA known to be specific to one particular organism; this is referred to as template DNA. The complementary strands of the double-stranded template are separated at 95°C, and the temperature is lowered slightly to allow two 10- to 30-base pair (bp) ssDNA primers to anneal to their complementary strands of the template. DNA polymerase binds the primer–template hybrid and initiates synthesis of DNA complementary to the template from the primer in a 5′–3′ direction. This cycle of heating to denature the double-stranded template DNA, followed by annealing and extension of the primers by DNA polymerase, is typically repeated approximately 30 times to yield literally billions of copies of the original template (Antwerpen et al., 2008).

In the original PCR process described by Mullis, *E. coli* DNA polymerase was used for DNA synthesis. The challenge presented by using the enzyme was that the 95°C melting step destroyed its polymerase activity, and the enzyme had to be replenished after the heating stage cycle. As a result, the original Mullis PCR process was very laborious and required vast amounts of DNA polymerase. The problem was resolved in 1986 by utilizing DNA polymerase from *Thermophilus aquaticus* bacterium that was discovered in Colorado hot springs. The bacterium was well-adapted to the extremely high water temperatures of the springs, and its DNA polymerase was found to endure the high temperatures required for PCR (Munster et al., 1986). Gelfand et al. successfully purified and cloned this DNA polymerase, now commonly referred to as Taq polymerase. The enzyme allows a series of 30–40 cycles of PCR amplification without the need of replenishing the polymerase and also reduces the associated potential for introducing contamination into the PCR assay. Moreover, because of its thermophilic origins, Taq polymerase functions optimally at approximately 72°C, allowing DNA synthesis to be performed at much higher temperatures than was possible with the *E. coli* enzyme. Because of the higher stringency of PCR primer annealing at the higher temperatures, the DNA template is copied with much higher fidelity, and the nonspecific products that had affected many previous PCR reactions are reduced (Saiki et al., 1988). One of the major disadvantages of Taq polymerase, however, is its low replication fidelity. Taq polymerase does not have a 3′–5′ exonuclease proofreading mechanism to replace base-pairing mismatches in the newly synthesized DNA strand; however, it does have a 5′–3′ exonuclease activity that allows real-time PCR (Luthra et al., 1998). The intensity of fluorescence emitted during the PCR reaction is monitored in real-time PCR, acting as an indicator of the degree of PCR amplification occurring during each PCR cycle. Thus, with newer real-time PCR instruments, reaction progress can be observed visually in real time.

The CDC and Department of Homeland Security Laboratory Response Network centers conduct polymerase chain reaction tests and time-resolved immunofluorescence assays to detect the ricin toxin in environmental specimens. Immunofluorescence is the immunoassay technique that uses a detector antibody or an antigen labeled with florescent dyes (Lim et al., 2005). Time-resolved florescence simply delays the time between excitation of the florescent dyes and quantifying resulting emission. The long-lived emissions of the florescent dyes are exploited to reject background signals such as sample autofluorescence as well as Rayleigh and Raman scatter (Marriott et al., 1991). Detection of the delayed emission leads to an increase in the signal-to-noise ratio of the dye, thereby lowering the detection limit of the assay. Time-resolved fluorescence also enhances separation of emission spectra from multiple dyes, thus improving assays that are multiplexed (Yan and Marriott, 2003).

Because PCR-based assays cannot directly detect the toxin protein, cell-based bioassays are sometimes used to confirm the intact toxin-killing activity of ricin in environmental samples (Brzezinski and Craft, 2007).

Many of these technologies have the potential to quickly and conveniently determine whether an unknown sample possesses ricin activity and could greatly aid in triage of victims after suspected exposure of ricin. Furthermore, it would enable determination of the effective decontamination for a site. Technology of this nature may also greatly aid in an initial forensic investigation by immediately determining potency, and field-forward personnel could estimate the purity, grade, and the age of the weapon. Finally, a rapid screening assay for RIP activity would also prove beneficial for the pharmaceutical industry as a screening tool for genetically engineered ricin developed for cancer chemotherapeutics, and for basic researchers attempting to identify and characterize new and novel RIPs.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The assays for ricin described in this chapter are based on either ELISA detection of the toxin or DNA-based detection. Largely ignored is the fact that the lectin portion (B-chain) of the intact toxin could also serve as an analytical target. Carbohydrate-binding lectins bind to carbohydrate ligands with weak binding affinities, ranging in molar to sub-millimolar concentrations, even when

preserving high specificities of the interaction. Lectins have the ability to distinguish a wide range of carbohydrate structures on the surface of cells. Their importance in cell recognition as related to mitogenesis, agglutination, and direction of cell cytotoxicity has been established (Sharon and Lis, 1989). This specificity has been exploited to differentiate various cancer cells (Welty et al., 2006). Our understanding of the adsorption and distribution of the ricin toxin within the target organism could be greatly enhanced through the use of such techniques.

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CHAPTER

28

Botulinum Toxin*

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INTRODUCTION

Botulism is a disease caused by anaerobic, spore-forming bacteria found in soil. Disease results from the actions of chemical toxins produced by these bacteria. The most common forms of human botulism include foodborne, infant, and wound. The main etiology of botulism in humans is foodborne; this form is caused by eating foods contaminated with botulinum spores, which germinate and multiply into bacteria to produce neurotoxins in the food. Commonly contaminated foods include improperly preserved home-processed foods such as honey, corn, green beans, and beets. Less frequent sources are fish products and commercially processed foods. Infant botulism is often associated with eating honey contaminated with spores, but new evidence suggests that soil and dust brought into the house from the outside may be a significant source of botulinum spores. Wound botulism occurs when spores contaminate a wound, germinate, and produce toxins that are absorbed into the bloodstream. Regardless of the form of botulism, disease results from the intoxicating effects of potent neurotoxins.

Botulinum neurotoxins (BoNTs) comprise a family of seven distinct neurotoxic proteins produced by immunologically discrete strains of the anaerobic bacteria. These spore-forming, Gram-positive bacteria secrete deadly toxins with an estimated human LD_{50} of 1–3 ng/kg (Simpson, 2004; Sobel et al., 2004). In fact, BoNTs are the most potent substances known to humankind. Due to their extremely high potency, ease of production, and previous history of weaponization, the BoNTs have been designated as category A threat agents by the US Centers for Disease Control and Prevention (CDC). Category A agents are defined by the CDC as those that "result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption and require special action for public health preparedness."

While there are currently seven known antigenic serotypes of BoNT, only serotypes A, B, and E are predominantly associated with human intoxication. Intoxication by BoNTs leads to bilateral flaccid paralysis, involving skeletal muscle and structures innervated by autonomic fibers (Simpson, 1986; Shapiro et al., 1998). Death is inevitable if the intoxication is left untreated. The toxicity of BoNTs leading to flaccid paralysis of skeletal muscle is due to their ability to block acetylcholine (ACh) release from peripheral cholinergic nerve endings. Paralysis could persist for weeks to months (depending on the serotype), and the available treatment consists of supportive care including fluids, total parenteral nutrition (TPN), and mechanical ventilation. Death occurs when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation or when patients succumb to secondary infections following long periods of intensive care (Hatheway et al., 1984; Shapiro et al., 1998).

BoNTs pose a serious concern to our national security. The toxins are highly lethal, easy to isolate, and easy to deliver by terrorists. Activities of hostile nations, international terrorists, and antigovernment groups make the threat of BoNTs a serious problem for both our military and civilian populations. Development of BoNTs as weapons of mass destruction began over 50 years ago. Nonstate-sponsored terrorists have an interest in BoNTs; members from the Aum Shinrikyo cult attempted to disperse BoNT in Tokyo and at US military installations throughout Japan on at least three occasions in the 1990s (Brackett, 1996). Furthermore, in the years following Operation Desert Storm in the first Gulf War (1990–1991), it was discovered that Iraq produced thousands of liters of concentrated BoNT in their weapons program.

* Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army or the US Food and Drug Administration.

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Moreover, approximately half that volume was already loaded into military weapons. The BoNT generated by Iraq has yet to be fully accounted for. An act by terrorists to release BoNT into a civilian population through contamination of products of consumption or inhalation would pose a serious threat to national security and public safety. Wein and Liu (2005) modeled the threat of deliberate BoNT release into a milk-processing facility. The actions by such a terrorist group could lead to massive casualties. Another study estimated that dispersal of BoNT via inhalation could kill 10% of an exposed population within 0.5 km downwind of the incident.

BACKGROUND

Toxin Structure and Molecular Function

Background

The highly potent neurotoxins synthesized by the *Clostridium botulinum* microorganism and several related clostridial species (*C. baratii*, *C. butyricum*, and *C. argentinense*) are the causative agents of botulism, a potentially lethal disease historically associated with the ingestion of contaminated food products. Seven different BoNTs, designated A through G, are currently known to be produced by various strains of clostridial bacteria; these neurotoxins are antigenically distinct but comparable in basic structure. The BoNTs are members of a superfamily of homologous proteins that also include tetanus neurotoxin. BoNTs are generated as single-chain polypeptides that are then posttranslationally modified (i.e., proteolytically nicked) to yield a disulfide bond-linked dichain structure composed of a heavy chain (H-chain or HC) and a light chain (L-chain or LC). Enzymes synthesized by these microorganisms themselves often mediate this cleavage, although the gastrointestinal enzymes of the host can also generate the dichain structure from the ingested toxin. The three-dimensional dichain protein structure of the purified toxin is provided (Figure 28.1).

Function of Heavy and Light Chains

The HC and LC of BoNTs each play critical roles in toxicity. HC is thought to mediate binding and internalization of the toxin at peripheral nerve synapses. LC, the toxic moiety, inhibits neurotransmitter exocytosis

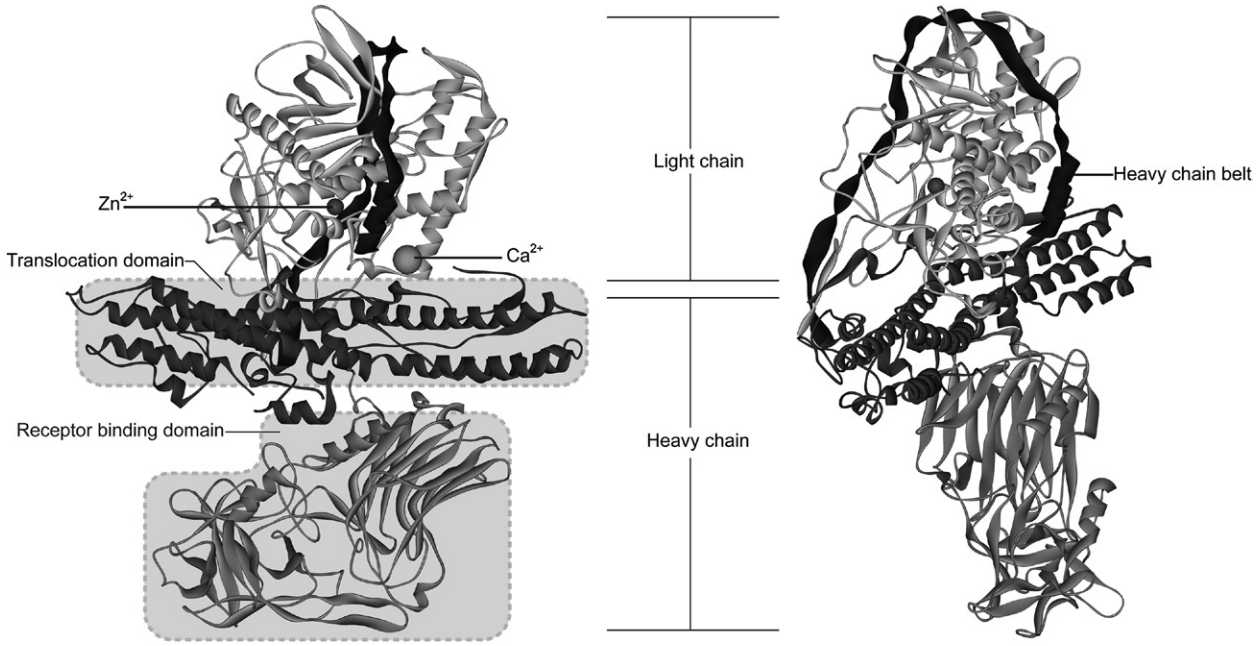

FIGURE 28.1 Three-dimensional structure of botulinum toxin serotype A (BoNT/A). BoNT/A (1296 amino acids), rendered as a ribbon structure, is depicted in two views. BoNTs are synthesized as a single polypeptide and nicked by bacterial proteases to form a dichain molecule. The 50-kDa LC, with 448 residues, and the 100-kDa HC, with 848 residues, are linked by a dilsulfide bond. All BoNTs comprise three major domains: a receptor-binding domain (C-terminal end of HC), a translocation domain (N-terminal end of HC), and a zinc-binding metalloprotease domain on LC. All seven BoNTs exhibit conserved sequences, but they are also antigenically distinct at the same time. The LC seems to be held in place by the translocation belt of HC (Brunger et al., 2007). The belt spans residues 492–545 in BoNT/A and 481–532 for BoNT/B and wraps around the catalytic domain of the LC. Brunger et al. (2007) suggest that the belt acts as a surrogate pseudosubstrate inhibitor of the LC protease and acts as a chaperone during translocation across the endosome membrane into the cytosol. The belt occludes access to the active site of LC, thereby holding the unreduced holotoxin in its catalytically inactive state. The sphere represents the bound Zn^{2+} at the LC active site. The structure of BoNT/A holotoxin was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Berman et al., 2000; PDB ID: 2nz9; Garcia-Rodriguez et al., 2007) and rendered using Accelrys DS Visualizer 2.0 software.

through its zinc-dependent endoproteolytic activity. The LCs of the various BoNT serotypes differ in their distinct molecular targets within the peripheral cholinergic nerve terminals (Schiavo et al., 1992, 1993a,b; Blasi et al., 1993). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects, despite their distinct targets.

BoNT serotype A is the most well characterized of the different serotypes in terms of both structure and function. Early biochemical efforts led to its crystallization, and this crystalline form was used in numerous animal studies on the pathogenesis of botulism. Crystalline type A toxin has a sedimentation constant of 19S (around 900 kDa), which is far larger than the combined size of the HC and LC components (Simpson, 1981). This large progenitor toxin form was shown to dissociate under moderately alkaline conditions, releasing the derivative (7S) or neurotoxin component (Heckly et al., 1960; Sugii et al., 1977a,b; Chen et al., 1998). The derivative neurotoxin has a molecular weight of 150–160 kDa, representing the combined size of the H- and L-chains. Additional work led to the finding that the dichain molecules comprising the various neurotoxins are often released as higher-order polypeptide complexes. The crystalline type A progenitor toxin consists of the 7S neurotoxin and one or more non-covalently linked accessory proteins. These nontoxic components of the progenitor toxin complex were later identified as three different hemagglutinins (HAs) and a nontoxic nonhemagglutinin (NTNH) protein.

Accessory Proteins of the Progenitor Toxin Complex

The accessory proteins of the progenitor toxin serve to enhance the stability of the toxin to ensure uptake from the gut. The NTNH component of the multimeric type A toxin complex is encoded by a single gene upstream of the neurotoxin locus, while three different HA proteins have been characterized in association with BoNTs. All *C. botulinum* serotypes have been shown to produce neurotoxin complexes with the NTNH and HA proteins.

Overview of BoNT Action

After gaining entry to the lymphatics and circulation via the gastrointestinal tract, BoNTs function as potent neuromuscular blockers. The cellular and molecular mechanisms involved in toxin absorption, transit to specific target tissues, escape from the vasculature, and uptake within peripheral cholinergic nerve terminals have yet to be fully characterized. However, each of the neurotoxins has been shown to block vesicular neurotransmitter release at peripheral cholinergic synapses through the endoproteolytic cleavage of proteins associated with the exocytosis machinery (Schiavo et al., 1993a,b, 1995,; Blasi et al., 1993). At peripheral cholinergic nerve endings, BoNT binding to high-affinity receptors leads to acceptor-mediated endocytosis and low pH-induced translocation across the endosomal membrane into the cytosol. The carboxy-terminal region of the toxin HC appears to mediate binding at the nerve synapse, while the amino-terminal domain controls translocation. The LC is held in close association with the HC by an amino acid belt. The LC of each toxin functions as a zinc-dependent endoprotease, cleaving at least one of three soluble *N*-ethylmaleimide-sensitive fusion (NSF) protein attachment receptor (SNARE) proteins involved in neurotransmitter release.

Stimulus-evoked, calcium-dependent release of ACh from the cholinergic synapse normally occurs through the formation of a fusion complex between ACh-containing vesicles and the intracellular leaflet of the nerve terminal membrane (Arnon et al., 2001). This synaptic vesicle (SV) fusion complex consists of several proteins of the SNARE family, including a 25-kDa synaptosomal associated protein (SNAP-25), vesicle-associated membrane protein (VAMP, or synaptobrevin), and the synaptic membrane protein syntaxin. Other SNARE proteins have been identified as components of membrane transport systems in yeast and mammals but have not been implicated as targets for BoNTs. Meanwhile, type A and E neurotoxins cleave SNAP-25, while types B, D, F, and G act on VAMP and type C1 toxin cleaves both syntaxin and SNAP-25. Neurotoxin-mediated cleavage of any of these substrates disrupts the processes involved in the exocytotic release of ACh and leads to flaccid paralysis of the affected skeletal muscles.

Clinical Forms of Botulism in Humans and Animals

Exposure to BoNTs can produce lethal disease in humans and other animal species. Six different clinical forms of human botulism have been described in the literature (see Table 28.1), including (i) foodborne botulism, (ii) infant botulism, (iii) wound botulism, (iv) an adult form of infant botulism, (v) inadvertent systemic botulism, and (vi) inhalation botulism (reviewed by Cherington, 1998; Middlebrook and Franz, 2000; Arnon et al., 2001). Botulism is the result of either an infectious process involving elaboration of toxin from the colonizing clostridial organism, or a noninfectious process. Infant and wound botulism are the most prevalent infectious forms of botulism. Although rare, an adult form of infant botulism has been documented; gastrointestinal colonization in adults may be enabled by alterations in normal gastrointestinal flora resulting from antibiotic treatment (Cherington, 1998). Foodborne and inhalational have noninfectious etiologies and are the result of ingesting or inhaling preformed toxin. Although only one inhalational botulism incident has been reported

TABLE 28.1 Clinical Forms of Botulism

	Infectious			Noninfectious		
	Infant	**Wound**	**Adult Colonization**	**Foodborne**	**Inhalational**	**Inadvertent Systemic**
Cause	Colonization of immature intestinal tract	Wound colonization resulting from contact with contaminated material	Intestinal colonization secondary to disruption of normal intestinal flora	Ingestion of preformed toxin in contaminated food products	Respiratory exposure to toxin aerosols or droplets	Systemic toxin uptake after therapeutic toxin administration
Susceptibility	Young infants (2–4 months of age) prior to establishment of normal intestinal flora	Self-administering users of IV drugs (often black tar heroin)	Antibiotic-treated patients	All exposed individuals	All exposed individuals	Patients treated with local toxin injections

in humans, this incident demonstrates that humans are susceptible to respiratory intoxication similar to that which has been experimentally produced in many laboratory species (reviewed by Middlebrook and Franz, 2000). Finally, the emergence of a multitude of therapeutic applications for BoNTs has led to infrequent cases of inadvertent systemic botulism resulting from local toxin injection (reviewed by Arnon et al., 2001).

Infectious Forms of Botulism

Infant Botulism

Infectious botulism is a consequence of ingesting or inhaling clostridial spores that colonize the large intestines, germinate, and elaborate toxin into the bloodstream. Infant and wound botulism are the most prevalent infectious forms of botulism. Infant botulism comprises the majority (72%) of reported human botulism cases in the United States, while most of the remaining cases involve foodborne and wound botulism (Mackle et al., 2001). Infants are especially susceptible to infant botulism. Infants were found to be uniquely susceptible to gastrointestinal colonization due to a lack of well-established competing gut flora (Arnon, 1995). While infant botulism can be acquired by inhaling spores, this differs markedly from inhalational botulism, which results from inhaling preformed aerosolized toxin and not spores. Clostridial spores do not pose a threat in older infants or most adults (Arnon, 1995, 1998; Cox and Hinkle, 2002).

Wound Botulism

Wound botulism involves growth of *C. botulinum* spores in a contaminated wound with *in vivo* toxin production (Weber et al., 1993). It accounts for less than 25% of all botulism cases. The majority of wound botulism cases are caused by serotype A and the remainder by serotype B (Shapiro et al., 1998). The neurological symptoms of wound botulism differ little from those of foodborne botulism except for the general absence of gastrointestinal symptoms. From its discovery in 1943 until 1996, only 111 incidents of wound botulism were documented (Merson and Dowell, 1973; CDC, 1998; Shapiro et al., 1998); among the 100 laboratory confirmed cases, 83 cases were type A, 16 cases type B, and one a mixture of type A- and B-producing organisms (CDC, 1998). Risk factors for wound botulism include deep wounds, avascular areas, compound fractures, and crush injuries of the hand. Although a rare form of naturally occurring BoNT intoxication, it most recently occurred in Maryland as a result of a construction worker receiving a contaminated, compound fracture of the femur after falling into an excavated pit (Hilmas, personal observation). Wound botulism also occurs in intravenous (IV) drug users as a result of bacterial colonization at needle puncture sites or nasal/sinus lesions secondary to cocaine snorting (MacDonald et al., 1985). From 1986 through 1996, 78 cases of wound botulism were reported, and the majority of cases were linked to black tar heroin, introduced intravenously.

Child or Adult Botulism from Intestinal Colonization

Gastrointestinal colonization in adults or children by *Clostridia* bacteria does not typically take place except in circumstances where the normal flora has been altered by antibiotic treatment (Cherington, 1998). Botulism results from *in vivo* production of toxin, analogous to the pathogenesis of infant botulism (Chia et al., 1986). Support for this form of botulism is provided by the demonstration of prolonged excretion of toxin and *C. botulinum* in stool, by the demonstration of *C. botulinum* spores but not preformed toxin in suspected foods, or both.

Noninfectious Forms of Botulism

Foodborne Botulism

Worldwide, BoNT intoxication is most commonly associated with food poisoning. In the early nineteenth century, the effects of botulism were observed

to be associated with the consumption and handling of meat products. Thus, German physician Justinus Kerner described what he termed "sausage poisoning" (Erbguth, 2004). It was later in the nineteenth century that the term *botulism* was used, from the Latin *botulus*, meaning "sausage." Foodborne botulism results from ingesting preformed toxin in food contaminated with toxin spores. Inadvertent and inhalational botulism, two other noninfectious forms of botulism, also involve exposure to preformed toxin. Outbreaks of foodborne botulism in the United States result from eating improperly preserved home-canned foods (CDC, 1995). The majority of cases of foodborne botulism are due to serotypes A, B, and E. From 1990 through 1996, type A accounted for 44.6% of foodborne outbreaks in the United States, followed by type E (35.7%) and type B (12.5%) (CDC, 1998). The prompt recognition of such outbreaks in the United States and early treatment with serotype-specific botulinum antitoxin has limited the number of casualties, severity of the disease, and the case-to-fatality ratio. Mortality from foodborne botulism has declined from 60% (CDC, 1998) in 1950 to less than 10% of clinical cases (Shapiro et al., 1998).

Inhalational

Because humans are relatively resistant to gut colonization by the *C. botulinum* microorganism, oral and inhalational exposures to preformed neurotoxin are likely to present the greatest threats with respect to intentional dissemination. The ability for inhaled BoNTs to produce illness has been documented in humans and in several experimental species. Only one incident involving inhalational intoxication in humans has been reported. Three laboratory workers presented with physical and neurological symptoms after accidental respiratory exposure to aerosolized type A toxin (Middlebrook and Franz, 2000; Arnon et al., 2001). These patients were all successfully treated with antiserum, gradually recovering from their weakness and visual disturbances over the next several days. After inhalational exposure, the neurotoxins are absorbed from the respiratory tract into the lymphatics and circulation for transport to peripheral cholinergic synapses (reviewed by Simpson, 2004). The pathogenesis following neurotoxin absorption is thought to be similar for both the respiratory and gastrointestinal exposure routes. Thus, the primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism.

Inadvertent Systemic Botulism

The therapeutic indications for BoNTs are numerous. They are used in the treatment of ophthalmological disorders (strabismus, Duane's syndrome, esotropia/exotropia), movement disorders (focal dystonias, blepharospasm), spasticity, neuromuscular disorders, pain (headache, myofacial pain), disorders of the pelvic floor (anal fissures), ear/nose/throat disorders, cosmetic applications (wrinkles), and hyperhidrosis. The recent explosion in new indications for BoNTs in the treatment of a wide range of medical conditions also brings the possibility of medical errors in BoNT dosing. Systemic botulism may result from injection of excessive doses of the potent neurotoxin. The most infamous case of systemic botulism involved the paralysis of four Florida patients, including a doctor, who were treated with BoNTs for wrinkles. The physician used formulations of type A from Toxin Research International, Inc., which were approved by the US Food and Drug Administration (FDA). The research grade type A neurotoxin was apparently sold to the doctor and reconstituted to be thousands of times more potent than the typical dose used in BOTOX® for paralyzing facial muscles. Later testing estimated that the raw bulk toxin that was used contained between 20,000 and 10 million units of botulinum toxin. In comparison, a typical vial of BOTOX from Allergan, Inc. contains only 100 units (CIDRAP, 2004). All three patients and the physician were injected with the toxin preparation; they developed severe systemic botulism requiring mechanical ventilation. While all four survived the superlethal dose of type A toxin, several of the patients experienced a syndrome involving chronic gastrointestinal symptoms and discomfort months after exposure.

Human Intoxication

The basic syndrome of BoNT intoxication is similar for all naturally occurring forms, as well as for inhalation exposure, and does not vary appreciably among serotypes (Hatheway et al., 1984; Simpson, 1986; Jankovic and Brin, 1997). Based upon documented laboratory evidence, human BoNT intoxication is caused by exposure primarily to serotypes A, B, E, and (to a much lesser extent) to serotype F; disease manifests mostly as a result of foodborne, infant, and wound botulism (Simpson, 1986). BoNTs are also lethal when inhaled in aerosolized form, although this is not generally observed in nature.

The various toxin serotypes are usually associated with analogous clinical presentations. Paralysis proceeds in a descending fashion after an initial bulbar involvement. The earliest symptoms of botulism typically include visual disturbances, followed by dysphagia, dysphonia, and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Jankovic and Brin, 1997). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to the lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death unless appropriate supportive care is provided (Shapiro et al., 1998).

EPIDEMIOLOGY

Foodborne Botulism

Human foodborne botulism outbreaks have typically been linked to the consumption of toxin-contaminated home-prepared or home-preserved foods (Maselli, 1998). The vast majority of foodborne botulism cases are attributed to toxin types A, B, or E. Maselli (1998) reports that type B is the most prevalent (52%) in the United States, followed by type A (34%) and type E (12%), while the CDC (1998) suggests 37.6% of all foodborne botulism outbreaks since 1950 were caused by type A, 13.7% by type B, 15.1% by type E, 0.7% by type F, and 32.9% were unidentified with respect to toxin type. Outbreaks of type F and G botulism are rare (Sonnabend et al., 1981; Maselli, 1998; Richardson et al., 2004), and only anecdotal reports of isolated type C1 and D botulism cases can be found in the published literature (e.g., Lamanna, 1959).

The natural epidemiology of foodborne botulism provides additional insight into the similarities and discrepancies between the human disease and that represented in various animal models. In the United States, around 25% of reported human botulism cases are classified as foodborne and 72% are infant (Mackle et al., 2001). Human type A and B foodborne botulism cases occur worldwide and constitute the vast majority of reported human intoxications (Maselli, 1998). The majority of other botulism cases are attributed to serotype E and are typically associated with the consumption of contaminated seafood. Generalizations have been made regarding the geographic distribution of the most common *C. botulinum* strains within the United States. Most human foodborne botulism outbreaks occurring west of the Mississippi are due to type A toxin; type B strains are more prevalent east of the Mississippi, while type E strains are typically isolated to Alaska and the Pacific Northwest (Arnon et al., 2001; Richardson et al., 2004).

Several clinical and epidemiological reports have evaluated the worldwide geographic distributions of human foodborne botulism cases. A review of 13 outbreaks between 1970 and 1984 identified geographic differences in the toxin serotypes associated with human foodborne botulism cases. Type B botulism was predominant in Portugal, Spain, France, and several other European countries (Lecour et al., 1988). Interestingly, the low mortality rate associated with human type B foodborne botulism (8.8%, versus 24.3% for type A and 30.8% for type E in the United States from 1950 to 1979) did not correlate with the high oral toxicity for type B toxin in mice (Ohishi et al., 1977; Sugii et al., 1977a–c). Serotype E was linked to botulism outbreaks in select regions such as the Baltic countries (Lecour et al., 1988) and typically resulted from the consumption of contaminated fish (Maselli, 1998).

Type F toxin was associated with only two reported outbreaks of human foodborne botulism prior to 1998 (Maselli, 1998). The first of these outbreaks occurred in Denmark (on the island of Langeland) and was attributed to a contaminated liver paste product (Richardson et al., 2004). The second outbreak, in 1966, affected three individuals in California and was associated with homemade venison jerky (Richardson et al., 2004). While a few other type F botulism cases have been reported, they are generally thought to have resulted from intestinal colonization and type F toxin production by another related species, *C. baratii* (Richardson et al., 2004). A recent report of a type F botulism case in California provided some additional insight into this uncommon toxin serotype and the associated clinical disease (Richardson et al., 2004). The patient described in this report presented with typical signs and symptoms, including ptosis, dysphagia, and weak extremities. Although the source of the ingested toxin was not conclusively determined, the exposure was tentatively linked to shellfish consumption, and type F toxin was subsequently detected in the patient's stool (Richardson et al., 2004). Human type F botulism cases may have been underreported in the past since some laboratories did not test culture isolates for the presence of *C. baratii*, which also produces type F toxin.

Type G toxin-producing clostridial organisms (*C. argentinense*) have been detected in several soil samples from a South American cornfield (Maselli, 1998), but only one reported outbreak of type G botulism (in Switzerland) has been identified in the published literature (Sonnabend et al., 1981). Certain aspects of this outbreak draw questions as to whether it was truly associated with type G intoxication. Type G organisms were isolated from all four affected adults and an 18-week-old infant, suggesting that the intoxications were due to ingestion and subsequent colonization by type G spores (Sonnabend et al., 1981). Type G toxin was detected at low levels of 2–7 mouse intraperitoneal lethal dose 50 ($MIPLD_{50}$)/mL in the serum of three out of the four lethally intoxicated adults, all of whom died suddenly sometime after the presumed foodborne intoxication. Type A toxin was also detected in two of these individuals, suggesting that the intoxications may have involved colonization either by a mixed set of clostridia or by a unique strain producing multiple toxins (Sonnabend et al., 1981). Alternatively, detection of dual serotypes could have been an artifact of the culture or testing methods. Soil samples taken from the area indicated the presence of only type A clostridial organisms (Sonnabend et al., 1981). Regardless, the occurrence of human type G botulism is rare, and the relative susceptibility of humans to colonization and intoxication from this serotype is not clear.

Species-specific patterns of susceptibility to different toxin types are common in both naturally occurring and

experimental foodborne botulism. These differences do not necessarily facilitate identification of the most appropriate animal models from the human condition, but they may help to eliminate highly variant species. For example, mink were reported to be relatively resistant to toxin types A, B, and E (Yndestad and Loftsgard, 1970), which are responsible for the vast majority of human botulism outbreaks (Maselli, 1998; Arnon et al., 2001). Weanling pigs, on the other hand, were shown to be moderately resistant to types A, C1, E, and F and highly resistant to type D toxin (Smith et al., 1971). Experimental and epidemiological studies have identified one persistent difference in the epidemiology of botulism in humans compared to many other animal species. Few reports citing human outbreaks of type C and D are available. One of these reports mentions two type C outbreaks and one type D outbreak in humans but provides no source for these cases (Lamanna, 1959). More recent reports of human type C or D botulism have not been found in the literature, and it is widely assumed that human foodborne intoxications are rarely, if ever, associated with these toxin types. In contrast, naturally occurring botulism of both types is quite common among domestic and wild animal species, and several studies have established the susceptibility of various laboratory species to experimental type C and D botulism.

The authors of this chapter have studied the effect of C and D toxin serotypes, as well as A, B, and E, on human intercostal muscle (Hilmas, unpublished data). All serotypes showed a similar ability to produce complete muscular paralysis in *ex vivo* human intercostal muscle. Intercostal muscle was excised from patients receiving a thoracotomy and intercostal muscle flap procedure. The muscle was removed tendon to tendon by surgical excision without electrocautery and dissected into multiple bundles with their associated intercostal nerves. The nerve–muscle units were placed in a vertical twitch bath and stimulated at 0.03 Hz (0.2-ms pulses of supramaximal strength) using a novel nerve clamp electrode to illicit an indirect muscle twitch. Potent toxins (1 nM) from various serotypes were added to the bath after confirming the stability of control muscle responses. In each case, twitch tensions declined to negligible amplitudes by 1 h after direct toxin application to the tissue bath.

Several nonhuman primate species are known to be susceptible to type C1 and D toxins both in nature and as experimental models. A large natural outbreak of type C botulism was reported in a troop of captive *hamadryas* baboons in 1989 (Lewis et al., 1990). The outbreak resulted in the deaths of 36 animals, including 3 adult males, 6 subadult males, 17 adult females, and 10 subadult females. Additional animals displayed mild to moderate symptoms that resolved themselves over a period of several days (Lewis et al., 1990). As with human foodborne botulism, various age groups and both sexes were affected, and no macroscopic lesions were apparent. Serum samples and gastric contents taken from ill animals contained type C1 toxin, although the source of the toxin was not identified (Lewis et al., 1990). The authors speculated that humans are probably also susceptible to type C1 toxin. The reason for the relative lack of human type C botulism cases remains unknown. It has been suggested that serotype C is often associated with carrion, providing a possible explanation for the absence of reported human cases. At least two other outbreaks of naturally occurring type C botulism in nonhuman primates were previously reported, one in squirrel monkeys (*Saimiri sciureus*) and capuchin monkeys (*Cebus capucinus* and *Cebus olivaceus*) (Smart et al., 1980) and the other in gibbons (*Hylobates lar*) (Smith et al., 1985).

In addition to nonhuman primates, most other animal species that show some sensitivity to botulinum intoxication are in fact susceptible to toxin serotypes C1 and D. Several rodent species are susceptible to oral intoxication with most botulinum toxins, including types C1 and D (Jemski, 1961a,b; Cardella et al., 1963; Sergeyeva, 1966; Sugiyama et al., 1974; Smith, 1986; Gelzleichter et al., 1999; Middlebrook and Franz, 2000). The majority of botulism outbreaks in cattle have also been attributed to toxins C1 and D. Cattle intoxication is typically associated with the ingestion of contaminated bones and other carcass remains; their apparent susceptibility to type C and D botulism might simply be due to frequent ingestion of decaying material that is primarily contaminated with these toxin types. A recent study indicated that cows are also uniquely sensitive to IV injection of type C1 toxin (Moeller et al., 2003). The high susceptibility of cattle to type C botulism is not dependent on exposure route, although the specific factors contributing to their sensitivity are not known.

A recent outbreak of type C botulism among farmed mink and foxes in Finland underscores the need to consider not only the quantitative susceptibility of various species to the toxins but also the potential epidemiological significance of interspecies differences in dietary patterns. Lindstrom et al. (2004) reviewed the Finland incident, which was the largest documented type C botulism outbreak in fur production animals. Over 52,000 animals developed illness after consumption of feed product that was contaminated with over 600 MLD of type C1 toxin per gram. This feed consisted of acidified slaughter by-products from poultry, beef, and fish (Lindstrom et al., 2004). According to national regulations, these by-products were acidified with an organic acid to yield a final pH of 4.0 or lower. Such processing would inhibit the growth of many microorganisms but would not necessarily result in significant toxin inactivation. Over 44,000 of the 52,000 affected animals died, and the death rate among all potentially exposed animals was almost 22% (Lindstrom et al., 2004).

The large number of animals affected and the high lethality associated with the outbreak could be considered indicative of the high susceptibility of the affected species to foodborne type C botulism. This high susceptibility might appear to be in stark contrast to that of humans due to the scarcity of type C cases in humans. However, the Finland outbreak provides a clear indication that dietary differences between species may play a significant role in these epidemiological patterns. Humans would be far less likely to consume slaughter by-products (including intestinal tissues), as opposed to higher-quality beef, poultry, and fish products. Moreover, the preparation of such products for human consumption would generally involve cooking rather than acidification. Thus, the influence of dietary habits must be taken into consideration when evaluating interspecies differences in epidemiological patterns for the various toxin serotypes.

It remains possible that humans generally do not consume the types of foods that are typically subject to contamination with type C1 and D toxins. Some researchers continue to speculate that humans are likely to be susceptible to both serotypes because they lead to botulism in monkeys, both in nature and after experimental oral exposure. Alternatively, humans might display a unique pattern of physiological susceptibility to the different toxin types. Lack of human susceptibility to type C1 and D intoxication could be attributed either to poor absorption of these specific toxins from the human gastrointestinal tract or to resistance of human cholinergic nerve terminals to the activity of these toxins. One cell culture study provided some support for the latter explanation. Type C1 neurotoxin was shown to bind with high efficiency to mouse neuroblastoma cells and to hybridomas of mouse neuroblastomas and rat gliomas, but not to human neuroblastoma cell lines (Yokosawa et al., 1989). Yokosawa et al. (1989) suggested that reduced binding of type C1 toxin to human versus mouse neuroblastoma cells could provide one explanation for the lack of type C botulism cases in humans.

Another potential explanation for the unique epidemiology of human botulism was provided in a study of botulinum toxin binding and transcytosis across polarized monolayers of two human colon carcinoma cell lines (T-84 and Caco-2). Substantial binding of iodinated BoNT/A and BoNT/B to human colon carcinoma cells was observed, while minimal binding of type C1 neurotoxin was detected (Maksymowych and Simpson, 1998). Both type A and B neurotoxins were also efficiently taken up, transcytosed, and released, by the polarized human carcinoma cells, whereas minimal transcytosis of type C1 neurotoxin was observed. The patterns of neurotoxin transcytosis (A and B, but not C1) observed in these human gut epithelial cell lines correlate with human susceptibility to foodborne botulism (Maksymowych and Simpson, 1998). The authors speculated that since human tissues are fully sensitive to the neuromuscular blocking properties of C1 neurotoxin (Coffield et al., 1997; Eleopra et al., 2004), the relative absence of human foodborne type C botulism could be due to the inability of this toxin to penetrate from the gut to the general circulation. Human susceptibility to type C1 and D neurotoxins remains unclear; however, clarification of this issue will be important in interpreting data derived both from *in vitro* studies on toxin transcytosis and from animal models for oral intoxication.

PATHOGENESIS

Overview of Pathogenesis

BoNTs are a group of immunologically distinct but closely related bacterial proteins that act as potent inhibitors of synaptic transmission in skeletal muscle. Inhibition of ACh release from the presynaptic terminal of the neuromuscular junction (NMJ) is thought to be the sole mechanism involved in the toxins' lethal action (Simpson, 1986) and therefore the cause of botulism. The pathogenesis of intoxication is not completely understood but is generally thought to involve a multistep process to interrupt the normal vesicular release of ACh from the presynaptic motor nerve terminal. In a process of transcytosis, ingested or inhaled BoNT must first cross a barrier (either intestinal or pulmonary epithelial cells) to gain access to the circulation (see intestinal absorption of BoNT; Figure 28.2). Once in the circulation, BoNT travels to its major target, the presynaptic membranes of alpha motor neurons at NMJs and neuroeffector junctions. Toxin binding through its HC to a cell surface receptor on the presynaptic motor nerve ending is followed by internalization via an endocytotic vesicle, acidification of the endosome, conformational change allowing cleavage of the enzymatically active LC from a bound HC, and release of LC toxin into the cytoplasm. Here, the LC cleaves one of the integral members of the SNARE complex (SNAP-25, VAMP, or syntaxin), proteins involved in exocytosis of ACh (Simpson, 1986, 2004; Black and Dolly, 1986; Blasi et al., 1993; Montecucco et al., 1994; Schiavo et al., 1995). BoNT thereby prevents docking of SVs with presynaptic plasma membrane by selective proteolysis of synaptic proteins. Each stage in BoNT action provides a potential point for pharmacological intervention.

Toxin Stability

Biological Stability of the Toxins in the Gastrointestinal Tract

A major factor to consider in botulism is the stability of both the organism and the toxin. A variety of

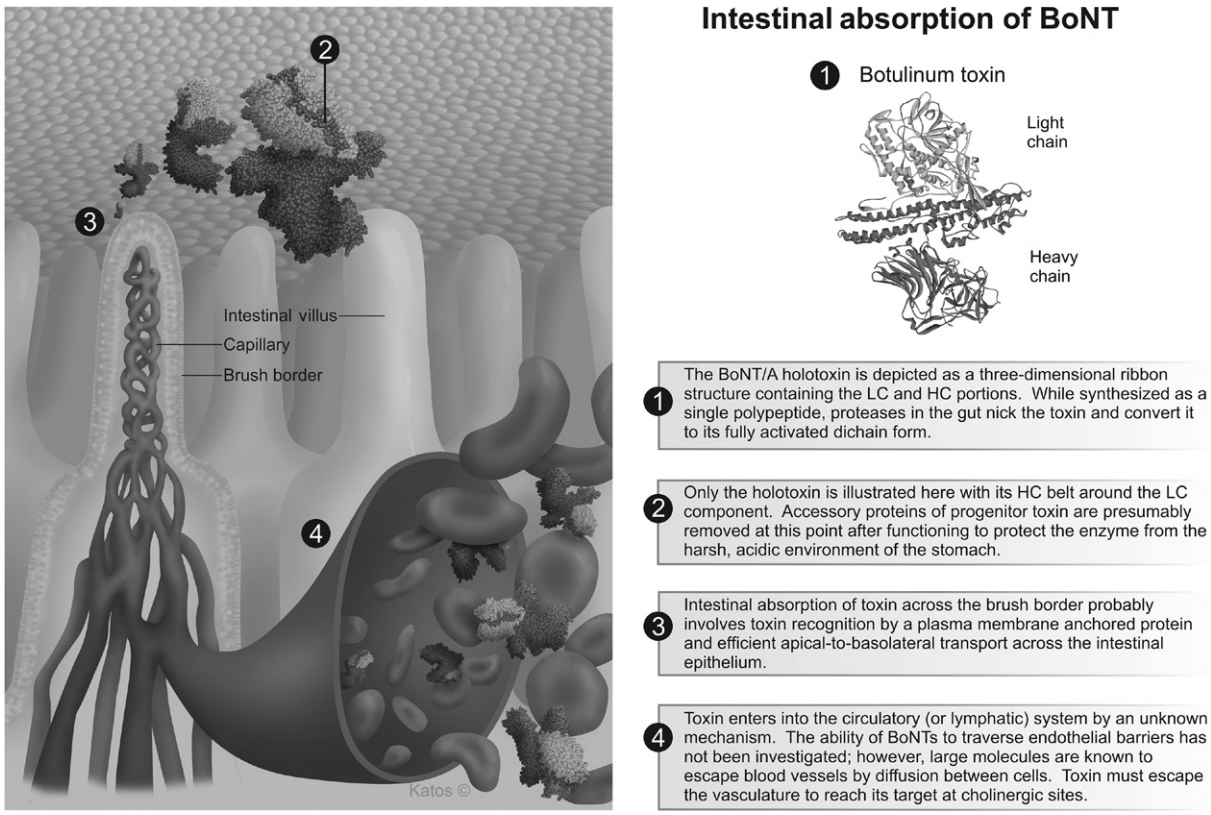

FIGURE 28.2 Intestinal absorption of BoNT. Neurotoxin present in the gut lumen as a result of foodborne (ingested toxin) or infant botulism (toxin synthesized by clostridial spores) must cross the epithelial membrane to reach the circulatory and lymphatic systems. The toxin presumably binds to an as-yet-unidentified receptor on the intestinal villus epithelium that is linked to an efficient transport process. Progenitor toxin contains nontoxic HA and NTNH accessory proteins, which are probably shed from the protein prior to entry across the intestine brush border. Progenitor toxin is thought to be too large for any significant rate of paracellular diffusion (Simpson, 2004). The ability of botulinum holotoxins without accessory proteins to traverse endothelial barriers has not been investigated; however, large molecules are known to escape blood vessels by diffusion between cells. Toxin escapes the circulatory system and reaches peripheral (and possibly central) cholinergic sites. These include NMJs, ganglia of the sympathetic and parasympathetic nervous system, postganglionic parasympathetic sites, and postganglionic sympathetic sites that release ACh. *Source: Illustrations are copyright-protected and printed with permission by Alexandre M. Katos.*

factors can affect the stability of ingested BoNTs within the gastrointestinal tract. The oral potency of the toxins is closely related to their ability to withstand the conditions found in these biological compartments prior to absorption into the lymphatics and general circulation. The stability of BoNT preparations, therefore, has been examined in the gastrointestinal compartments of intoxicated animals, as well as under different enzymatic and acidic conditions *in vitro*. Some of the earliest work on BoNT intoxication indicated that the stability and resulting potency of type A toxin vary both qualitatively and quantitatively in different rodent species (Minervin and Morgunov, 1941).

Several groups have evaluated the influence of ingested foods on the gastrointestinal stability and potency of BoNTs. Lamanna and Meyers (1959) reported that the ingestion of protein- or fat-containing foods prior to oral type A exposure in mice resulted in moderate increase in toxicity. The mechanisms by which food intake enhanced toxin potency were not clarified; however, the relatively small observed increases (twofold) could have been due to normal experimental variation in determining oral toxicity values. The same study demonstrated that fluorescein-labeled type A toxin was quickly destroyed in the stomachs of mice. Crystalline and purified toxins form stable complexes with albumin and other proteins found in food and serum (Lamanna and Meyers, 1959). Albumin was later shown to prevent loss of potency when type A toxin was exposed to a wide range of pH values (Zacks and Sheff, 1967). This observation was consistent with the more recent finding that the enzymatic activity of BoNT/A was enhanced in the presence of albumin (Schmidt and Bostian, 1997).

Subsequent studies expanded upon this early work by investigating the stability of other toxin types and preparations in solutions having different pH values and other conditions similar to those encountered in the gastrointestinal tract. Type C1 toxin was stable in most acidic and basic environments, as significant inactivation (as indicated by loss of toxicity in a mouse lethality assay)

was observed only following exposure to extreme pH values (i.e., pH 1.8 and pH 12) (Halouzka and Hubalek, 1992). Progenitor type E toxin was more stable than its derivative (purified) form, which was subject to rapid inactivation when exposed to pH values less than 4.0 (Sakaguchi and Sakaguchi, 1974). This study demonstrated that type E progenitor toxin dissociated either during or after gastrointestinal absorption in mice, as only the derivative component could be detected in the blood and lymph following oral administration of the progenitor form.

Similar findings on the relatively high stability of progenitor versus derivative forms of the other toxin serotypes have been reported in other studies. Types A, B, and F progenitor toxins were more stable under conditions of low pH, as well as more resistant to digestion by pepsin and papain, than their corresponding derivative toxins (Sugii et al., 1977a,b).

The derivative forms of toxins A and B were almost completely inactivated after 10 min of peptic digestion at pH 2.0, while the progenitor forms retained over 60% toxicity after an 80-min treatment (Sugii et al., 1977a). Crystalline type A toxin was shown to be partially resistant to proteolysis by trypsin, retaining 25% of the potency of control-treated toxin even after a 72-h trypsin digestion at 37°C (Coleman, 1954). The crystalline toxin was more readily inactivated by digestion with pepsin at pH 1.4 and chymotrypsin at pH 6.5. Interestingly, another group reported that the potency of type A toxin was weakened by 80% after a 5-h incubation in phosphate buffer (pH 7.5), while toxins C1 and D maintained 100% toxicity under the same conditions (Miyazaki and Sakaguchi, 1978). These findings demonstrate both serotype- and enzyme-dependent effects on the *in vitro* stability of the BoNTs that are also likely to affect their persistence in the gastrointestinal tract. A similar pattern of stability among the various BoNT forms in gastrointestinal juices isolated from different animal species has been demonstrated (Sugii et al., 1977a). The progenitor forms for all toxin serotypes retained significant toxicity in comparison to their derived holotoxin counterparts.

Epitope mapping experiments suggested that the nontoxic component of the intact progenitor toxin complex covers a large portion of the binding domain of the neurotoxin (Chen et al., 1998). Toxin interaction studies also revealed that the purified neurotoxin adheres to lipid monolayers while the progenitor complex is not subject to significant adsorption to the same monolayers. This observation led to speculation that the protective nontoxic components (HA and NTNH) may also facilitate progenitor toxin transit through the gastrointestinal tract by minimizing neurotoxin adherence to lipid membranes (Chen et al., 1998). On the other hand, toxicity studies suggest that adherence to lipid membranes is not critical for neurotoxin absorption since the intact progenitor complex is generally much more potent by the oral route than the purified neurotoxin. Moreover, the neurotoxin has been shown to protect the agglutination capacity of the associated nontoxic HA components within the progenitor toxin complex (Chen et al., 1998).

Importantly, the nontoxic HA components of the progenitor complex appear to protect the neurotoxin from proteolysis and degradation under pH extremes while the agglutinating activity of the nontoxic component is maintained by the presence of the neurotoxin. The type A progenitor toxin complex contains several HA components that might contribute to protecting the neurotoxin from degradation. One of these HA components, referred to here as *HA-33* (or *HA1* in some studies), was shown to interact directly with type A neurotoxin and to significantly increase its resistance to enzymatic proteolysis *in vitro* (Sharma and Singh, 2004). The authors of this work hypothesized that HA-33 provides protection against enzymatic degradation either by blocking the accessibility of protease-sensitive sites on BoNTs or by inducing structural changes within the neurotoxin itself.

Collectively, these studies offer important insight into the relative stability of the BoNTs within the gastrointestinal tract based upon their resistance to inactivation under various enzymatic conditions. In general, toxin stability directly correlates with the presence of the accessory HA and NTNH components of the multimeric progenitor toxin complex. These proteins appear to function in protecting the neurotoxin from degradation or inactivation. The various toxin serotypes also display unique resistances to enzymatic digestion although the basis for these differences is not known.

Oral Intoxication: Toxin Absorption from the Gastrointestinal Tract

Role of Progenitor Toxin Accessory Proteins

The role of the nontoxic accessory proteins within the progenitor toxin complexes is not fully understood. They appear to function in protecting the ingested toxins from degradation and in facilitating absorption from the gastrointestinal tract. Functional characterization of the HA and NTNH proteins has been advanced by biochemical techniques for generating toxin preparations containing only select components of the progenitor complex. These 7S toxins are relatively sensitive to proteolytic degradation and denaturation in the stomach (Schiavo et al., 1992). The auxiliary HA and NTNH proteins within the multimeric complex have been shown to dramatically increase the stability of the associated neurotoxin during transit through the gastrointestinal tract (Ohishi et al., 1977; Sugii et al., 1977a–c). The multimeric complex is then thought to readily dissociate either in the intestine or after absorption into the circulation.

Most studies suggest that the accessory proteins do not appear to have any involvement in the activity of the toxins at peripheral nerve terminals. Thus, the HA and NTNH components are likely to be dispensable in disease pathogenesis after parenteral or respiratory exposure, where the toxins bypass the harsh conditions of the gastrointestinal tract.

Role of Enterocytes

Both absorptive enterocytes and Peyer's patch-associated M cells have been implicated in toxin transcytosis from the gastrointestinal tract after oral exposure. Peyer's patches are collections of lymphoid tissue that are part of the gut-associated lymphoid tissue. M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. Park and Simpson (2003) indicate that knockout mice deficient in Peyer's patch-associated M cell complexes are still susceptible to both oral and respiratory botulinum intoxication and the development of HC-specific antibody responses. Based upon these results, the authors suggest that M cells are not likely to be involved in toxin uptake and processing from the respiratory tract. In addition, both cell types have comparable transcytosis rates (M cells are five times as efficient in transcytosis than intestinal enterocytes), but enterocytes greatly outnumber M cells; therefore, gastrointestinal enterocytes are the predominant cell types involved in toxin uptake and processing from the gastrointestinal tract.

Maksymowych and Simpson (1998) used transwell culture systems with various transformed epithelial cell lines to evaluate the fate of the HA components of type A progenitor toxin complex. Radiolabeled preparations of both BoNT/A and HA were taken up by cultured T-84 human colon carcinoma cells by bulk endocytosis. However, efficient delivery across the T-84 cells was observed only for the neurotoxin.

Respiratory Intoxication

The potential threat posed by aerosolized botulinum toxins is emphasized by their ease of production, their extremely high potency relative to other biological toxins, and their use in various weaponization programs over the past several decades (Arnon et al., 2001). This threat, along with the relative lack of information on respiratory toxicity and pathogenesis in humans, has fueled research on inhalational botulism in several animal models, including mice, rabbits, guinea pigs, mongrel dogs, and rhesus monkeys.

Toxin Absorption from Respiratory Tract

The relative persistence and absorption of the toxins following experimental respiratory exposure have been investigated in a few animal species. An early literature review suggests that type A toxin is more potent in mice by the respiratory route than by subcutaneous (s.c.) administration but less potent by the intraperitoneal (i.p.) route (Morton, 1961).

Guinea pigs were shown to be highly sensitive to inhaled botulinum toxins when compared to other rodent species. Respiratory penetration and retention of inhaled toxin are higher in guinea pigs than mice (Lamanna, 1961). Toxin could be detected in the lungs of guinea pigs after intranasal (i.n.) administration of only two mouse lethal doses of type E toxin, although detection in the blood or liver required higher doses (Sergeyeva, 1962, 1966). Guinea pigs were also reportedly more susceptible than mice to type A toxin by inhalation because shorter incubation periods were observed in guinea pigs prior to the onset of acute disease (Iakovlev, 1958).

Although inhalational botulinum intoxication was investigated in other animal species, these studies have not provided specific data on toxin absorption. The behavior of BoNTs in the respiratory tract was only recently investigated. Park and Simpson (2003) studied the properties of pure BoNT/A neurotoxin both *in vivo* and *in vitro* using mice and pulmonary cell culture models, respectively. Mean survival times were compared in mice receiving various doses of pure BoNT/A either i.n. or i.p. Pure BoNT/A was found to be a potent i.n. poison, although the toxicity (as determined by mean survival time) associated with i.p. administration was somewhat higher. Mean survival times in mice were less than 100 (i.p.) or 600 min (i.n.) after administration of 0.1 μg pure toxin; 75 (i.p.) or 400 min (i.n.) for 1 μg toxin; and 120 min (i.n.) for 10 μg toxin (Park and Simpson, 2003). As seen with oral and parenteral routes, a linear relationship existed between the log of the intranasal dose administered and the geometric mean survival time. The HA and NTNH component of the progenitor toxin did not enhance toxicity, establishing different requirements for the stability and absorption of inhaled versus ingested toxin (Park and Simpson, 2003).

Transwell experiments were also performed to investigate BoNT/A transcytosis across a human pulmonary adenocarcinoma cell line (Calu-3), the MDCK cell line, and a primary rat alveolar epithelial cell line (Park and Simpson, 2003). Efficient BoNT/A transcytosis in both directions across polarized Calu-3 monolayers was observed, while toxin transcytosis occurred at a much lower rate across MDCK cells. These findings were in agreement with previous work demonstrating that the efficiency of BoNT/A transcytosis across MDCK monolayers was much lower than that observed across gut epithelial cells (Maksymowych and Simpson, 1998). BoNT/A transcytosis was also observed across primary rat alveolar cells, although at a slightly slower rate than that seen for the human adenocarcinoma cells (Park

and Simpson, 2003). While the LC of BoNT/A was not essential for transcytosis, HC apical–basolateral (A–B) and basolateral–apical (B–A) transcytosis rates were somewhat lower than those of intact BoNT/A for both Calu-3 cells (HC 53% lower than BoNT/A for A→B; 45% lower for B→A) and rat alveolar cells (HC 62% lower for A→B; 17% lower for B→A). The transcytosis process was shown to involve an active-energy-dependent mechanism and was significantly inhibited by toxin preincubation with immune serum (Park and Simpson, 2003).

An important caveat to consider when evaluating the relevance of these *in vitro* studies is the use of pulmonary adenocarcinoma and alveolar epithelial cell lines in modeling respiratory absorption. It is generally believed that systemic absorption of inhaled particles is more likely to occur within the distal regions of the respiratory tract; therefore, particles must pass through thinner membranes in the deep lung and are less susceptible to nonabsorptive particle clearance (Lamanna, 1961). Some potential also exists for significant particle absorption from the nasopharyngeal and tracheobronchial regions of the respiratory tract; the cell lines utilized in these *in vitro* studies clearly do not account for this absorption potential. Importantly, investigators in the field have recently sought to characterize the specific cell types involved in toxin absorption from the respiratory tract.

M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. The studies of Park and Simpson (2003) indicate that M cells are not the major players in transepithelial transport of toxin across the respiratory epithelium. Additional studies directly investigating the absorption of inhaled BoNTs do not exist.

Toxin Binding and Uptake into Target Tissues

The remaining steps of BoNT pathogenesis following neurotoxin absorption are thought to be similar for both the respiratory and gastrointestinal exposure routes. After oral or inhalational exposure, the neurotoxins are absorbed from the gut or respiratory tract, respectively, into the lymphatics and circulation for transport to peripheral cholinergic synapses (Simpson, 2004). Figure 28.3 illustrates the NMJ, a major target for the actions of BoNTs. BoNTs are taken up presynaptically at the endplate region of NMJs (Verderio et al., 1999) and at other cholinergic synapses. Toxin binding involves high-affinity presynaptic receptors. These receptors have recently been identified as a combination of polysialogangliosides, SV protein 2 (SV2), and synaptotagmin (Verderio et al., 2006). Each serotype displays an affinity for a unique combination of receptors. For example, BoNT/B recognizes synaptotagmin II (and I) and ganglioside lipids (Dong et al., 2003) (see Figure 30.3). BoNT/A involves recognition of SV2C, SV2A, and SV2B (Dong et al., 2003); binding to SV2C also involves a lipid. After toxin binding, the complex is internalized by what is believed to be a clathrin-mediated endocytotic process.

TOXICOKINETICS

The onset of symptoms in botulism depends upon the amount of toxin ingested or inhaled and the related kinetics of absorption. Time to onset can range from as short as 2 h to as long as 8 days, although symptoms typically appear between 12 and 72 h after consumption of toxin-contaminated food (Lecour et al., 1988; Arnon et al., 2001). In a review of 13 foodborne botulism outbreaks involving 50 patients from 1970 to 1984, the incubation period ranged from 10 h to 6 days (Lecour et al., 1988).

Foodborne Toxicity

Toxin Persistence in Circulation and Transit to Target Tissues

Case reports of human foodborne botulism incidents offer some information on toxin persistence and transit after oral exposure in humans. Koenig et al. (1964) reported that circulating toxin was detected in five out of six patients suffering from type E botulism after consuming contaminated fish by the mouse lethality assay on serum samples collected from the patients from 1 to 10 days after foodborne exposure. Serum from one of the patients who rapidly succumbed to disease contained approximately 8 MIPLD$_{50}$/mL; extrapolation of this value yields an estimate that 20,000–24,000 human LD$_{50}$s were in this individual's circulation (Koenig et al., 1964). The toxin isolated from the serum of these clinically ill patients was not further activated *in vitro* by trypsin treatment. This observation was in agreement with other studies demonstrating cleavage of the single-chain prototoxin to the active dichain form within the gastrointestinal tract. Importantly, circulating toxin was not detected in a patient with minimal disease 11 days after ingestion of contaminated food (Koenig et al., 1964). Six out of seven individuals who had consumed the contaminated fish but did not develop clinical illness also lacked circulating toxin. Circulating toxin, therefore, was detected much more consistently in symptomatic patients associated with this outbreak than in subjects who were unaffected after toxin ingestion (Koenig et al., 1964).

Koenig et al. (1964) also reviewed previously published literature on the detection of circulating toxin in

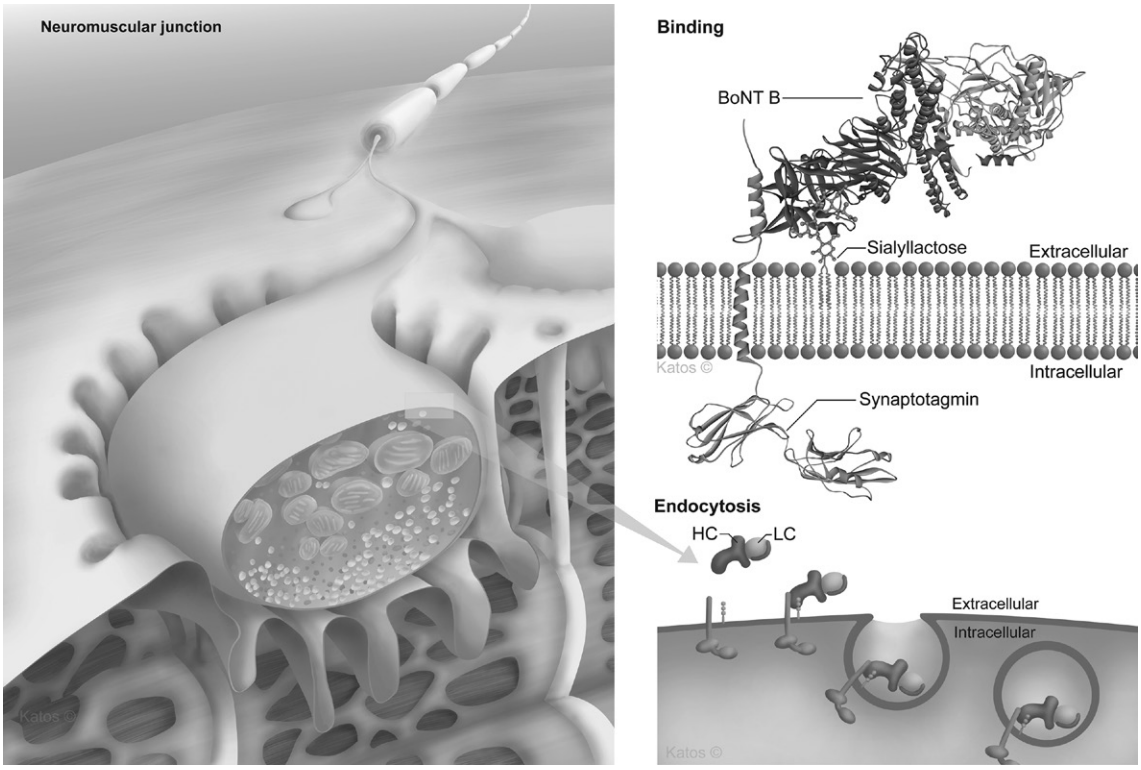

FIGURE 28.3 Toxin binding and internalization at the NMJ. Left panel: A mammalian NMJ is illustrated with the alpha motor neuron innervating skeletal muscle at specialized junctional folds in the membrane (Couteaux, 1973). Invaginations of the T system are also illustrated at the level of transition between the A and I bands. The axon loses its myelin sheath and dilates to establish irregular contact with the muscle fiber. Muscle contraction begins with the release of ACh from SVs (tiny spheres) at the motor endplate region. ACh binds to postsynaptic, muscle-type nicotinic ACh receptors and causes an increase in the permeability of the sarcolemma. This process is propagated to the rest of the sarcolemma and ultimately to the sarcoplasmic reticulum (SR) by the T system. An increase in SR permeability liberates calcium ion (Ca^{2+}) stores, resulting in the sliding of illustrated muscle filaments and muscle contraction. BoNTs bind and internalize at the presynaptic side of the NMJ (Verderio et al., 1999). Release of BoNTs into the cytosol results in inhibition of ACh release and flaccid paralysis of innervated muscle. Top-right panel (Toxin binding): A three-dimensional ribbon structure of BoNT/B is illustrated. The receptor-binding domain of BoNT/B HC binds to synaptotagmin (Syt-II) and ganglioside (sialyllactose) receptors of the presynaptic motor endplate. Each BoNT serotype binds to a different set of receptors in the membrane (Verderio et al., 2006). Bottom-right panel (endocytosis): Receptor-mediated endocytosis of BoNT/B holotoxin is illustrated in this panel. The remaining steps in BoNT toxicity involve acidification of the endosome, pH-induced conformational change in the toxin, translocation of BoNT LC across the endosomal membrane with the aid of BoNT HC, and proteolytic degradation of target SNARE proteins by LC. *Source: Illustrations are copyright-protected and printed with permission by Alexandre M. Katos.*

botulism patients. Circulating toxin (primarily serotype B) had been detected in select patients from 2 to 25 days after consumption of contaminated food and was rarely detected in type A botulism patients (Koenig et al., 1964). The authors suggested that serotype-specific differences in the persistence of circulating toxin might be attributed to their unique avidities to target tissues. Circulating toxin is generally detected only at very low levels at or immediately prior to death in lethally intoxicated patients (Ono et al., 1970).

Efforts have been made to determine the kinetics of the accessory components of the progenitor toxin complexes after systemic absorption of BoNTs. Iida et al. (1970) found that circulating type E toxin was shown to exist in the 7S form after oral administration of the progenitor toxin to rabbits, suggesting that the larger toxin complex dissociated at some point during or after absorption from the gastrointestinal tract. Similar findings were reported on the absorption and persistence of progenitor type A toxin in the rat; the mean sedimentation value of toxin in the lymph after intraduodenal instillation was 7.9S, significantly lower than that of the crystalline toxin (Heckly et al., 1960).

Inhalation Toxicity

Toxin Persistence in Circulation and Transit to Target Tissues

Very limited data are available on the persistence of BoNTs in circulation following inhalation exposure in any animal species. These data indicate that circulating toxin can be detected soon after exposure but is

subsequently cleared rapidly from the circulation. Park and Simpson (2003) reported on the time course for appearance (and amount) of either purified BoNT/A or type A HC in the circulation of mice after intranasal exposure. Maximum serum levels were observed at 2 h postexposure for both proteins although the peak values were higher for BoNT/A than for HC. Rapid clearance was observed over the next few hours.

An earlier study showed that type A toxin could be detected primarily in the lungs and liver rather than the serum of guinea pigs after i.n. exposure (Sergeyeva, 1962). The same group reported on the correlation between administered toxin dose and detection of toxin in the blood, lungs, and liver of guinea pigs intoxicated via the i.n. route (Sergeyeva, 1966). Type E toxin was detected in the lungs of guinea pigs after i.n. administration of two lethal doses, while toxin appeared in the blood or liver only following i.n. administration of at least five lethal doses. The organ distribution patterns were similar in guinea pigs after inhalation exposure to types A, B, or C toxins (Sergeyeva, 1966). These studies did not address the potential for persistent toxin detection in the lymph after inhalational intoxication, despite the fact that other routes of exposure result in significant absorption into the lymphatics.

While scant literature is available on persistence and distribution after inhalation exposure, several studies have evaluated the systemic behavior of parenterally administered toxins. One group investigated toxin persistence in serum and tissue distribution in white mice following IV administration of 1,000 lethal doses of ^{35}S-labeled type B toxin (Pak and Bulatova, 1962). Mice were sacrificed at 20, 60, and 150 min after toxin administration, and blood and tissues were harvested for toxin distribution analysis. These mice showed symptoms of severe intoxication, including atypical breathing patterns and paralysis, at 150 min postexposure. Toxin levels (as determined by radioactivity) were highest in the lung 20 min after toxin injection, followed by the liver, heart, kidneys, intestines, and brain (Pak and Bulatova, 1962). Radioactivity levels in the blood, as well as the liver, heart, intestines, and brain, were further reduced after 60 min post-toxin injection. Serum toxin concentrations were lower than those detected in any other tissue at all times (Pak and Bulatova, 1962). The authors concluded that the toxin rapidly escaped from blood to various other tissues, suggesting the capacity for unimpeded passage of the toxin through the vasculature and cellular membranes.

Somewhat slower kinetics for toxin clearance from the circulation were observed in dogs following parenteral [IV, i.p., or intramuscular (i.m.)] exposure to type A toxin (House et al., 1964). Serum toxin persistence was evaluated in mongrel dogs receiving 8,000–10,000 mouse units/kg of type A toxin. Peak serum toxin levels were detected 5 h after i.p. administration (13% of injected dose), 12 h after i.m. administration (9% of the injected dose), and within only 3 min after IV administration (79% of the injected dose) (House et al., 1964). The relative clearance kinetics were slower after i.m. and i.p. exposure than for IV administration, as serum toxin levels were identical 22 h after injection via all three rounds (approximately 6% of the injected dose). Some serum toxin activity could be detected by the mouse lethality assay for 2–4 days after parenteral administration. Serum toxin patterns were also evaluated in rhesus monkeys following IV administration of type A toxin (Stookey et al., 1965). Serum toxin levels dropped by about 50% of maximum within 16–24 h after IV injection, and previous exposure did not affect toxin clearance rates after the administration of subsequent doses.

Another study investigated circulating toxin levels in weanling pigs (5–12 weeks old) following parenteral administration of toxin types A, B, C1, and D (Smith et al., 1971). Toxin was cleared from circulation less than 24 h after IV injection of type B (560 $MIPLD_{50}$/kg), type C1 (5,000 $MIPLD_{50}$/kg), or type D (60,000 $MIPLD_{50}$/kg) toxin. In contrast, toxin could consistently be detected in the serum over the entire 4-day period prior to death in pigs injected with serotype A (21,400 $MIPLD_{50}$/kg) (Smith et al., 1971). Serum toxin levels were 100 MIP_{50}/mL at 24 and 48 h after injection of type A toxin, 30 $MIPLD_{50}$/mL after 3 days, and 10 $MIPLD_{50}$/mL after 4 days. These findings indicated serotype-specific differences in the persistence of circulating BoNTs, at least in systemically intoxicated pigs.

Although these studies provide some insight into the persistence of circulating toxin after parenteral administration, they do not necessarily reflect the behavior of absorbed toxin after respiratory exposure. The route of administration may not have a significant impact on the behavior of toxin once absorbed into the serum and lymph, but the patterns and kinetics of absorption into the circulation might be quite different after inhalational versus parenteral exposure. Respiratory exposure could lead to a different proportion of toxin taken up into the circulation, lymphatics, or both over a given time period than that seen after systemic injection. Such discrepancies might affect both the quantitative persistence of circulating toxin and its transit to peripheral target tissues. At this point, information is not yet available on the homing and distribution of toxins to target nerve tissues. The available literature also provides no insight on the mechanisms by which toxin is removed from the circulation, either through extravasation and uptake in target tissues or by metabolic processes. In the future, such data will be important in characterizing the pathogenesis of botulism after respiratory intoxication and other routes of exposure.

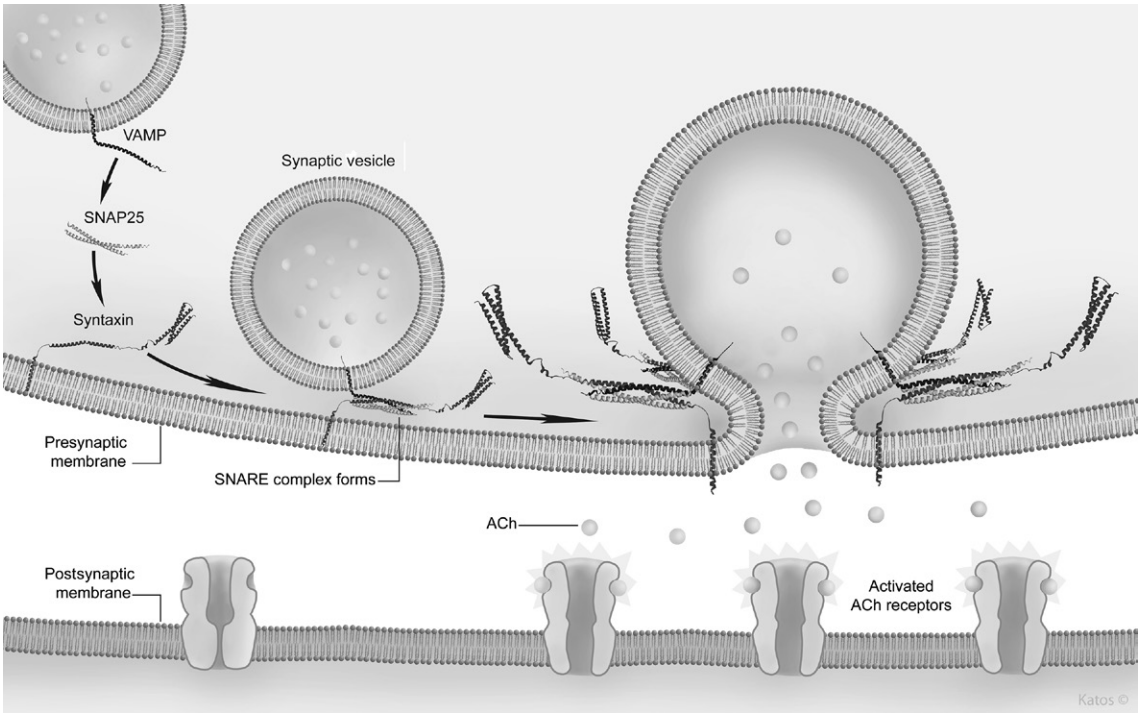

FIGURE 28.4 Neuromuscular transmission in the absence of BoNT. A nerve impulse is transmitted to the effector (muscle) cell by neurotransmitter liberated at the synapse. When the action potential arrives at the axonal terminus to depolarize the presynaptic membrane, Ca^{2+} ions enter through voltage-dependent Ca^{2+} channels. Ca^{2+} ions facilitate the fusion of SVs, containing the neurotransmitter ACh, with the presynaptic membrane. Three SNARE proteins (syntaxin, synaptobrevin, and SNAP-25) are critical for SV fusion. So long as the SNARE complex is intact, ACh releases by exocytosis, diffuses across the synaptic cleft, and binds to postsynaptic muscle-type nicotinic ACh receptors. Binding of ACh makes the sarcolemma of the muscle cell more permeable to sodium, which results in membrane depolarization. Excess ACh is hydrolyzed by the enzyme cholinesterase bound to the synaptic cleft basal lamina. ACh breakdown is necessary to avoid prolonged activation of ACh receptors. *Source: Illustrations are copyright-protected and printed with permission by Alexandre M. Katos.*

MECHANISM OF ACTION

ACh release from presynaptic vesicles depends upon a propagated action potential, localized depolarization at the presynaptic motor endplate, proper SNARE complex formation (i.e. SNAP-25, syntaxin, and synaptobrevin), and SV docking with the presynaptic membrane (see neuromuscular transmission in the absence of BoNT; Figure 28.4). Regardless of the exposure route, BoNTs lead to inhibition in the release of ACh from peripheral cholinergic nerve terminals resulting in flaccid paralysis (Simpson, 1986; see Figure 28.5). The specific target for BoNT/A and /E is the 25-kDa vesicle-docking protein SNAP-25; BoNT/A cleaves the last 9 residues, whereas BoNT/E cleaves a larger 26-residue fragment from the C-terminus of this protein (Blasi et al., 1993; Montecucco et al., 1994). The target of BoNT/B is the small transmembrane protein synaptobrevin/VAMP located on the surface of small SVs (Schiavo et al., 1995). The enzymatically active portion of the 150-kDa BoNT is the 50-kDa LC; the role of the 100-kDa HC involves binding to cholinergic nerve endings and intracellular penetration via receptor-mediated endocytosis (Simpson, 1986, 2004; Montecucco et al., 1994).

Heavy Chain

The HC of the BoNTs has been shown to mediate toxin binding and internalization at cholinergic nerve terminals (Daniels-Holgate and Dolly, 1996; Simpson, 2004). The mostly β-strand-containing carboxy-terminus of the HC appears to be directly involved in toxin binding, while the mostly α-helical amino-terminal region mediates translocation across the endosomal membrane (Simpson, 2004). Through mechanisms that have yet to be fully characterized, the toxins gain entry into the nerve terminal through receptor-mediated endocytosis followed by pH-induced translocation from the endosome to the cytosol. The ability of the Hn region to form transmembrane ion channels raises the possibility that they are intimately involved in translocating the toxic moiety into the cytoplasm (Koriazova and Montal, 2003). Once translocated into the cytosol, the toxic fragments exert their paralytic effects by inhibiting ACh

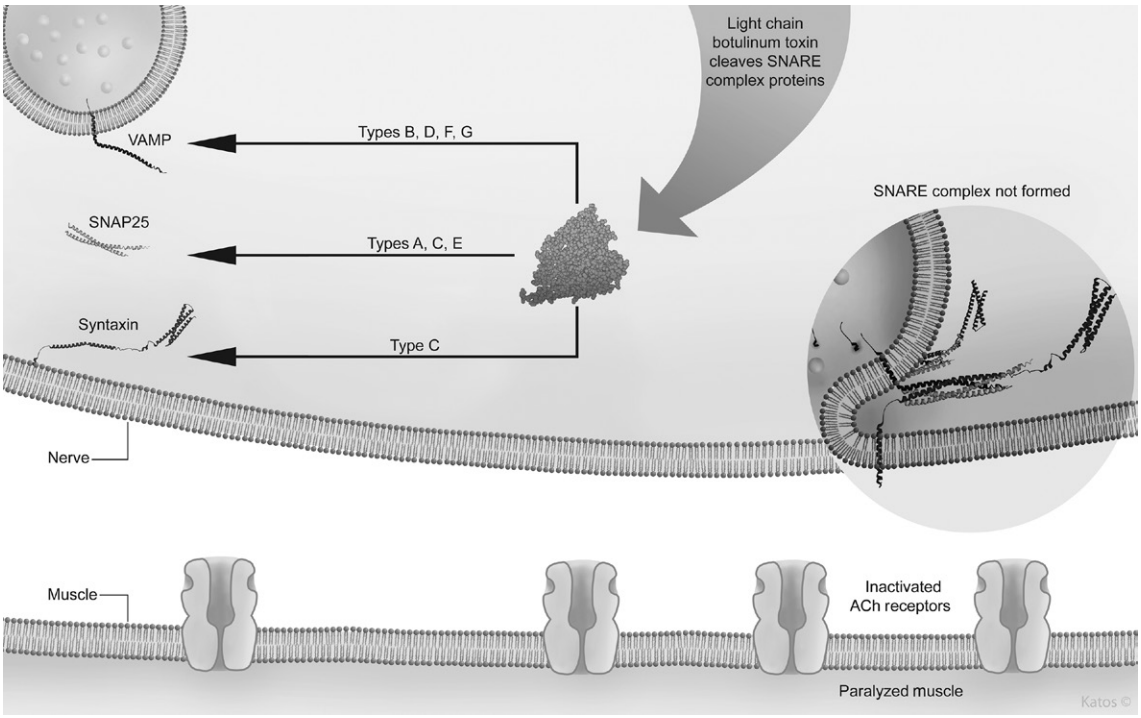

FIGURE 28.5 Neuromuscular transmission in the presence of BoNT. The catalytic LCs of the various serotypes cleave specific SNARE proteins. The SNARE complex does not form if any of the proteins are cleaved. In the presence of BoNT LC inside the axonal terminus, SVs will not fuse with the presynaptic membrane, ACh will not be released, and the muscle will not contract, resulting in paralysis at the NMJ. *Source: Illustrations are copyright-protected and printed with permission by Alexandre M. Katos.*

release from NMJs as well as other peripheral cholinergic sites, including sympathetic and parasympathetic ganglia and postganglionic parasympathetic synapses (Lamanna, 1959; Simpson, 2004).

Light Chain

These paralytic effects have been attributed to the proteolytic activity of BoNT LC on protein substrates required for vesicular exocytosis. BoNT LC inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various neurotoxin serotypes possess distinct molecular targets within the peripheral cholinergic nerve terminals (Schiavo et al., 1992, 1993a,b, 1995). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects.

The intracellular proteins SNAP-25, syntaxin, and synaptobrevin (or VAMPs) normally interact with each other in mediating neurotransmitter release from cholinergic and other nerve terminals (see Figure 28.4). Toxin types B, D, F, and G cleave the VAMPs, while types A, C1, and E act on SNAP-25; type C1 toxin also cleaves syntaxin (Dong et al., 2003). The functions of the various neurotoxins are even more specialized, in that one toxin type can cleave its substrate at a different site than that targeted by other toxin serotypes. For example, BoNT/A cleaves SNAP-25 between residues 197 and 198, resulting in the loss of 9 amino acids, while BoNT/E cleaves the same protein between residues 180 and 181, thereby removing 26 amino acids (Schiavo et al., 1993a).

Although the LCs of both BoNT/A and /E target SNAP-25, these two serotypes exert significantly different potencies and paralytic profiles in cultured neurons and *in vivo*. A potential molecular basis for this discrepancy was established by the finding that these neurotoxins target different cleavage sites within the SNAP-25 protein. BoNT/A cleavage generates a 197-residue truncated protein (P197) by cleaving the last 9 amino acids from the C-terminus of SNAP-25 while BoNT/E cleavage produces a 180-residue species (P180) by removing the final 26 residues (Schiavo et al., 1993a). A series of studies by Keller et al. (1999) and Keller and Neale (2001) provided additional insight into the molecular mechanisms associated with the potent and persistent action of type A neurotoxin relative to type E.

Serotype-specific cleavage events provide insights into the differential activities of the toxins at nerve terminals. In some cases, substrate cleavage studies also revealed important information regarding interspecies differences in the activity of certain toxins. BoNT/B was shown to block neuromuscular transmission by cleaving VAMP proteins between residues Q76 and F77 in humans and mice (Bakry et al., 1997). However, the rat

VAMP1 (synaptobrevin) protein sequence differs at this critical cleavage site, in that the glutamine at position 76 is replaced by a valine, rendering the region more resistant to proteolysis by BoNT/B (Bakry et al., 1997; Verderio et al., 2006). On the other hand, rats and mice were shown to have similar susceptibilities (body weight adjusted) to i.m. injection of type A toxin; rats have also been shown to be much more resistant than mice to type F toxin (Kauffman et al., 1985).

The specific paralytic profiles associated with each of the BoNTs are typically attributed to their unique proteolytic activities within the nerve terminal. These activities are known to be mediated by the LC components of the various neurotoxins. The various nontoxic components within the multimeric progenitor toxin complexes have traditionally been considered accessory proteins that primarily function to increase neurotoxin stability and, in some cases, to facilitate absorption. Yet studies over the past few years have suggested a potential role for the HA constituents in enhancing the endopeptidase activity of the LC (Cai et al., 1999; Sharma and Singh, 2004). It is widely believed that pure BoNT/A requires either proteolytic nicking or chemical reduction for significant SNAP-25 cleavage activity. However, new evidence suggests that the type A progenitor toxin complex is apparently highly active even in nonreduced form (Cai et al., 1999). Further research is needed to substantiate this preliminary work and to establish a more detailed understanding of the prerequisites for LC proteolytic activity.

A recent study by Sharma and Singh (2004) provided additional support for the expanded roles of at least one neurotoxin-associated protein within the type A progenitor complex. The HA-33 component, representing 25% of the accessory protein content of progenitor neurotoxin, significantly increases the proteolytic activity of both BoNT/A and /E *in vitro* and in rat synaptosome preparations. The addition of HA-33 to nonreduced BoNT/A leads to a 21-fold increase in GST-SNAP-25 fusion protein cleavage activity *in vitro* and a 13-fold enhancement of endopeptidase activity in rat synaptosomes (Sharma and Singh, 2004). Similar enhancement of proteolytic activity was seen when HA-33 was added to BoNT/E both *in vitro* and in rat brain synaptosomes.

The enhancement of SNAP-25 cleavage activity by HA-33 in rat brain synaptosomes was taken as evidence that the neurotoxin and the accessory protein both gain entry to the nerve terminal (Sharma and Singh, 2004). The possibility that an accessory component of the progenitor toxin complex could exert direct effects on LC endopeptidase activity within the nerve terminal could have important implications with respect to neurotoxin function *in vivo*.

Two recent reports revealed additional layers of complexity regarding the mechanisms involved in the distinct durations of action associated with the different toxin serotypes. Fernández-Salas et al. (2004) investigated the subcellular localization of BoNT/A, /B, and /E LC-GFP fusion proteins following overexpression in several different mammalian cell lines. The LC/A fusion protein was shown to localize within discrete plasma membrane compartments in both neuronal (PC12) and nonneuronal (HEK293, HeLa, and HIT-T15) cell lines, while LC/B was detected throughout the cell and LC/E was primarily found within the cytosol.

TOXICITY

Lethality

BoNTs are the most potent substances known to humans. A comparison of the lethal nature of BoNTs in relation to other toxic chemicals and substances discussed throughout this book is provided in Table 28.2. The toxicity associated with oral exposure of a given species to BoNTs is significantly lower than that resulting from parenteral administration (see Table 28.3). The

TABLE 28.2 Comparison of Toxic Chemicals and Substances

Chemical or Toxin	Mouse IV LD_{50}[a] (mg/kg)	Chemical or Toxin	Mouse IV LD_{50}[a] (mg/kg)
Botulinum toxin	0.00001	Strychnine	0.41
Batrachotoxin	0.002	Potassium cyanide	2.60
Anthrax lethal toxin	0.003–0.005	Mustard[b]	3.30
Ricin	0.005	Aflatoxin	9.5
Tetrodotoxin	0.01	Heroin	21.8
Saxitoxin	0.01	CR[c]	37
VX	0.012	Marijuana[d]	42
Abrin	0.02	BZ[e]	46
GD	0.066	CS[f]	48
GB	0.10	Caffeine[g]	62
GA	0.15	CN	81
TCDD[h]	0.182	Thujone[i] (absinthe)	134.2
Capsaicin	0.40	PAVA[j]	224

[a] *Intravenous dose that is lethal to 50% of mice.*
[b] *Mustard gas, 1,1′-thiobis[2-chloroethane].*
[c] *Riot control agent [dibenz-(b,f)-1,4-oxazepine].*
[d] *Delta-3,4-trans-tetrahydrocannabinol.*
[e] *Incapacitating agent, 3-quinuclidinyl benzilate.*
[f] *Riot control agent (o-chlorobenzylidene malononitrile).*
[g] *Riot control agent (chloroacetophenone).*
[h] *2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin (TCDD), a contaminant of the defoliant and herbicide Agent Orange.*
[i] *Constituent of wormwood absinthe, a popular emerald liquor, 4-methyl-1-(1 methylethyl) bicycle [3.1.0] hexan-3-one.*
[j] *Riot control agent (pelargonic acid vanillylamide).*

TABLE 28.3 Comparison of the Lethality of Serotypes A–G by Various Routes of Administration in the Guinea Pig[a]

Route of Intoxication	Botulinum Toxin Serotype						
	A	B	C	D	E	F	G
Oral	717	306	177	436	–	–	–
i.p.[b]	3.1[c]–5.2[d]	4.2[d]–6.5[c]	1.6[d]–3.2[c]	3.0[e]–6.4[c]	34.3[d]–78[e]	–	40–100[f]
i.m.[g]	4.3[c]	6.9[c]	3.1[c]	8.7[c]	102[c]	–	–
SC[h]	6[i]–30[j]	–	–	3[k]	100[l]	30–30[k]	–
Aerosol	141	350	87	186	778	–	–

[a]*The doses are normalized to mouse i.p. LD_{50} units.*
[b]*Intraperitoneal administration.*
[c]*Gelzleichter et al. (1998a).*
[d]*Cardella et al. (1963).*
[e]*Lamanna (1961).*
[f]*Cicarelli et al. (1977).*
[g]*Intramuscular administration.*
[h]*Subcutaneous administration.*
[i]*Morton (1961).*
[j]*Sergeyeva (1962).*
[k]*Dolman and Murakami (1961).*
[l]*Sergeyeva (1966).*

susceptibility of various animal species to parenteral intoxication does not provide adequate indication of their sensitivity to gastrointestinal exposure (Lamanna, 1961). The estimated human LD_{50} of approximately 1 ng/kg for parenteral botulinum intoxication is similar to that reported for most laboratory animals (Arnon, 1995; Middlebrook and Franz, 2000). In contrast, the relative susceptibilities of humans and other animal species to oral intoxication vary significantly (Morton, 1961). A recent clinical review of human botulism reported that the ingestion of as little as 0.05–0.1 μg of BoNT/A may be sufficient to cause death in humans (Cherington, 1998). Human lethal doses have also been extrapolated from primate studies, yielding an oral lethal dose of approximately 70 μg for crystalline type A toxin for a 70-kg human (Arnon et al., 2001). The lethal human respiratory dose is estimated to be 0.7–0.9 μg and the IV or i.m. dose is 0.05–0.15 μg (Middlebrook and Franz, 2000; Arnon et al., 2001).

Oral Toxicity

An earlier report suggested that humans are more susceptible than monkeys to type A toxin by the oral route based on toxin dose estimates in foodborne botulism case studies (Morton, 1961). This same study also summarized the susceptibility of numerous other animal species to oral botulinum intoxication. Mice, monkeys, and guinea pigs were considered highly susceptible, while chickens, rabbits, horses, dogs, rats, and cattle were classified as more resistant and ferrets, minks, and hogs were deemed resistant. The oral lethal doses of type A toxin in guinea pigs (1000–3000 MIPMLD) and monkeys (2000 MIPMLD) (Morton, 1961) are similar to the estimated oral lethal dose for humans (7000 MIPMLD).

Morton (1961) provided evidence for an estimated human oral lethal dose of much less than 3500 MIPMLD for type B toxin. This estimate was based on an earlier report describing a fatal type B human botulism case resulting from the ingestion of 3500 MIPMLD in toxin-contaminated cheese (Meyer and Eddie, 1951). A man weighing 104 kg consumed approximately 70 g of contaminated cheese; repeated tests of the cheese indicated that it contained only 50 MLD/g of type B toxin. The patient first developed somewhat atypical disease symptoms of nausea, vomiting, diplopia, dysphagia, phagodynia, and instability within 7 h of exposure (Meyer and Eddie, 1951). The man was later hospitalized and developed symptoms more characteristic of foodborne botulism within 18–20 h. He died 57 h after toxin ingestion despite receiving 35,000 units each of both type A and B antitoxins (Meyer and Eddie, 1951). Therefore, it was determined that 3500 MIPMLD is much greater than the minimum lethal dose of type B toxin for humans due to the rapid onset of illness and severity of disease (Morton, 1961). The same study reported a woman who died from botulism 42 h after consuming a small piece of toxin-contaminated pear.

Inhalation Toxicity

Naturally occurring botulism cases in humans and other animal species are almost exclusively associated

with the ingestion of toxin- or spore-contaminated foods. The level of knowledge in the published literature on toxin absorption following inhalational exposure is, therefore, much more limited than that associated with gastrointestinal intoxication. The potencies of inhaled BoNTs have been investigated in several experimental animal species. In a review of the early literature on this topic, Morton (1961) reported comparatively similar ratios (5.9:1) of oral to respiratory toxicity (the comparative lethal doses for toxin administered via the oral versus the respiratory route) for type A toxin in guinea pigs and mice. Iakovlev (1958) concluded that guinea pigs were more susceptible than mice to type A toxin by inhalation because they succumbed to intoxication after a shorter incubation period (1–2 days, versus 3–4 days for mice). Several technical reports established more specific guinea pig inhalation toxicity data for serotypes A through E (Jemski, 1960, 1961b).

Clinical Toxicity

The natural occurrence of human foodborne and infant botulism translates into a wealth of information on the clinical signs and symptoms of disease. This information can be compared to the array of physiological and pathological findings in various species of experimental animals after oral administration of BoNTs. The ability of inhaled BoNTs to produce illness has also been documented in humans and in several experimental species. The primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism; however, infants display a unique clinical picture of botulism. In addition, the various toxin serotypes are usually associated with analogous clinical presentations, with the most severe cases of foodborne botulism being caused by the ingestion of type A toxin.

Exposure to BoNT via oral or inhalational routes results in symptoms indicative of an inactive peripheral cholinergic system due to inhibition of ACh release from the nerve terminal. The time to onset of disease depends on the amount of toxin ingested and ranges from several hours to a few days after oral exposure (Lecour et al., 1988; Arnon et al., 2001). Prominent signs and symptoms of intoxication common to all serotypes and various routes of exposure include the following, in order of descending frequency: dysphagia, xerostomia, diplopia, dysarthria, fatigue, ptosis of the eyelids, constipation, arm weakness, leg weakness, gaze paralysis, blurred vision, diminished gag reflex, nausea, facial palsy, dyspnea, emesis, tongue weakness, sore throat, dizziness, dilated or fixed pupils, abdominal cramps, reduced or failed reflexes, nystagmus (involuntary rapid eye movement), diarrhea, ataxia, and paresthesia (reviewed by Arnon et al., 2001).

Foodborne Botulism

Human foodborne botulism presents as an acute, symmetric, flaccid paralysis that generally involves multiple cranial nerve (CN) palsies initially, termed *bulbar involvement.* Early symptoms involve paralysis of the motor components of the CNs. The motor components are derived from cell bodies located in the brain, with axons that exit the cranium to control muscles, glandular tissue, or specialized muscle in the heart and gastrointestinal tract. Paralysis by BoNTs leads to ptosis and dilated pupils (CN III); disconjugate gaze and blurred vision (CN III, IV, VI); facial droop or palsy (CN VII); dysphagia, dysarthria, and absence of gag reflex (CN IX, X); tongue weakness (CN XII); and weakness of neck strap muscles (CN XI). Botulism patients typically develop difficulty in seeing, speaking, or swallowing in the early phases of intoxication. As paralysis extends caudally, toxic signs and symptoms include loss of head control, hypotonia, generalized weakness, and flaccid paralysis or floppy appearance (infants and children). In infants and young children exposed to BoNT, floppy appearance and constipation may be the only presenting signs to warrant a diagnosis of BoNT exposure, since obtaining a reliable history may not be possible in this population.

Loss of the gag reflex and dysphagia may require intubation and mechanical ventilation. Deep tendon reflexes are often lost during later stages of intoxication, and death in untreated patients results from airway obstruction or inadequate tidal volume (Arnon et al., 2001). Respiratory failure is the most serious clinical manifestation of botulism, and the decline in mortality associated with foodborne botulism is primarily due to improvements in ventilatory support (Lecour et al., 1988). Around 60% of botulism patients in the United States require mechanical ventilation at some point during their hospitalization and treatment (Varma et al., 2004). In severe botulism cases (as in the previously mentioned case of the Florida physician involving research grade type A toxin instead of BOTOX for facial muscle paralysis), respiratory support may be required for prolonged periods of time, and autonomic dysfunction may persist for a period ranging from months to years (Mackle et al., 2001).

Other clinical forms of the disease share many of these signs and symptoms. The presentation and duration of the disease are coupled to the relative persistence of the toxin in blocking the release of ACh at peripheral nerve synapses. Although untreated botulism is potentially deadly, the availability of antiserum has dramatically reduced the mortality rates for the common clinical manifestations of the disease. Severe cases of foodborne botulism may still require ventilatory support for over a month, and neurological symptoms can sometimes persist for more than a year (Mackle et al., 2001).

Infant Botulism

The characteristic symptoms of infant botulism are poor sucking, constipation, generalized weakness, floppy appearance, and respiratory insufficiency (Cox and Hinkle, 2002). Infant botulism may quickly progress to respiratory failure if not treated. The development of the intestinal flora has been demonstrated to suppress the germination and growth of *Clostridium botulinum* spores in mice (Sugiyama and Mills, 1978). Ingestion of honey by infants is the classic scenario cited in infant botulism; therefore, honey is not recommended in this susceptible population (Arnon, 1998).

RISK ASSESSMENT

BoNTs present a very real threat to the public health and are the most toxic substances known to humans. In a military or bioterrorist incident, intoxication by BoNT is likely to occur by inhalation of aerosolized toxin or by ingestion of contaminated food or beverages (Franz, 1997; Sobel et al., 2004). Although the municipal water systems are considered to be safe from BoNT attacks, due to chlorination and dilution, it is not known whether current water treatments adequately decontaminate the toxin. Furthermore, bottled mineral water and milk (Sobel et al., 2004; Kalb et al., 2005) are obvious targets for terrorist groups. The vulnerability of the nation's milk supply was highlighted in a recent modeling study, where its complex distribution system would magnify the consequences of poisoning by BoNT (Kalb et al., 2005; Wein and Liu, 2005). BoNTs are a serious threat to the US national security due to their potency, remarkable stability, and persistence in the body.

Wein and Liu (2005) modeled a bioterror attack using BoNTs on the nation's milk supply. Modeling of toxins for dispersal into a liquid medium has been previously computed in a terrorist scenario (Dembek, 2005) involving a water fountain and contamination at a recreational center (CDC, 1999). Wein and Liu's assessment estimates the amount of toxin required, critically evaluates entry points into the milk supply industry, and details deficiencies in our current detection capabilities required to thwart such an attack (Wein and Liu, 2005).

The most prevalent BoNTs isolated in human botulism are serotypes A, B, and E. The ability of serotypes C and D, in addition to F, to paralyze human skeletal muscle should also be noted (Hilmas, unpublished). Complicating matters is the fact that all BoNTs remain stable in common beverages and retain significant potency for prolonged periods of time (>90 days) at room temperature and in biological fluids (human whole blood and serum) at physiological temperatures (Hilmas et al., 2006b; Williams et al., 2007). In addition, BoNTs possess a remarkable ability to remain within the nerve terminal for extended periods. Keller et al. (1999) showed BoNT protein detectable by western blot for 90 days in rat spinal cord cultures.

Stability of the BoNT protein should be considered in an assessment of the threat posed by intentional release of the toxins. In addition to the remarkable persistence of the toxin in biological fluids and beverages described previously, BoNT remains a potent environmental threat. BoNT/A was subjected to desiccation to simulate the residue of an intentional release. Following 28 days of drying, the toxin still possessed remarkable paralytic properties (Williams et al., 2007).

The duration of muscle paralysis following intoxication by BoNT/A exceeds that resulting from exposure to other BoNT serotypes (Keller et al., 1999; Fernández-Salas et al., 2004). The remarkable persistence of BoNT/A action has led to its widespread use in the treatment of disorders of muscle tone and movement (Jankovic and Brin, 1997). Although a long duration is desirable in clinical use, the prolonged action of BoNT/A would also make intoxication by this serotype difficult to treat, particularly if it is used as a bioweapon (Franz, 1997). The duration of intoxication by BoNT/E is relatively brief (several weeks), whereas BoNT/B is of intermediate duration (Keller et al., 1999). The basis for the differences in serotype persistence is currently unknown. In any case, a bioterrorist attack, involving the most lethal substance known to humankind, would overwhelm the limited resources (i.e., mechanical ventilators) available to treat botulism patients.

TREATMENT

There are currently seven known antigenic serotypes of botulinum toxin, designated with the letters A through G, whereby antitoxin to one type does not cross-neutralize any of the others. Only early administration of antitoxin antibody in cases of suspected botulism will minimize the neurologic damage but will not reverse any existing paralysis. Paralysis could persist for weeks to months, and the available treatment consists of supportive care including fluids, TPN, and mechanical ventilation.

Antitoxin

The administration of a heterologous antitoxin was one of the first therapeutic approaches developed for botulism patients and remains the most effective when initiated in the early stages of intoxication. The primary limitation of antitoxin treatment was established in some of the earliest published reports on experimental botulism. One of these reports evaluated the pathogenesis of oral intoxication and the efficacy of antitoxin therapy in

monkeys (Dack and Wood, 1928). Antitoxin treatment was not effective when administered after symptoms of botulism were already apparent, despite the fact that circulating toxin could still be detected in many of the animals.

Oberst et al. (1967) investigated the effectiveness of antitoxin therapy, artificial respiration, and supportive treatment in rhesus monkeys after IV type A toxin injection. These treatments were administered to the animals, either alone or in combination, after signs of intoxication were observed. Only 1 in 6 monkeys survived after receiving antitoxin injections alone as treatment for overt intoxication with 2.5 LD_{50} of type A toxin (Oberst et al., 1967). A combination of antitoxin therapy and supportive treatment initiated soon after the development of toxic signs protected 8 of 10 animals from death after IV injection of 4–5 LD_{50}. Artificial respiration prolonged survival in monkeys with respiratory paralysis but was ineffective as a primary treatment after lethal intoxication; no animals receiving only artificial respiration survived intoxication with 5–24 LD_{50} (Oberst et al., 1967). Untreated animals developed overt signs of intoxication within 20–38 h and died 32–135 h after toxin injection.

While antitoxin treatment was generally ineffective in experimental animals displaying significant clinical signs, several case studies of foodborne botulism indicated that antitoxin therapy remained potentially beneficial in humans even after the onset of illness. Iida (1970) reviewed the high efficacy of antitoxin therapy in type E botulism outbreaks associated with contaminated fish consumption in Japan. A mortality rate of only 3.5% was observed among 85 antitoxin-treated patients in 9 recent foodborne botulism outbreaks, while a rate of 28.9% was reported among 135 untreated patients in 19 previous outbreaks. Iida et al. (1970) noted that all moderately and seriously ill patients in a 1962 foodborne type E botulism outbreak survived after antitoxin treatment. Hatheway et al. (1984) reported on the effectiveness of trivalent (ABE) antitoxin therapy during a 1978 outbreak of type A botulism. Four of seven patients with confirmed disease from type A toxin ingestion were treated with two to four vials of trivalent antitoxin (Hatheway et al., 1984). All four treated patients survived, although one of these individuals continued to suffer from severe paralysis and required ventilatory assistance for several months.

The current CDC therapy for the public is an FDA-approved, bivalent, botulinum equine antitoxin against serotypes A and B. The trivalent antitoxin against types A, B, and E is no longer available. In cases of exposure to any of the other botulinum toxin serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) equine antitoxin, but the time required for typing a toxin subtype would limit its effectiveness in certain cases, such as an outbreak. A parenteral vaccine against the toxin is currently available, but the need exists for newer, nonparenteral vaccines that could be administered orally or via inhalation.

Treatment for Infant Botulism

Administration of equine antitoxin is not recommended for pre-exposure prophylaxis. The heterologous serum of antitoxin therapy can lead to a high frequency of adverse reactions. The equine antitoxin available for use in humans has been reported to cause adverse reactions, such as anaphylaxis, in over 20% of treated patients (Lewis and Metzger, 1980). This problem has been circumvented in the development of a safer approach to the treatment of infant botulism using plasma isolated from human subjects repeatedly immunized with pentavalent toxoid. Equine antitoxin is not used as a treatment for infant botulism due to the high risk of serious adverse reactions and the possibility of long-term sensitization to horse serum-based therapeutics (Arnon, 1998). An antiserum product termed BabyBIG (botulism immune globulin), derived from human volunteers immunized with pentavalent toxoid, is available for infant botulism patients. Intravenous BabyBIG therapy has proven extremely effective in counteracting the toxic effects of *C. botulinum* colonization in infants and in avoiding the risk of adverse reactions to equine antitoxin. It is also most effective when administered within 24 h of a high-dose aerosol exposure to the toxin (Gelzleichter et al., 1998a,b).

Vaccines

There are as yet no FDA-approved vaccines to prevent botulism. An investigational pentavalent botulinum toxoid (PBT) product, developed at Fort Detrick, Maryland, is available for persons at risk for botulism (i.e., laboratory workers and soldiers). While determined to be safe and immunogenic, PBT is not useful, nor is it recommended, for post-exposure prophylaxis. Antitoxin titers do not develop until a month after the third dose in the vaccine schedule. PBT is reserved for employees at high risk for BoNT exposure, not for the general population. Several factors limit the usefulness of PBT as a vaccine for inoculating the general population. These include a declining potency and immunogenicity in recent years, the need to take multiple doses to maintain titers, and the limited supply of the vaccine.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The toxicity of botulinum toxins leading to paralysis is due to their ability to block ACh release from peripheral cholinergic nerve endings (Simpson, 2004). Once

ingested or inhaled, the toxin binds to epithelial cells, transports to target tissues via the circulatory system, targets the NMJ, and penetrates cellular and intracellular membranes. BoNTs bind to the lipid bilayer of the neuronal cell surface, gain access by receptor-mediated endocytosis, and cleave polypeptides involved in exocytosis of ACh. As a result, botulism leads to a descending flaccid paralysis, starting usually in the bulbar musculature to involve deficits in sight, speech, and swallowing. Paralysis eventually progresses beyond CN palsies to include generalized muscle weakness and loss of critical accessory muscles of respiration. If untreated, death is inevitable from airway obstruction secondary to paralysis of pharyngeal, diaphragm, and accessory respiratory muscles, as well as loss of the protective gag reflex.

The CDC-recommended therapy for the public is a trivalent equine antitoxin against types A, B, and E. In cases of exposure to other BoNT serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) antitoxin. However, the antitoxins are in limited supply and would need to be retrieved from stockpiles. Therefore, the development of safe and effective post-exposure therapeutic compounds for BoNT intoxication is of paramount importance to serve the requirements of the military and civilian populations. In conjunction with drug discovery efforts, there is a parallel exigency to develop appropriate animal models to test the usefulness of various strategies for protection against BoNT intoxication.

Development of Animal Model Test Systems

Inadequacies of Current Animal Model Test Systems

Currently, a large number of animal models (mice, rats, guinea pigs, rabbits, and nonhuman primates) have been used for BoNT research, and it is not clear which species is the most appropriate. This is especially problematic since there are marked species differences in the relative potencies of the different serotypes and in their latency of action (the effect of BoNT/B in mice, rabbits, or guinea pigs versus rats; Erdal et al., 1995; Hilmas et al., 2006a). Mice, in particular, are desirable in BoNT research because they offer the most favorable balance between the scientific needs of the experiment and consistency with the existing literature. A variety of mouse strains and sexes have been used for other BoNT studies. The mouse LD_{50} is still used to quantitate the purity of BoNT batches and is the basis of the international standard used in serum neutralization assays of BoNT antitoxin. The mouse phrenic nerve–hemidiaphragm assay has been used to measure the effect of BoNTs on skeletal muscle contraction, and the doses necessary for inhibition are well characterized. The mouse has further advantages over other rodent species like rats.

Rats are not a valuable test system for BoNTs, as they are widely recognized as being insensitive to serotype B (Verderio et al., 2006). On the other hand, skeletal muscles of CD-1 mice, Hartley guinea pigs, and New Zealand white rabbits have similarities to humans, in that their muscles are sensitive to serotypes A, B, C, D, and E (Hilmas et al., 2006b). *In vivo* and *in vitro* physiological assessments of BoNT action in rat have also proved to show inconsistent and erroneous results. *In vivo* experiments using the rat extensor digitorum longus (EDL) muscle assay showed sensitivities of rat muscle to the B serotype at low doses (10 MU, corresponding to approximately 1–10 pM) (Adler et al., 1996), despite the wide body of literature on the rat to the contrary. In addition, *ex vivo* rat phrenic nerve–hemidiaphragm preparations are insensitive to BoNT/B, even at very high concentrations in the nanomolar range (Williams et al., 2007).

Another physiological model to evaluate therapeutic candidates against BoNT intoxication is the rat toe spread assay. The rat toe spread assay is problematic as a model test system, however. First, it will not allow for the evaluation of therapeutic candidates against the B serotype since rats are insensitive to BoNT/B. Second, the rat toe spread assay does not involve focal application of BoNT; neighboring muscles are paralyzed due to local diffusion of toxin from the site of i.m. injection. Toe spread in the rat is mediated predominantly by digiti minimi abductor muscles and to a lesser extent by the EDL, the actual muscle injected in the assay. Intramuscular injection of rat EDLs with BoNT will primarily paralyze the EDL and, to a lesser extent, the digiti mini muscles, the true abductors of toe spread, by local diffusion. Therefore, EDL muscles injected with BoNTs would tend to show an erroneously early recovery of toe spread as the primary effectors of toe spread recover sooner compared to injected EDL muscles. To date, there is no acceptable *in vivo* model to test the efficacy of inhibitory compounds.

Advantages of the Mouse Hemidiaphragm Assay

Current approaches to the inhibition of BoNT activity involve a number of strategies, each with potential advantages and disadvantages. Ultimately, model test systems that can incorporate each of these potential approaches are needed to evaluate the relative merit of potential therapeutic compounds. Since the presynaptic terminal is the primary target for BoNTs, a test system based on toxin action at presynaptic terminals is indicated. Such systems should permit testing of all relevant aspects of toxin (internalization, activity, and overcoming the inhibition of transmitter release), should be simple and reliable and should permit rapid evaluation of novel therapeutics or their precursor compounds.

Due to the high sensitivity of mammalian synapses to the actions of BoNTs, due in part to the presence

of high-affinity binding sites for toxin on the cell surface and to the intracellular presence of the appropriate enzymatic substrates, the test model systems should be of mammalian origin. Muscle is the ideal test system for BoNT since it is the most sensitive *in vivo* target for neurotoxin action. In addition, inhibition of the diaphragm muscle is the proximal cause of death in botulism (Simpson, 1986). Furthermore, a positive result with BoNT on muscle implies that the toxin is correctly folded and the binding, catalytic, and translocation domains are all intact. Enzyme-linked immunosorbent assays (ELISAs), on the other hand, detect only components of the toxin and may provide positive results when the toxin has in fact lost its ability to intoxicate (Kalb et al., 2005). The mouse phrenic nerve–hemidiaphragm assay is a favorable model test system to evaluate therapeutics against BoNT-induced paralysis.

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CHAPTER

29

Anthrax

Corey J. Hilmas and Jaime Anderson

INTRODUCTION

Anthrax is a virulent, contagious, and potentially fatal disease. The first accounts of anthrax infection were written by the Roman poet Vergil in early antiquity. Although its lethal effects were ascribed to the actions of an exotoxin more than a half century ago, the pathogenesis and mechanism of anthrax toxicity continue to be refined. Depending on the route of exposure, anthrax can cause a different disease, including inhalational, cutaneous, and oral/ingestional forms. Anthrax infection involves a complex set of steps in its pathogenesis from spore uptake by immune cells, germination, transport to local lymph nodes, production of deadly toxins, systemic spread, and, ultimately, death of the host. The details of each step are continually being debated. Even today, debate ensues regarding the mechanisms of macrophage killing by the toxins and the importance of other cell types involved in toxin-induced fatality.

Although anthrax infection causes high numbers of bacilli and overt septicemia, it is the exotoxins that are responsible for the intoxicating symptoms and death. Birth of the molecular age in modern research led to the identification and enzymatic characterization of three proteins that constitute two anthrax exotoxins. These are the protective antigen (PA), edema factor (EF), and lethal factor (LF). A better understanding of the precise mechanisms of toxicity initiated by anthrax toxins will uncover many of the unsolved mysteries surrounding *Bacillus anthracis* infection. Moreover, nature's clever engineering will be evident from the sometimes contradictory actions of the spore form, vegetative bacilli, and bacterial toxins.

HISTORY

Anthrax has plagued humans and animals since early recorded history. Scholars have attributed several plagues in antiquity to anthrax. The Plague of Athens (430–427 BC) and two of the plagues of Egypt (the fifth (death of livestock) and sixth plagues (boils)) during the time of the Israelites' captivity have both been ascribed to outbreaks of anthrax (McSherry and Kilpatrick, 1992). Publius Vergilius Maro or Vergil (70–19 BC) provided the earliest, definitive, and detailed descriptions of an anthrax epidemic in his four-volume *Georgics*, a narrative on agriculture and animal husbandry (Sternbach, 2003). Vergil described the same disease ravaging sheep, horses, cattle, dogs, and various other animals. In addition to signs of toxicity, he provided insight into knowledge of how the disease was transmitted, namely wool. He even noted the virulent and contagious nature of anthrax, as well as its ability to spread to humans (Dirckx, 1981).

> The pelts of diseased animals were useless, and neither water nor fire could cleanse the taint from their flesh. The sheepmen could not shear the fleece, which was riddled with disease and corruption, nor did they dare even to touch the rotting strands. If anyone wore garments made from tainted wool, his limbs were soon attacked by inflamed papules and foul exudates. (Dirckx, 1981)

During the course of the next 1500 years, Europe witnessed sporadic outbreaks of anthrax as they occurred in fourteenth century Germany and seventeenth century central Europe and Russia. The disease was classified as anthrax or charbon malin (Morens, 2003) in 1769 by the French physician Nicholas Fournier (Fournier, 1769; Morens, 2003). The name is derived from the black eschar lesions, the hallmark of cutaneous infection. Fournier also noted a link between those who worked with raw animal hair or wool and an increased susceptibility to disease. In the eighteenth century, an epidemic destroyed half of the sheep in Europe, possible evidence that anthrax was a major problem. Inhalation anthrax became known in the Victorian era as woolsorters'

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disease; however, infection was more often the result of contact with goat hair or alpaca than with wool.

In 1850, Pierre-Francoise Olive Rayer (Rayer, 1850) and Casimir-Joseph Davaine (Davaine, 1863) reported the presence of "small filiform bodies" in the blood of anthrax-infected sheep (Carter, 1988). By 1855, Franz Aloys Antoine Pollender confirmed this discovery and implicated their role in producing anthrax disease (Pollender, 1855). In 1858, Freidrich August Brauell noted the "bodies" to be absent from healthy animals or animals infected with diseases other than anthrax. Brauell also noted their inability to be transmitted from pregnant sheep to fetus (Brauell, 1857).

In the 1870s, Robert Koch, a Prussian physician, isolated the anthrax bacillus and traced the complete lifecycle using suspended-drop culture methods. Koch determined the bacillus could form spores that remained viable, even in hostile environments (Koch, 1876). Louis Pasteur provided demonstration of infectious disease transmission. He inoculated one cohort of cattle with live attenuated vaccine and a control cohort without vaccine. When all animals were injected with virulent anthrax, only the control cattle died.

Modern History: Weaponizing Anthrax and Terrorism

Research into the utilization of anthrax spores as a biological weapon began in the early twentieth century. During World War I, German development of *B. anthracis* and other disease-causing organisms gave rise to covert programs intended to infect livestock and animal feed to be exported to the Allies. These plans included contaminating feed for horses and cattle to be exported from the United States to England, infecting sheep from Romania to be exported to Russia, and exporting contaminated livestock from Argentina to various Allied nations (Merck, 1946; Hugh-Jones, 1992).

During World War II, the pace of anthrax and biological weapons research in general accelerated. Imperial Japan had a large, active bioweapons program that included a substantial anthrax component. The central Japanese research facility was located in Manchuria, known as Unit 731. It is believed that in excess of 10,000 prisoners of war died either by direct experimental exposure to *B. anthracis*, among other pathogens, or by execution after exposure (Harris, 1992, 1994). Much of the Japanese bioweapons research transitioned to the battlefield. Operations such as Nomonhan in 1939, where Japanese troops entered the Soviet Union to infect Russian herds, met with only partial success. As it turned out, Japanese troops were unprepared to operate in a biological weapons environment. Their actions resulted in many inadvertent friendly casualties (Harris, 1992).

The Allies were pursuing biological weapons programs at the same time as the Axis powers. In 1942, the United Kingdom conducted anthrax experiments off the coast of Scotland at Gruinard Island. British scientists working at the biological weapons laboratory at Porton Down had demonstrated the lethality and military utility of the bacillus. Spores persisted and remained theoretically capable of infection for decades afterwards. A subsequent decontamination effort took nearly 10 years to clean the island (Carter, 1992). The United States began developing anthrax as a biological weapon in 1943. A civilian agency, the War Reserve Service, constructed a research facility at Camp Detrick (later Fort Detrick in 1956) and conducted research on a number of pathogens, including *B. anthracis*.

In April and May 1979, an anthrax epidemic occurred in Sverdlovsk, a city of then 1.2 million people, 1,400 km east of Moscow. The Soviet medical community reported an outbreak in livestock south of the city, and human exposures by ingestion of infected meat and contact with diseased animals led to cases of gastrointestinal and cutaneous anthrax. According to Soviet medical reports, 96 cases of human anthrax were identified; of these, 79 were said to be gastrointestional and 17 were cutaneous. These cases resulted in 64 deaths, all reported to be gastrointestional exposures (Meselson et al., 1994). In 1986, the Soviet Union invited a group of American scientists to visit Russia and investigate the outbreak. In collaboration with the Russian clinicians who treated the victims, the panel concluded that the outbreak was the result of inhalation exposure attributable to the accidental release of an estimated 10 kg of military-grade anthrax from the Soviet military microbiological facility in Sverdlovsk. This event remains the largest documented outbreak of inhalation anthrax (Sternbach, 2003).

In 2001, the first case of intentional anthrax release in the United States occurred. In October and November of that year, 11 confirmed cases of inhalation anthrax, and 11 confirmed or suspected cases of cutaneous anthrax were reported in postal workers and others who handled mail that had been deliberately contaminated with anthrax spores (Abalakin et al., 1990). These contaminated letters were mailed anonymously to several news media and Federal government offices. The letters contained handwritten threats as well as cryptic references to the terrorist attacks on September 11 of that year. The anthrax spores were analyzed and determined to be of the "Ames" variety, the strain that originated in the United States and had been acquired by Army research institutes for vaccine development.

Anthrax remains both a serious public health hazard and a very real biological weapon threat. Deliberate release of an anthrax weapon in a populated area could have catastrophic implications. An economic model developed by the Centers for Disease Control

and Prevention (CDC) suggested a cost of $26.2 billion to treat 100,000 people exposed to anthrax (Kaufmann et al., 1997). A risk assessment, provided at the end of this chapter, serves to highlight the dangers of a realistic scenario involving anthrax spores.

EPIDEMIOLOGY

Persistence

B. anthracis can remain for extended periods of time in soil. The mechanism responsible for its persistence is unclear. Therefore, persistence may involve multiplication cycles and sporulation. It may involve multiplication of the organism in the soil or bacterial amplification in infected animals before soil contamination by the carcass. Spores may germinate simply on application of water to soil (Hanna and Ireland, 1999; Oncul et al., 2002).

Infection

Infection typically results from herbivores grazing on soil or feed contaminated with spores. Oral consumption may produce oropharyngeal or gastrointestinal infection, an invariably fatal condition. Terminally ill herbivores generally bleed from the nose, mouth, and bowel, resulting in further contamination of the soil or drinking source (Shafazand et al., 1999). Although the actual number of cells or spores shed by an infected animal is unknown, studies have shown that counts of 10^4–10^6 spores/g of soil can be found near infected carcasses (Turnbull et al., 1998).

Dissemination

Dissemination of anthrax spores may result from biting flies or vultures (De Vos, 1990). Flies and mosquitoes contaminated with the vegetative cells, as a result of feeding on blood, can remain infectious for hours. The infectious material is deposited onto leaves through defecation, leading to contamination of herbivore species such as cattle, sheep, horses, and goats. Vultures often feed on contaminated carcasses and disseminate the organism to other birds or common drinking sources shared by various animals.

Forms of Anthrax Disease

Anthrax in humans is associated with agricultural, horticultural, or industrial exposure to infected animals or animal products. Three forms of anthrax can be diagnosed in humans: cutaneous, gastrointestinal, and inhalational. Cutaneous anthrax infection can occur through handling of contaminated hides, wool, bones, and carcasses. Cutaneous anthrax is the most common form of natural human infection, consisting of more than 95% of anthrax cases, and is treatable if recognized early. Infection occurs as a result of direct contact with infected animals or animal products. The skin is typically damaged or abraded before establishment of successful skin lesions by the bacteria. The persons most at risk for a natural cutaneous exposure are industrial or agricultural workers, such as herders, butchers, slaughterhouse workers, or processing mill workers (Pile et al., 1998). Infection can also occur through an insect bite (Spencer, 2003). Although cutaneous anthrax is rarely fatal (mortality <1% in treated cases) (Anon, 2000), it can progress to a systemic infection with a mortality rate of 5% to 20% in untreated cases (Pile et al., 1998). In general, cutaneous anthrax is not as life-threatening as the inhalational form, which results in much higher mortality. However, it is still important to study dermal pathogenesis models, particularly because cutaneous anthrax cases may result from an aerosol release, a method most likely to be used by bioterrorists.

Inhalational anthrax may occur after inhaling aerosols of spores, formed from processing contaminated animal products (wool) or as the result of direct bioweaponization. Inhalational anthrax contributes to only 5% of all reported cases but is by far the most lethal form. The estimated mortality is approximately 90% in untreated patients (Meselson et al., 1994; Dixon et al., 1999; Friedlander, 1999; Atlas, 2002; Bales et al., 2002; Oncu et al., 2003). Inhalational anthrax is typically reported in industrial settings where animal products are handled in enclosed spaces, allowing for exposure to aerosolized spores. Individuals passing by these industrial facilities have been stricken with inhalational anthrax. In one study of 27 anthrax cases involving textile mills, 21 cases were cutaneous and six were inhalational. All but one of the inhalational cases was fatal. One case of inhalational anthrax occurred in a secretary at a goat hair–processing facility.

Gastrointestinal anthrax is far less common than inhalational anthrax, but the mortality rate is extremely high, from 50% to 75%, even with early treatment (Mansour-Ghanaei et al., 2002). Oropharyngeal or gastrointestinal forms of anthrax can result from ingestion of contaminated meat (Dixon et al., 1999; Friedlander, 1999; Atlas, 2002; Bales et al., 2002). Gastrointestinal anthrax has never been confirmed in the United States, but this may be the result of cases being unreported in rural communities, where physicians may not be aware of this form (Pile et al., 1998; Dixon et al., 1999; Friedlander, 1999; Shafazand et al., 1999; Atlas, 2002; Bales et al., 2002; Oncu et al., 2003). Two out of 53 persons in a 1998 Kazakhstan outbreak developed the gastrointestinal form, resulting from the consumption of contaminated raw meat. In Minnesota, several family members

consumed steer meat and fell ill with gastrointestinal symptoms (Bales et al., 2002). The meat was later confirmed to contain *B. anthracis*, but the bacteria could not be cultured to confirm the presence of the gastrointestinal pathogen. According to one case of a 15-year-old boy who was infected after ingesting half-cooked sheep meat, the incubation period for gastrointestinal anthrax varies from 2 to 5 days (Mansour-Ghanaei et al., 2002).

Worldwide, the annual incidence of human anthrax infection is estimated to be between 20,000 and 100,000 (Pile et al., 1998; Oncu et al., 2003). The majority are cutaneous anthrax. In the United States, less than one case is diagnosed per year, as compared with 127 cases per year diagnosed in the early twentieth century (Pile et al., 1998; Shafazand et al., 1999; Oncu et al., 2003). Occasionally, outbreaks of anthrax will occur as a result of breakdown in public health standards and practices or lack of public health services.

PATHOGENESIS

Overview

Themes common among all anthrax infections are the following: uptake by macrophages and other immune cells; germination to the vegetative form at or near the site of inoculation before transit to target tissues; time course of transport to target organs; organs targeted for toxicity; overwhelming septicemia; and release of soluble factors responsible for death. Two types of *B. anthracis* have been identified: a spore form and a vegetative form. Unless stated otherwise, the vegetative bacillus will be referred to as *B. anthracis* because this is the form that produces the deadly toxins; however, both forms are discussed at length. The spore form is essential for uptake by host cells.

Uptake of Spores

Anthrax infection typically results from entry of *B. anthracis* spores into the host through a minor abrasion, insect bite, ingestion of contaminated meat, or inhalation of airborne spores. These routes of exposure lead to cutaneous, intestinal/oropharyngeal, and inhalational anthrax diseases (Figure 29.1). Although *B. anthracis* is not categorized as an intracellular pathogen, it uses tissue macrophages as a sanctuary where the engulfed spores can germinate during the early phase of infection (Guidi-Rontani and Mock, 2002). Successful infection and eventual disease require uptake of spores. Although spores are resistant to phagosomal superoxide, they have evolved to recognize receptors contained on host phagocytic cells through their pathogen-associated molecular patterns (PAMPs). Whereas most studies have shown uptake of the bacterium by macrophages, one study showed that human dendritic cells can be triggered to internalize *B. anthracis* spores (Brittingham et al., 2005).

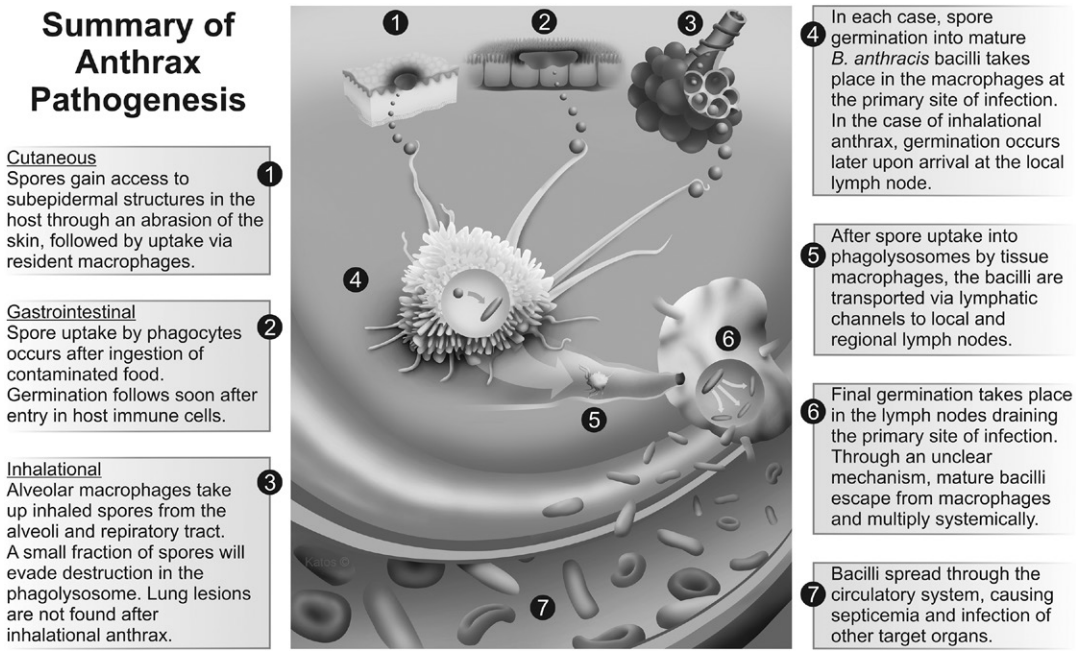


FIGURE 29.1 Overview of anthrax disease and pathogenesis. The steps of anthrax intoxication including spore uptake by macrophages, germination to the vegetative form, migration to lymph nodes, bacillus multiplication, release into the circulatory system, and septicemia are illustrated. *Source: Illustrations are copyright protected and printed with permission by Alexandre M. Katos.*

This led to the hypothesis that these cells may take part in bacterial transport to the lymph nodes, similar to macrophages. Spore PAMPs are antigenic determinants containing highly conserved molecules that interact with cell surface Toll-like receptors (TLRs) on tissue macrophages, dendritic cells, polymorphonuclear leukocytes (PMNs), and other cell types (Janeway and Medzhitov, 2002). PAMPs typically include lipopolysaccaride (LPS) from Gram-negative bacteria and lipoteichoic acid and peptidoglycan from the cell wall of Gram-positive bacteria such as *B. anthracis*. Anthrax spores can trigger a strong inflammatory response by activating TLR4 on antigen-presenting cells of the immune system at the entry site into the host (Hsu et al., 2004). In addition, anthrolysin O, a protein secreted by *B. anthracis*, is a potent agonist for TLR4 (Park et al., 2004).

Uptake via Lungs

Anthrax spores are approximately 1–2 μm in diameter, optimal for inhalation and deposition in the alveolar spaces (Brachman, 1980; Penn and Klotz, 1997). In the case of inhalational anthrax, inhaled spores reach the respiratory bronchioles and alveoli (Figure 29.2). Although most spores are internalized rapidly into phagolysosomes by resident macrophages in the alveolar space (Ross, 1957; Guidi-Rontani et al., 1999b), the exosporium layer of anthrax, discovered by Flügge (1886), prevents its degradation. It should be noted that the exosporium is not present on vegetative forms of anthrax bacteria and therefore only spores contain the antigenic determinants recognized by TLR4 receptors of immune cells. In any case, spores will escape destruction due to their resistance to superoxide and enzymes of the phagolysosome. Spore-bearing alveolar macrophages migrate along lymphatic channels to mediastinal, peribronchial, and tracheobronchial lymph nodes (Ross, 1957) while germinating *en route*.

Uptake via Skin

The cutaneous infection process initiates as a result of anthrax spores colonizing an abrasion in the skin (Figure 29.3). A small eruption or lesion develops into a painless, black eschar. Eschars usually develop within 2–5 days after exposure. During this stage of infection, low-level spore germination can occur at the primary site of infection, leading to localized edema and necrosis. Although this infection often remains localized, some patients experience systemic symptoms.

Systemic disease, a rare secondary occurrence of cutaneous anthrax, is likely attributable to phagocytosis by macrophages in the dermis that marginate out of lymphatic channels and blood capillaries (Figure 29.3). In cutaneous anthrax, germination typically occurs immediately inside the host macrophage. Macrophages

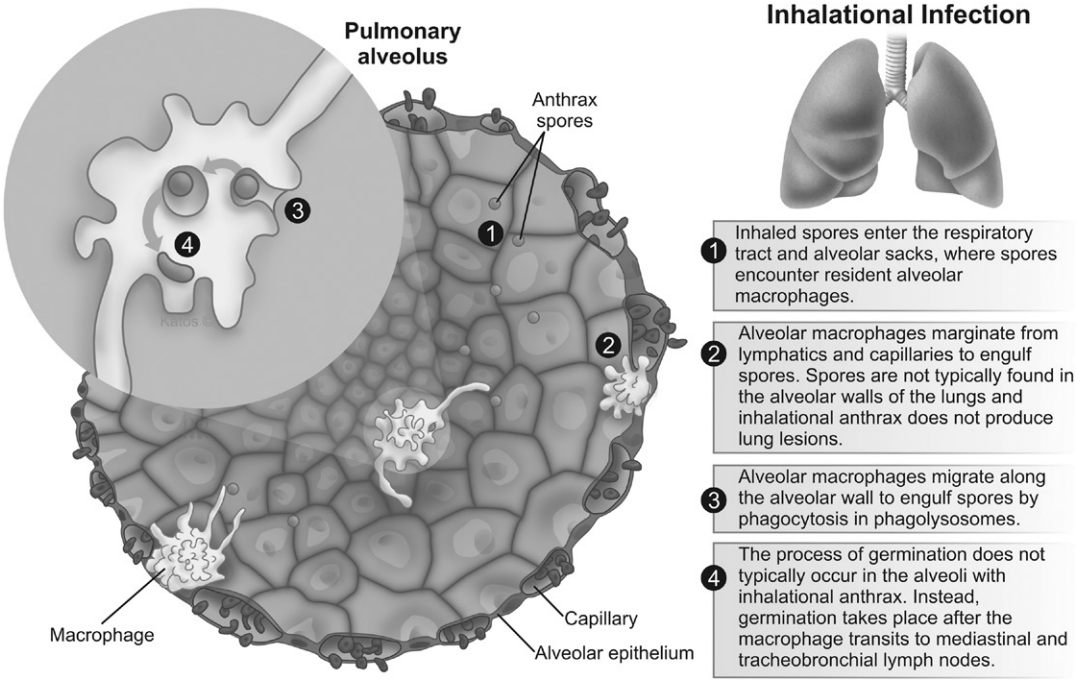


FIGURE 29.2 Spore uptake in inhalational anthrax disease. The steps involving spore deposition into a pulmonary alveolus, margination of alveolar macrophages out of capillaries into the alveolar space, spore uptake, and entry into lymphatic channels are illustrated. *Source: Illustrations are copyright protected and printed with permission by Alexandre M. Katos.*

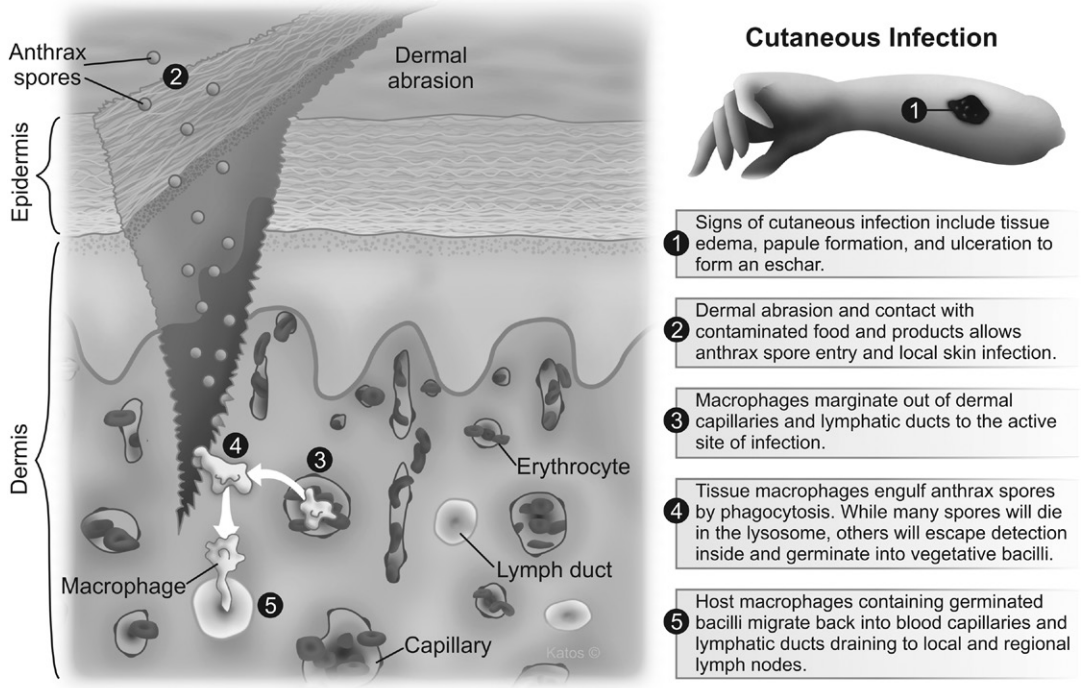


FIGURE 29.3 Spore uptake in cutaneous anthrax disease. The steps involving spore uptake at the primary site of local infection on the skin, germination inside the macrophage, and migration back into lymphatic ducts are illustrated. *Source: Illustrations are copyright protected and printed with permission by Alexandre M. Katos.*

carrying *B. anthracis* cells migrate back into lymphatic ducts *en route* to regional lymph nodes draining the primary site of infection (Dixon et al., 1999). The anthrax bacilli spread through the blood and lymph and proliferate to high concentrations, creating acute septicemia.

Uptake via Gastrointestinal Route

The infection process of gastrointestinal anthrax starts with ingestion of spore-contaminated food/drink or ingestion of inhaled spores. In general, gastrointestinal anthrax is similar to cutaneous anthrax, but gastrointestinal anthrax occurs in the intestinal mucosa. As in cutaneous anthrax, the organisms probably invade the mucosa through a preexisting wound or lesion, or possibly through interaction with a cell surface receptor on epithelial cells. Presumably, the mucosal lining is the entry point for the endospores (Figure 29.4), but the exact germination location is yet unknown in cases of gastrointestinal anthrax infection. The bacteria may spread from the mucosal lesion to the lymphatic system by way of macrophages migrating to the primary site of infection. Germination can occur inside host macrophages after uptake before macrophage migration back to lymphatic channels draining to lymph nodes (Figure 29.4). Ulcer formation is a typical symptom during gastrointestinal anthrax, and ulcers may be present at various locations along the gastrointestinal tract from the oral cavity to the cecum. However, it is not known whether ulceration occurs only at sites of bacterial infection or if it is caused by the anthrax toxins and therefore has a less restricted occurrence (Dixon et al., 1999).

Spore Function

Several studies have examined the molecular mechanisms by which *B. anthracis* spores undergo phagocytosis, germination, and subsequent escape to mediate systemic infection. Time-lapse microscopy suggested that the number of spores per macrophage can affect whether any spores are able to outlast the macrophage's inhibitory action of the bacteria. Macrophages that have engulfed a larger number of spores are more likely to have a few that survive to germinate and become vegetative bacilli (Ruthel et al., 2004). Inside the macrophage, the spores must first avoid cellular nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. This enzyme, which is present in the phagosomal membrane, initiates the reduction of oxygen to superoxide anion through NADPH oxidation. The superoxide anion is further converted to other reactive oxygen species (ROS), including hydrogen peroxide. To date, no *B. anthracis* enzymes have been discovered that are involved in the removal of ROS (Guidi-Rontani and Mock, 2002).

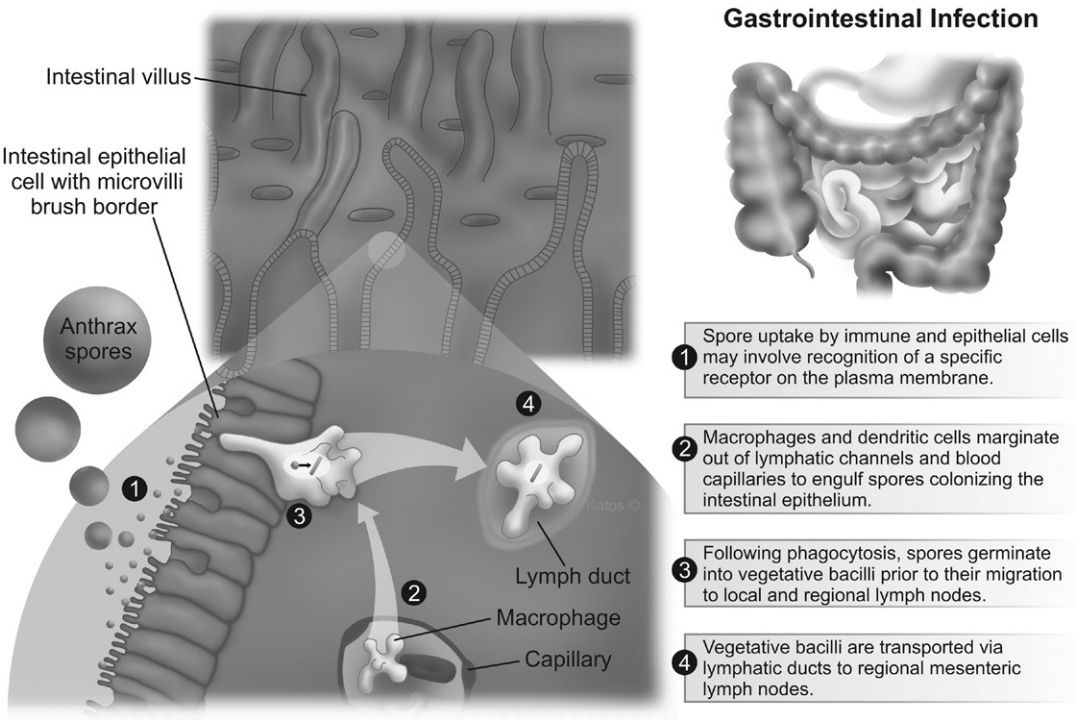


FIGURE 29.4 Spore uptake in gastrointestinal anthrax disease. The steps involving spore colonization of the gastrointestinal mucosa, uptake by phagocytes, germination, and lymphatic spread are illustrated. *Source: Illustrations are copyright protected and printed with permission by Alexandre M. Katos.*

After surviving the initial membrane oxidative burst, the anthrax spores must contend with the acid environment of the phagolysosome to further germinate and multiply. Several pathogens have shown an ability to evade phagolysosomal activity; however, the mechanism by which anthrax spores avoid hydrolysis in the phagolysosome is unclear (Guidi-Rontani and Mock, 2002).

Anthrax spores promote expression of interleukins (ILs) and other pro-inflammatory cytokines in macrophages and dendritic cells (Pickering et al., 2004). Macrophages are important mediators of the inflammatory response and produce tumor necrosis factor alpha (TNFα), IL-1β, and IL-6 in response to infection. Although some spores will be killed by immune cells, many will evade the host cell detection only to initiate germination. The purpose of promoting inflammation here by anthrax spores is to increase their chance for internalization by tissue macrophages and dendritic cells to germinate into the vegetative form of the bacteria (Welkos et al., 1989).

An intact mitogen-activated protein kinase (MAPK) cascade and pro-inflammatory response by macrophages is also necessary for host cell migration to regional lymph nodes and recruitment of additional macrophages to the primary site of infection. Without functional immune cells, the toxin would not be able to enter lymphatics or the general circulation. In fact, dendritic cells that engulf *B. anthracis* spores change their pattern of chemokine receptor expression. Specifically, they lose tissue-retaining receptors (CCR2 and CCR5) and upregulate lymph node homing receptors (CCR7 and CD11c) (Brittingham et al., 2005).

Time Course of Spore Germination

In cutaneous and gastrointestinal anthrax, spore germination takes place at or near the inoculation site after uptake by the macrophage or dendritic cell. It is the vegetative form, not the spore, that produces the deadly factors. After transformation to the vegetative bacilli, these bacteria are free to mediate toxicity at the inoculation site in skin and gastrointestinal tract (Beatty et al., 2003). In inhalational anthrax, germination does not occur until the spores have been transported to the local lymphatics and mediastinal lymph nodes (Riedel, 2005). In the case of inhalational anthrax, spores are not believed to germinate at the site of infection in the alveoli or bronchioles. Instead, they germinate *en route* or after migrating to local nodes of the lymphatic system.

Spore Germination

Although the anthrax endospore has no measurable metabolism, spore germination is a predictor of productive anthrax infection. Similar to endospores from other

species, anthrax spores seem to represent a biologically inert organism with the ability to transform into one of the most lethal organisms on earth. Germination inside the host immune cell is the key step toward this transformation from benign bystander to active infection. The spores have little or no water, no ATP production, no macromolecular synthesis, and no active enzymes. Thus, endospores can remain stable in the environment under adverse conditions for decades. Once inside a host cell, spores start to germinate and initiate early synchronous *de novo* expression of genes vital for infection as well as expression of genes necessary for vegetative growth (Hanna and Ireland, 1999; Oncul et al., 2002).

Several putative germination proteins have been identified (Guidi-Rontani et al., 1999a,b), but their roles in spore germination have yet to be clarified. L-alanine appears to be essential in initiating germination (Foster and Johnstone, 1990), but the mechanism that leads to spore germination is unclear. A germinant receptor locus (*ger S*) essential to *B. anthracis* germination has been identified; without it, the organism could not germinate in macrophages (Ireland and Hanna, 2002). Once the spore has germinated, the vegetative bacilli are free to synthesize deadly bacterial toxins; however, the precise manner in which vegetative bacilli or spores break free from immune cells is still poorly understood. A recently proposed model suggests that an interaction between anthrax toxin from newly germinated spores inside the phagolysosome of macrophages and anthrax toxin receptor 2 promotes escape of the bacilli from the cells (Banks et al., 2005).

Vegetative Anthrax and its Capsule

The role of the vegetative bacillus is in stark contrast to that of the spore form (Table 29.1). The vegetative form functions mainly to produce the deadly toxins lethal toxin (LT) and edema toxin (ET). Germination of *B. anthracis* within macrophages is closely followed by expression of the toxin genes (Guidi-Rontani et al., 1999a). *B. anthracis* contains no capsule *in vitro*, but capsule synthesis begins on host infection (Preisz, 1909). Host signals, including carbon dioxide concentrations greater than 5%, are thought to induce transcription of capsule and toxin genes (Uchida et al., 1997). *In vitro* studies by Ezzell and Abshire (1995) suggest that encapsulated vegetative cells appear within 30 min after germination *in vitro*. The capsule enables vegetative forms to survive as extracellular pathogens and to avoid phagocytosis by macrophages (Guidi-Rontani and Mock, 2002). *B. anthracis* isolates lacking capsules that are significantly less virulent. Vegetative cells move through the bloodstream and lymphatics to cause systemic infection.

TABLE 29.1 Differences Between Spore and Vegetative Forms of *B. anthracis*

Spore Form	Vegetative Form
• Contains an exosporium which promotes immune host detection and uptake • Exosporium promotes internalization by macrophages • Requires phagocytosis, internalization by macrophages, and germination to become pathogenic • Does not produce anthrax toxins • Requires target host cells to remain intact so they can circulate to local lymph nodes • Promotes TNF-α and cytokine production • Enhances host inflammatory response • Can remain dormant for months	• Contains poly-D-glutamic acid capsule, essential for virulence and avoidance of immune detection • Capsule avoids phagocytosis by macrophages • Survives as extracellular pathogen within the body of the host • Produces LT and ET to kill the target host cell • Kills the host cell • Inhibits TNF-α and cytokines • Suppresses the immune system • Unable to survive outside the host for any appreciable length of time

Systemic Infection and Septicemia

Significant numbers of bacilli in the blood are characteristic of the late stages of infection (Shafazand et al., 1999; Atlas, 2002; Oncu et al., 2003; Riedel, 2005). Without an activated immune response due to the release of soluble anthrax toxins that suppress immune function, *B. anthracis* is free to multiply to high concentrations. It is currently unclear whether *B. anthracis* multiplies within the macrophage, as some studies suggest, or the blood stream. Cultured blood from infected rabbits and guinea pigs suggest a continuous increase in bacterial counts until the final hours of life; numbers increase sharply to 10^7 and 10^9 organisms per milliliter of blood for rabbits (Bloom et al., 1947) and guinea pigs (Smith and Keppie, 1954), respectively. Death was shown to be dependent on bacteremia, but death was not shown to be a consequence of mechanical obstruction by large bacterial counts. Regardless of the route of infection, systemic spread results in similar colonization. The lungs and gastrointestinal tract are particular targets of system infection by mature bacilli, with the spleen, brain, liver, and almost any other organ being colonized within hours or days (Riedel, 2005). *B. anthracis* has been isolated from stool specimens of both animals and humans, reflecting its presence in the bowel (Beatty et al., 2003). Although septicemia is important, death is the direct result of soluble factors (discussed later) secreted by the vegetative

bacilli that weaken the immune response and initiate cell death.

Anthrax Infection Cycle

An infection cycle for *B. anthracis* has been proposed previously (Hanna and Ireland, 1999). Phagocytosis of spores by macrophages, germination, vegetative cell proliferation, and toxin release occur within hours after exposure to *B. anthracis* in a productive infection. During the middle stages of infection, the bacilli grow extracellularly and express toxins and other virulence factors. During the final stages of infection, the bacilli, having depleted nutrients from the host, form endospores, which return to the environment for the next cycle of infection (Hanna and Ireland, 1999; Oncul et al., 2002). The precise sequence of events leading to successful establishment of infection is not completely understood. After germination, anthrax bacilli multiply in the lymph nodes, causing an immediate lymphadenitis and hemorrhagic mediastinitis, and spread throughout the body via the circulatory system (Albrink, 1961).

Release of Soluble Factors

B. anthracis possesses four known virulence factors, including an antiphagocytic capsule of the vegetative form, LF (90 kDa), EF(89 kDa), and a PA (83 kDa). These virulence factors acting together play a key role in pathogenesis but are not toxic when present individually. In combination, PA, LF, and EF induce a dangerous cascade of events on cell entry. As early as 1953, toxic factors have been implicated as the cause of death from anthrax infection (Smith et al., 1953, 1955). It has since been shown that these three factors combine to form the two protein exotoxins of anthrax LT and ET.

PA binds to an ill-defined cell receptor/lipid raft, mediating the entry of the other two components inside the cytoplasm via the endosomal pathway. EF is a Ca^{2+}/calmodulin-dependent adenylate cyclase involved in producing a generalized accumulation of fluid into the interstitium, characteristic of anthrax. LF is a proteolytic enzyme involved in the inactivation of MAPK kinases (MAPKK), key players in secondary messenger signal transduction cascades. Both toxins are translocated into the cytosol of target cells by way of endosomes through a pore formed from a heptamer of PA molecules. They interfere with vital cellular responses to bacterial infection, disabling host immunity and promoting bacterial dissemination. As the disease progresses, the toxins accumulate to higher levels, causing respiratory distress, shock, widespread hemorrhage, and death.

TOXICOKINETICS

Inhalational Anthrax

To date, mice, rats, guinea pigs, rabbits, nonhuman primates, dogs, swine, and sheep have been used in inhalational animal model studies for *B. anthracis*. Routes of administration have included aerosol, intranasal, and intratracheal methods. In addition, various animal species differ in their natural resistance to infection (Welkos and Friedlander, 1988). Some of the most common findings in human inhalational anthrax concern the respiratory tract. A majority of patients experience mediastinal widening (70%), pulmonary infiltrates (70%), and pleural effusion (80%). These signs demarcate inhalational anthrax cases from influenza-like illnesses (Oncu et al., 2003). Pathological findings from inhalational anthrax patients in a bioterrorism-related outbreak cited hemorrhage and necrosis in mediastinal lymph nodes, hemorrhage or inflammation of the pleurae and interhilar septae, and prominent intra-alveolar macrophages or inflammation in the lung parenchyma. There were no skin lesions. Bacilli were located in the lung, mediastinal tissues, and thoracic tissues, as identified by staining (Guarner et al., 2003).

Early studies with mice were unable to identify the vegetative form of *B. anthracis* in the alveolar walls of the lungs. Aerosol exposure was achieved by exposing mice (mouse strain unreported) to clouds of spores (Albrink, 1961). Other aerosol studies involving mouse models indicated evidence of the spore form in lung sections but not the vegetative form (Barnes, 1947). The Barnes study highlighted the fact that not all of an aerosolized dose ends up in the lungs; spores can be swallowed to pass through or infect the alimentary tract.

Intratracheal administration of anthrax has demonstrated a respiratory lesion in mice (Lyons et al., 2004). Inoculation of the Ames strain (5,000 versus 10,000 versus 50,000 spores) in Bagg Albino (BALB)/c mice does not produce signs of early pulmonary lesions. Significant numbers of anthrax colony-forming units were found in lung-associated lymph nodes harvested 5 h after inoculation in a dose-dependent manner (Lyons et al., 2004). Airway damage did occur 24 h after inoculation with all anthrax doses; signs of toxicity include widespread edema vacuole formation, degeneration, airway epithelial cell sloughing, and necrosis. After 48 h, bacterial rods (vegetative form) were located within alveolar capillaries, suggesting active bacterial uptake from the respiratory tract.

Drysdale et al. (2005) investigated the role of *B. anthracis* capsule in uptake from the respiratory tract. These investigators deleted the capsule operon *capBCAD* in

mutant strains of *B. anthracis* and inoculated female BALB/c mice intratracheally with approximately 4×10^4 mutant spores. All capsule-deficient strains germinated in the lungs (Drysdale et al., 2005), indicating no role for the capsule at this particular stage in pathogenesis. This contradicts the accepted hypothesis that germination takes place in the lymph nodes for inhalational anthrax exposure.

In the only intranasal mouse model, Guidi-Rontani et al. (1999b) demonstrated uptake of *B. anthracis* by alveolar macrophages. A dose of 2.5×10^7 Sterne strain spores was administered to Balb/c mice. Bronchial alveolar lavage (BAL) fluids were subsequently collected at 1, 3, and 24 h after inoculation. Germination of spores in the fluid was assessed by exposing the material to heat (65°C) for 30 min. Ungerminated spores are resistant to this treatment. Germination was significantly evident in the alveolar macrophage fraction of BAL fluid by 24 h after inoculation. Therefore, the uptake of bacteria by alveolar macrophages occurs rapidly in this system (Guidi-Rontani et al., 1999b).

To date, evidence of respiratory lesions in humans does not exist. Spore cloud exposure studies by Ross (1957) determined the pathogenicity of anthrax in guinea pigs. Guinea pigs were exposed to high numbers of M.36 strain spores for 20 min and killed at various time points to assess spore location. After 1 h, spores were found in alveolar macrophages. Free spores were not, however, isolated from surrounding lymph nodes at this early time point. However, after 18–24 h, bacilli were observed throughout the lymphatic system. The results of these studies led to the idea that spore germination takes place in regional lymphatic nodes (i.e., tracheobronchial nodes) before spreading to the rest of the body via the blood circulatory system (Ross, 1957).

In a guinea pig/aerosol model, 6×10^5 spores of the vaccine anthrax strain STI were detected in the lungs 1 h after infection from a dose of 2.43×10^6 spores. *B. anthracis* was not detectable in the tracheobronchial lymph nodes until 2 days after infection. On day 36 after infection, lung levels were down to 10^3 spores and tracheobronchial lymph nodes were again negative.

Cutaneous Anthrax

Dermal *B. anthracis* pathogenesis has been studied in mice, rats, hamsters, rabbits, guinea pigs, nonhuman primates, and dogs. Several methods of inoculation have been tested, including subcutaneous, intradermal, epicutaneous, footpad, and scarification. Data on uptake/absorption from the skin, persistence in the circulation, transit to target tissues, and lethality are discussed. The only animal models that have been used in bacterial uptake studies after dermal inoculation to date are the mouse and rabbit.

An extensive study of the interaction of *B. anthracis* with mouse skin after epicutaneous inoculation was conducted by Hahn et al. (2005). C57BL/6 and DBA/2 mice were inoculated with the Sterne strain. Epicutaneous inoculations were performed by applying an inoculum of 10^7 spores onto the shaved, tape-stripped, or abraded area of the mouse. A difference in foci development was observed, according to skin treatment before inoculation. Shaved-only inoculation sites did not have foci of vegetative bacilli. In contrast, abraded inoculation sites had readily apparent foci. Germination and proliferation occurred at the skin surface and in the epidermis and hair follicles. Hair follicles had deeper foci of infection, more than 200 μm below the skin surface. In animals inoculated with 2×10^8 spores onto unshaved skin, foci appeared only in the hair follicles and not in the epidermis or dermis (Hahn et al., 2005). According to reviews of human infection, germination and proliferation also occur, to some degree, at the site of inoculation (Cranmer and Martinez, 2001). Proliferation also occurs in the draining lymph nodes near the site of inoculation (Anon, 2000). Zaucha et al. (1998) subcutaneously inoculated New Zealand White rabbits with $43–1.56 \times 10^5$ CFU of the Ames strain of *B. anthracis*. At the inoculation site, the main sign of bacterial uptake involved dermal and subcutaneous edema, signs observed in human cutaneous anthrax.

Gastrointestinal Anthrax

Despite the rarity in documented human cases of gastrointestinal anthrax (Beatty et al., 2003), this form is common in underdeveloped areas of the world where infected carcasses are consumed (Sirisanthana and Brown, 2002). Gastrointestinal anthrax infection carries a 25% to 60% mortality rate (Mansour-Ghanaei et al., 2002). Gastrointestinal anthrax can be divided into intestinal and oropharyngeal forms. In the intestinal form, after an incubation period of 1–7 days, there is severe abdominal pain, hematemesis, melena and/or hematochezia, ascites, and watery diarrhea. Intestinal anthrax carries a greater risk of mortality. In contrast, the milder oropharyngeal form can be contracted after consumption of infected cattle and water buffalo. In this form, there is marked neck edema and ulcerative lesions in the oropharynx. Clinical signs include nausea, loss of appetite, emesis, and fever. In one natural outbreak of oropharyngeal anthrax, only 3 out of 24 patients died (Anon, 2000; Pile et al., 1998).

Limited data regarding the pathogenesis of gastrointestinal *B. anthracis* infection in any animal model system are available. Barnes (1947) observed that 1 h after inhalational exposure to anthrax spores, the majority of spores were found in the stomach. Therefore, regardless of the route of exposure, there is a high risk of spores

transiting to the gastrointestinal tract. Unfortunately, further pathophysiology was absent from the study.

The guinea pig is the only animal model to date for which there are data after oral exposure. There is a need for more oral animal models of *B. anthracis* infection. Gastrointestinal anthrax is perhaps far underdiagnosed in humans; however, it remains an important disease because of the significant risk of ingesting spores after exposure to inhalation anthrax. Because there are limited data regarding pathogenesis of gastrointestinal anthrax in humans, evaluation of future animal models will be difficult. Stability of the organism as a function of pH in the various compartments of the gastrointestinal tract, method of inoculation, presence of stabilizers, and gastrointestinal physiology will become important factors to analyze for oral anthrax.

Aloni-Grinstein et al. (2005) assessed the stability of spores and vegetative cells in the gastric fluid and gastrointestinal tract. Female Hartley guinea pigs were administered 5×10^9 spores or 5×10^8 vegetative cells of the anthrax MASC-13 variety orally. This vaccine strain is nontoxigenic, devoid of capsule, and lacking a nonfunctional form of PA. Bacteria in feces and gastric fluid were charted according to days after ingestion. Gastric fluid incubations were conducted at 37°C, with fluid taken from the guinea pig stomach. Anthrax spores exhibited much greater stability to the harsh environment created by the gastric mucosa than their alternate form. Vegetative cells were barely detectable on day 1 after ingestion in feces and gastric fluid. There are no animal models or human studies that have examined bacterial persistence in circulation and in transit to target tissues after oral exposure to *B. anthracis*. Similarly, there are no animal models that examine oral lethality, clinical signs, or epidemiology after oral exposure.

MECHANISM OF TOXICITY

The significance of the capsule in virulence was demonstrated early last century when anthrax strains lacking a capsule were shown to be avirulent (Bail and Weil, 1911; Bail, cited by Sterne, 1959). The genes encoding synthesis of the capsule were found to be encoded on a 110-kilobase (kb) plasmid. Anthrax strains lacking the plasmid no longer produced the capsule and were attenuated (Ivins et al., 1986), confirming the role of the capsule in pathogenesis.

The capsule contains a polymer of poly-D-glutamic acid, conferring resistance to phagocytosis by macrophages (Keppie et al., 1963). This linear polymer of the capsule is weakly immunogenic. The negatively charged capsule enables the bacterium to inhibit phagocytosis of bacilli by macrophages (Sterne, 1937; Keppie et al., 1963). In conjunction with LF and EF, whose target cells include macrophages, dendritic cells, and other immune cells, the capsule allows *B. anthracis* to grow virtually unimpeded in the infected host. The capsule functions as a "one-way" filter, allowing bacilli to diffuse the other three virulence factors through the capsule, resulting in host cell intoxication without causing self-harm.

Protective Antigen

B. anthracis secretes three plasmid-encoded soluble toxin proteins collectively referred to as anthrax toxin; these are PA, LF, and EF. LF and EF function individually and in combination as catalytic enzymes in susceptible host cells. In an unusual twist of nature, they both have evolved to share PA as a common receptor binding moiety for translocation into the cytosol of the host. PA is a dominant component of the three-part protein toxin secreted by *B. anthracis* (Petosa et al., 1997). The mature form of PA, a secreted 735-amino acid protein, has a molecular weight of 83 kDa. The ribbon structure is illustrated in Figure 29.5 along with a detailed description of its functional domains. PA forms a membrane-insertion heptamer that translocates other toxic enzymes (EF and LF) into the cytosol of host cells.

The mechanisms by which *B. anthracis* toxins work at the cellular level are illustrated in Figure 29.6 and are described elsewhere in great detail (Duesbery and Vande Woude, 1999; Mock and Fouet, 2001). In this model, PA released from the vegetative form of the bacilli binds to cell surface receptors, namely tumor endothelium marker (TEM) 8 and capillary morphogenesis protein (CMG) 2, which are expressed as different isoforms by many cell types, including immune cells (Collier and Young, 2003; Baldari et al., 2006). On binding to the receptor, PA is cleaved into a 20-kDa C-terminal domain (PA_{20}) and a 63-kDa fragment (PA_{63}) by furin or similar host cell surface–associated protease (Molloy et al., 1992). The PA_{20} fragment is released, resulting in spontaneous oligomerization of truncated PA (PA_{63}) into heptamers that bind to EF and LF. It was once thought that a PA heptamer complex may bind up to seven molecules of LF and/or EF (Duesbery et al., 1998; Mock and Fouet, 2001); however, evidence suggests that LF or EF binding sites on the PA_{63} heptamer span the interface between adjacent PA_{67} subunits. Therefore, it is believed that a single heptamer can interact with a maximum of three EF or LF molecules (Baldari et al., 2006). The heptamer PA-LF and PA-EF complexes enter lipid rafts, illustrated as a shaded disc in Figure 29.6, in the membrane. Binding and oligomerization trigger a receptor-mediated endocytotic event followed by internalization of the hetero-oligomeric toxin complex in a membrane-bound vesicle via clathrin-dependent, receptor-mediated endocytosis. The internalized vesicle becomes an acidic endosome. The acidic pH of the endosome triggers a conformational

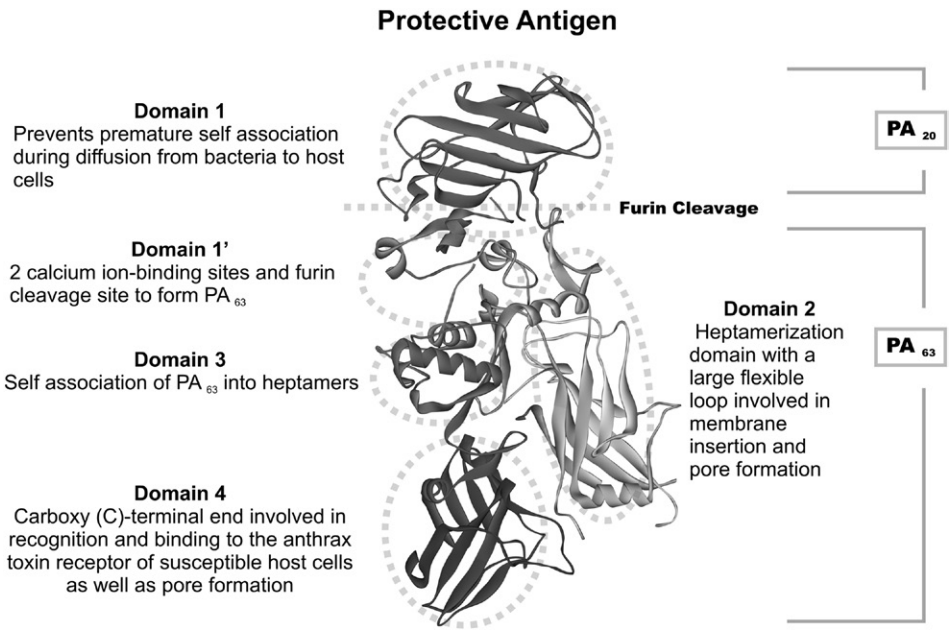


FIGURE 29.5 Three-dimensional ribbon structure of PA. PA, a 83-kDa protein with 735 residues, is organized into antiparallel β-sheets comprising four major domains (Petosa et al., 1997). Domain 1 contains the first 258 residues and the furin enzyme cleavage site between residues 164 and 167. Before translocation of soluble factors into the host cell, PA is cleaved by a cell-surface protease (furin enzyme) into PA20 (20-kDa portion containing residues 1–167) and PA63. PA20 is responsible for maintaining PA as a soluble monomer and preventing premature self-association (Collier and Young, 2003). Domain 1′ (residues 168–258) designates the N-terminal end of PA63. This domain contains two charged calcium (Ca^{2+}) atoms that function to maintain PA63 in a conformation capable of self-association into heptamers and binding to the host ligand anthrax toxin receptor (Petosa et al., 1997; Collier and Young, 2003; Gao-Sheridan et al., 2003). Domain 2, comprising residues 259–487, is illustrated with a β-barrel core structure and large flexible loops. This structure enables membrane insertion and pore formation (Petosa et al., 1997; Benson et al., 1998). Domain 3, comprising residues 488–595, is involved in self-association of PA63 into heptamers. Domain 4 contains the remaining C-terminal end of PA63 (residues 596–735) and binds to the anthrax toxin receptor on the host cell membrane (Singh et al., 1991; Collier and Young, 2003). The various domains of PA63 enable the protein anchor to the plasma membrane of the host, form heptamers, bind EF or LF, and translocate these soluble factors into the cytosol through a pore. The structure of PA was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Petosa et al., 1997; PDB ID: 1acc; Berman et al., 2000) and rendered using Accelrys DS Visualizer 2.0 software.

change in the complex, leading to insertion of a flexible loop of each PA molecule into the lipid bilayer to form a pore. Formation of the pore allows for translocation of LF and EF out of the late endosome and into the cytoplasm of the host cell. Once inside the cytoplasm, LF and EF reach their respective targets.

Structure and Activity: Edema factor (EF)

EF (89 kDa) is a calmodulin (CaM)-dependent adenylate cyclase, increasing intracellular cyclic adenosine monophosphate (cAMP) levels in the infected host cell cytosol. The first 261 N-terminal residues of EF are responsible for CaM binding (Duesbery and Vande Woude, 1999). The catalytic domain resides in amino acids 265–570 of the EF peptide sequence (Escuyer et al., 1988). The ribbon structure of EF is illustrated in Figure 29.7. The N-terminal sequence of EF is highly conserved to a similar region in LF; this domain is essential for binding to PA. Fusion of this highly conserved N-terminal sequence to other toxins, such as Shiga and diphtheria, can cause toxic effects in mammalian cells (Arora and Leppla, 1994). ET, the combination of PA and EF, causes edema when injected into the skin of experimental animals (Stanley and Smith, 1961). Injection of EF alone, in contrast, has no toxic activity.

Structure and Activity: Lethal Factor (LF)

LF has a molecular weight of 90 kDA and is one of the key agents of anthrax disease. The structure of LF is illustrated in Figure 29.8. LF consists of several domains, including a PA-binding domain, a Zn^{2+}-binding domain, an imperfect repeat region, and a catalytic domain. LF functions as a highly specific Zn^{2+}-binding metalloprotease that can cleave MAPKKs near their amino (N)-termini. This cleavage reaction can potentially inactivate one or more host cellular signaling pathways (Pannifer et al., 2001). Similar to EF, the LF N-terminal residues (1–254) serve as the binding domain for PA (Arora and Leppla, 1994). Adjacent to the PA-binding domain is a region of imperfect repeats containing 19

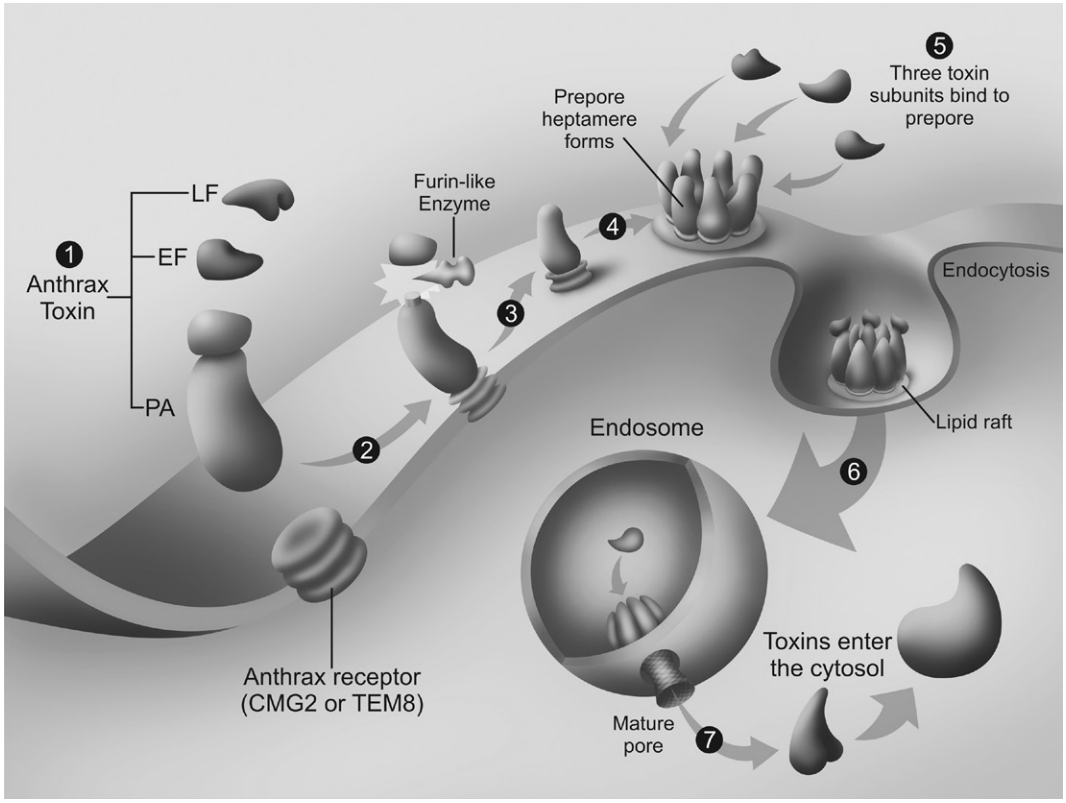


FIGURE 29.6 Anthrax toxin entry. Cellular entry of LF and EF into susceptible host cells involves an unusual mechanism for sharing PA. Binding of PA to the host cell, cleavage to form PA63, heptamerization of PA in the plasma membrane, organization into lipid rafts, binding of EF and LF to PA, internalization, PA pore formation, and translocation of anthrax-soluble factors across the vesicle membrane are illustrated. *Source: Illustrations are copyright protected and printed with permission by Alexandre M. Katos.*

amino acids each. This region was demonstrated to be essential for toxic activity. Deletion of the first of the four imperfect repeats of residues 308–383 eliminated LF toxicity. The LF catalytic domain resides in the C-terminus, where a zinc-metalloprotease consensus sequence has been identified within residues 686–692 (Klimpel et al., 1994). The zinc metalloprotease activity is responsible for the cytotoxicity of LF. LF binds Zn^{2+} (Klimpel et al., 1994), and mutations in LF that decrease zinc binding are poorly cytotoxic to cultured cells (Klimpel et al., 1994).

LF interferes in the MAPK pathway, which relays environmental signals to the machinery required for transcription in the nucleus and therefore modulates gene expression and protein synthesis. Specifically, LF inhibits MAPKKs; the identified substrates for LF enzymatic activity are MAPKK1, MAPKK2, MAPKK3 (Duesbery et al., 1998; Pellizzari et al., 1999), MAPKK4, MAPKK6, and MAPKK7 (Vitale et al., 2000). MAPKK5 has never been demonstrated to be cleaved by LF. MAPKK cleavage occurs within the N-terminal proline-rich region preceding the kinase domain, subsequently inhibiting protein–protein interactions essential for assembly of host cell signaling complexes (Hammond and Hanna, 1998).

Mechanism of Toxicity: Edema Toxin (ET)

ET does not produce major tissue damage. In fact, its major role is to impair phagocyte function (Leppla, 2000). This is consistent with other toxins that function to elevate cAMP concentrations. ET inhibits phagocytosis of spores by human PMNs similar to LF; this is in contrast to spores which promote immune cell uptake (see Table 29.1). Increased intracellular cAMP induced by EF inhibits neutrophil chemotaxis, phagocytosis, superoxide production, and microbicidal activity (Turk, 2007). EF has been shown to inhibit TNFα and to increase IL-6 production (Hoover et al., 1994). Increased cAMP levels also block LPS-induced activation of extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) MAPK pathways in monocytes through protein kinase A (PKA) activation. This might explain suppression of cytokine production by EF (Willis and Nisen, 1996). EF also activates guanine nucleotide-exchange proteins directly activated by cAMP 1, known as Epac1, through elevated cAMP levels. Epac1 inhibits phagocytosis by both alveolar macrophages and monocyte-derived macrophages (Bryn et al., 2006). EF also inhibits T-cell proliferation and subsequent cytokine production

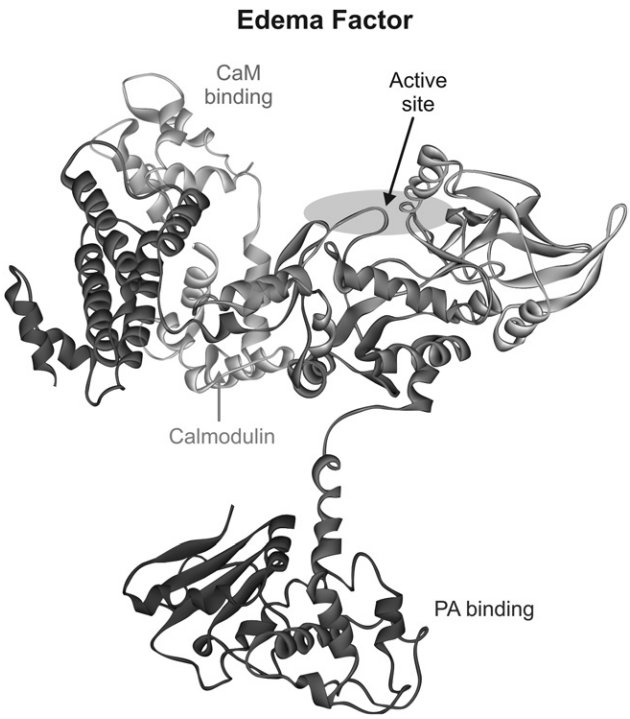


FIGURE 29.7 Three-dimensional ribbon structure of EF. The purified and crystallized structure of EF has been previously reported (Shen et al., 2005). EF is a calmodulin (CaM)-activated adenylyl cyclase and is another key factor in anthrax pathogenesis. EF contains a finger-like projection comprising a CaM-binding region, an active site to bind Mg^{2+} and 3′ deoxy-ATP, and a separate PA63 binding region. The structure of EF with bound CaM is illustrated. CaM binding causes a conformational change and activation of EF. The PA63 binding region of EF is separate from the remainder of the protein. It is shown in the same approximate position as the homologous PA-binding domain of LF in Figure 29.8 for comparison. The structure of EF was provided free of copyright restrictions from the RCSB PDB (Berman et al., 2000; PDB ID: 1xfv; Shen et al., 2005) and rendered using Accelrys DS Visualizer 2.0 software.

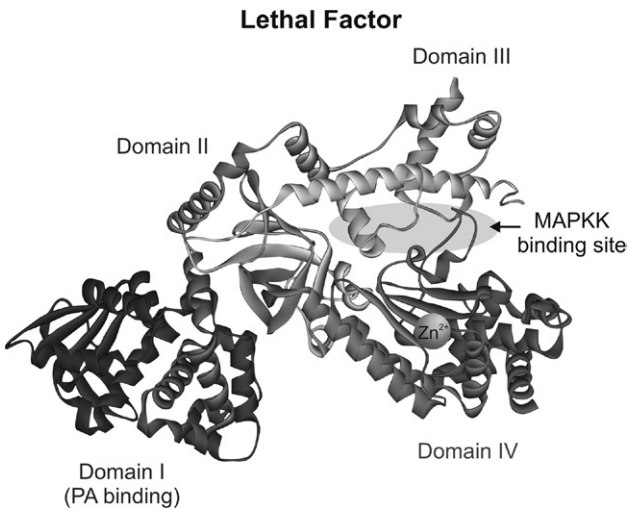


FIGURE 29.8 Three-dimensional ribbon structure of LF. The purified and crystallized LF has been reported (Bernardi et al., 2000; Pannifer et al., 2001). LF contains four recognized regions, termed domains I to IV. Residues 28–263 comprising domain I are illustrated as being entirely separate from the rest of the protein. Domain I binds the membrane-translocation component of PA63 (Lacy et al., 2002). It is homologous to the same PA binding domain of EF. Domains II to IV function together to create a long, deep pocket that holds the 16-residue N-terminal tail of MAPKK before the cleavage reaction takes place. Members of the MAPKK proteins are the only known cellular substrates of LF. The cleavage reaction removes the docking sequence for the downstream MAPK and therefore blocks cellular signaling via the MAPK pathway. Domain II has an ADP-ribosyltransferase active site. Domain III contains an α-helical bundle, and domain IV contains both a Zn^{2+}-binding motif and a catalytic center of the protease. The structure of LF was provided free of copyright restrictions from the RCSB PDB (Berman et al., 2000; PDB ID: 1jky; Pannifer et al., 2001) and rendered using Accelrys DS Visualizer 2.0 software.

through inhibition of ERK and JNK MAPK pathways (Comer et al., 2005). Therefore, EF impairs the adaptive immune response as well. EF affects other cell types; it causes endothelial cell barrier dysregulation, coagulopathy, and RBC death (Banks et al., 2006).

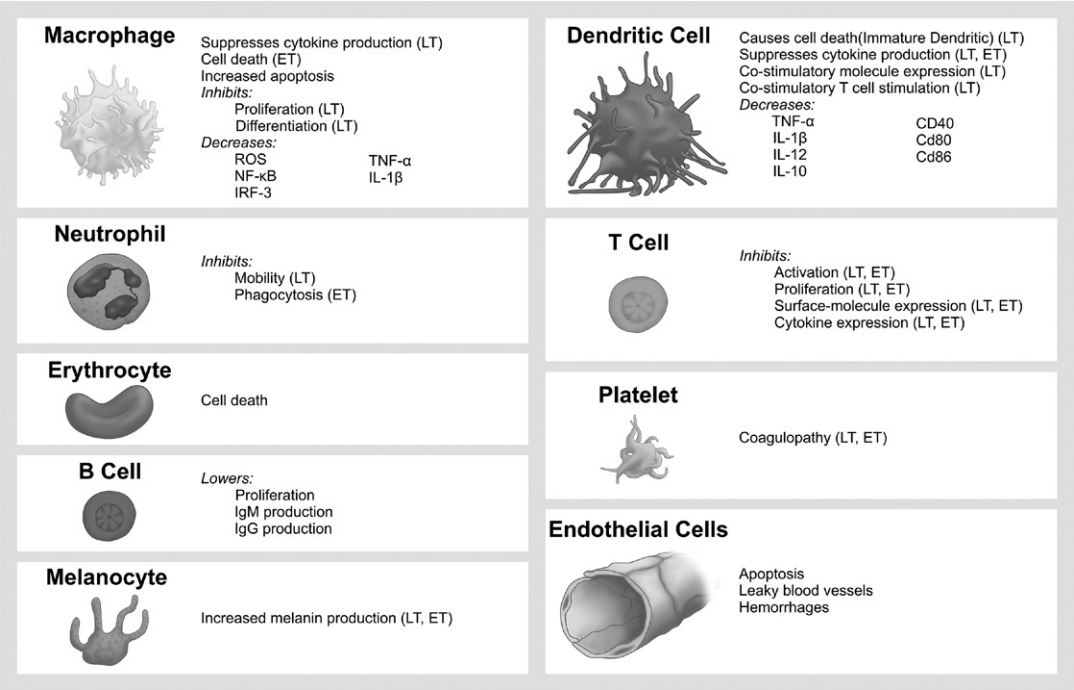
Mechanism of Toxicity: Lethal Toxin (LT)

The mechanism of death accredited to LT is unclear. A summary of the cellular targets and effects of lethal and ETs is provided in Table 29.2. LF has similar actions on phagocytes; it disables phagocytosis by macrophages and dendritic cells. Once internalized, LF cleaves the N-termini of MAPKKs, kinases for MAPKs and ERKs, and MEKs (MAPKs and ERKs) (Collier and Young, 2003; Turk, 2007). As a result, LF blocks three critical cell signaling pathways downstream of MAPKKs and MEKs. These include the ERK1/2, JNK, and p38 MAPK pathways. In addition, LF has additional targets within host cells.

There is evidence that LT suppresses pro-inflammatory cytokine production in macrophages and decreases TNFα (Pellizzari et al., 1999). These reports, which suggest impairment of innate immunity in the host cell, are in contrast to those that suggest LT functions to increase cytokines by macrophages (Hanna et al., 1993). LT has also been shown to cause an increase in ion permeability and rapid depletion of ATP in J774 macrophage-like cells, leading to cell lysis by osmotic mechanisms (Hanna et al., 1992). Inhibition of MAPKKs may block induction of NF-κB target genes, causing apoptosis of activated macrophages (Park et al., 2002).

LF effects on monocytes and macrophages are multiple. LF seems to decrease innate immune responses by blocking maturation of monocytes, which differentiate into both macrophages and dendritic cells and promote

TABLE 29.2 Effects of Anthrax Bacilli and Toxins on Various Cell Types



Source: Adapted from Koo et al. (2002), Baldari et al. (2006), Banks et al. (2006), and Turk (2007).
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death of activated macrophages (Banks et al., 2006). LF causes reductions in pro-inflammatory cytokines and inhibits the ability of dendritic cells to activate T cells *in vivo* (Agrawal et al., 2003). This negates an important mechanism of inducing adaptive immunity. Alileche et al. (2005) demonstrated that LF can be cytotoxic to human and murine dendritic cells. Cytotoxicity was demonstrated to be through either a caspase-dependent apoptotic mechanism in the case of human dendritic cells or a necrotic pathway. LF alters PMNs, important mediators of the adaptive and innate immune response. LT also slows neutrophil mobility (During et al., 2005).

LF promotes lysis of red blood cells (RBCs) when PMNs are present (Banks et al., 2006). It is believed that LT induces these immune cells to release toxic factors that are hemolytic, causing RBC lysis. Vascular damage is pathognomonic of anthrax disease. Signs of endothelial damage include hemorrhages and leaky blood vessels, leading to vascular collapse, shock, and death. LF appears to induce a caspase-dependent apoptotic pathway in endothelial cells derived from large vessels or so-called human umbilical vein endothelial cells. LF has been shown to cause similar apoptosis in small vessels, namely neonatal dermal vascular endothelial cells (Kirby, 2004).

Interactions Between LT and ET

Because their expression is coordinately regulated, both anthrax toxins circulate together during infection (Turk, 2007). These toxins have distinct mechanisms of toxicity, yet they target similar cell types. Both LF and EF cause endothelial cell barrier dysregulation, coagulopathy, RBC death, inhibition of neutrophil mobility and phagocytosis, and alterations in cytokine modulation. Acting together, EF and LF inhibit superoxide production by neutrophils and cytokine production by dendritic cells (Tournier et al., 2005). EF upregulates PA receptors, TEM 8 and CMG 2, in macrophages and increases their sensitivity to LF cytotoxicity (Comer et al., 2005). Finally, the appearance of black pigmentation characteristic of anthrax disease might be explained by the combined effects of LF and EF on melanocytes. LF has been shown to produce melanin in melanoma cell lines, an effect enhanced by the addition of EF (Koo et al., 2002).

TOXICITY

Cutaneous Anthrax

Cutaneous anthrax is the most common form of anthrax encountered worldwide, consisting of more

than 95% of anthrax cases. The most obvious sign of bacterial uptake/absorption from the skin in human anthrax cases is the developing papule. This papule progresses to become vesicular in nature and then ulcerative. Finally, a black eschar forms, which is the hallmark of human cutaneous anthrax infection (Bell et al., 2002). A more detailed look at the lesion discloses subepidermal edema, vessel thrombosis, tissue destruction, and hemorrhagic interstitium (Oncu et al., 2003).

Symptoms of cutaneous anthrax infection begin with a painless papule at the site of infection 3–5 days after exposure. After 24–36 h, the papule progresses to a vesicle 1–2 cm in diameter. Once the lesion ruptures, it slowly erodes, leaving a necrotic ulcer with a black central scab, termed an eschar. Eschar lesions typically form at exposed areas of the body (arms, hands, neck, and face). The formation of edema around the eschar lesion is also characteristic of cutaneous anthrax. Two to three weeks later, the eschar sloughs off the skin. Other signs and symptoms of cutaneous disease in humans include fever, headache, malaise, toxemia, and regional lymphadenopathy. Painful swelling of regional adrenal glands can occur.

Inhalational Anthrax

Signs and symptoms of inhalational anthrax follow a biphasic course. The initial phase is characterized by fever, malaise, and unproductive cough characteristic of an upper respiratory infection. After this flu-like phase, the patient will typically recover after 2–4 days. The second phase proceeds rapidly with the following constellation of signs and symptoms: acute dyspnea, pleural effusion, fever, progressively worsening respiratory failure, cyanosis, circulatory collapse, shock, and death, if left untreated. Death occurs 24 h after the onset of this second phase secondary to toxemia and suffocation (Dixon et al., 1999; Friedlander, 1999; Atlas, 2002; Bales et al., 2002). Although the lung is the primary site of infection here, inhalational anthrax is not considered a true pneumonia. In most, but not all cases, there is no infection in the lungs (Albrink, 1961). An infectious dose is estimated at 8,000–50,000 spores via aerosol (Franz et al., 1997).

Gastrointestinal and Oropharyngeal Anthrax

Although extremely rare, gastrointestinal anthrax has a high estimated mortality rate (25–60%). Symptoms occur 1–7 days after ingestion, presenting as either oropharyngeal or intestinal anthrax. Oropharyngeal anthrax presents as tongue lesions, lymphadenopathy, fever, and dysphagia. Intestinal anthrax presents 2–5 days after infection with abdominal pain, fever, nausea, emesis, and diarrhea. Abdominal pain and diarrhea are a direct result of ulceration, edema, and hemorrhaging of the gastrointestinal tract due to EF and LF (Friedlander, 1997). Therefore, diarrhea may be bloody with extensive fluid loss and circulatory collapse. If treatment is not started early, then toxemia and shock may develop, resulting in death. Oropharyngeal anthrax produces a milder infection than the intestinal form and leads to a better outcome. Treatment for either form reduces symptoms and typically leads to full recovery 10–14 days after infection.

Meningitis

Meningitis is a complication of all anthrax infections and is associated with high mortality. Anthrax meningitis occurs through either a hematogenous or lymphatic spread from the infection site to the central nervous system (CNS) (Sejvar et al., 2005). High bacterial counts from anthrax bacteremia allow for *B. anthracis* to cross the blood–brain barrier into the CNS to infect the meninges and cerebrospinal fluid (CSF). Anthrax meningitis is most common with inhalational anthrax and is seen in ~50% of those cases. Development of anthrax-associated meningitis is a very grave sign and invariably fatal. Even with antibiotic treatment, death occurs approximately 1–6 days after signs and symptoms of meningitis appear (Dixon et al., 1999), and the mortality rate is estimated to be 94% (Sejvar et al., 2005).

Symptoms at the onset of anthrax meningitis include fever, headaches, nausea, vomiting, chills, malaise, agitation, and nuchal rigidity (Sejvar et al., 2005). Delirium, coma, refractory seizures, and neurological degeneration occur within 2–4 days (Sejvar et al., 2005). Neurological degeneration can be seen with signs that include cranial nerve palsies, myoclonus, fasciculations, decerebrate posturing, and papilloedema (Sejvar et al., 2005). A notable feature of anthrax meningitis infection includes subarachnoid and intraparenchymal hemorrhages. Hemorrhage can be observed in the CSF, as well as polymorphonuclear pleocytosis, an increase in protein concentration, and a decrease in glucose concentration. *B. anthracis* can be observed in the CSF, meninges, subarachnoid space, and brain parenchyma.

DETECTION AND DIAGNOSIS

Detection

Detection and diagnosis of anthrax infection can be difficult. Diagnosis is made predominantly on a positive history of exposure to contaminated animal products and a physical examination of the presenting signs and symptoms. Tests to detect *B. anthracis* are typically never ordered unless there is evidence of exposure. The disease typically progresses to an advanced stage before initiating appropriate treatment. The formation of a black eschar with

hyperemic and edematous borders is a hallmark of cutaneous anthrax infection. Unfortunately, the black eschar occurs in the later stages of infection. The appearance of painless, pruritic papules with edematous borders is suggestive of possible cutaneous anthrax infection. Further tests to identify *B. anthracis* as the infectious agent should be attempted but should not delay initiation of antibiotic treatment. Evidence of pulmonary involvement on chest X-ray can provide suspicion of inhalational anthrax. The common triad of findings is mediastinal widening, pulmonary infiltrates, and/or pleural effusions (Friedlander, 1997; Dixon et al., 1999). Without a proper diagnosis, progression of some forms (i.e., inhalational anthrax) to the second stage of infection is nearly 100% fatal.

Depending on the form of disease suspected, certain specimens should be collected. If cutaneous anthrax is suspected, then swabs of the lesion are warranted. A sterile, dry swab should be used to collect vesicular fluid if the lesion is in the vesicular stage. If the lesion is in the eschar stage, then the edge of the eschar should be lifted and the swab should rotate underneath for 2–3 s. However, if the lesion is not in either the vesicular or eschar stage, the base of the ulcer can be swabbed with a sterile swab moistened with saline. Blood cultures should be collected regardless of the route of exposure or signs of disease (Beatty et al., 2003). If gastrointestinal anthrax is suspected, then ascitic fluid should be taken, and if oropharyngeal anthrax is suspected, then swabs from oropharyngeal lesions should be taken (Beatty et al., 2003). If pleural effusions are seen, then pleural fluid should be collected, and if meningeal signs/symptoms are present, then CSF should be collected.

Diagnostics

Laboratory tests for anthrax can include Gram staining, differential plating, γ-bacteriophage plaque assay, blood cultures, motility tests, enzyme-linked immunosorbent assays (ELISAs), and fluorescent covalent microsphere immunoassay (FCMIA). Although microbial tests take 24 h to perform, often delaying the diagnosis, these tests may be necessary to confirm a diagnosis of anthrax. If there is a high index of suspicion for anthrax, then initiation of therapy should not be delayed for results of these confirmatory tests.

Microbiological Tests

Simple tests to rule out anthrax infection are Gram staining of cultured bacteria, differential plating, and a γ-bacteriophage plaque assay. Gram staining is nearly always used in the identification of bacteria (CDC, ASM, APHL, 2002). *B. anthracis* will appear as large, Gram-positive rods in short chains. The size of the bacterium is approximately 1–1.5 by 3–5 μm. *B. anthracis* capsules will not absorb India ink stain and will cause the bacilli to appear as clear zones on a black, stained background. *B. anthracis* can be distinguished from other *Bacillus* species by culturing the organism on blood agar plates. It is the only *Bacillus* species that does not cause hemolysis. In addition, *B. anthracis* cells are lysed by the bacterial virus γ-bacteriophage to form small plaques on nutrient agar plates.

Blood cultures should follow normal laboratory protocol. Specimens collected for cutaneous anthrax can be plated on 5% sheep blood agar (SBA), MacConkey agar (MAC), or any media normally used for surface wounds (CDC, ASM, APHL, 2002). Stool specimens from gastrointestinal anthrax cases should be plated on phenylethyl alcohol agar, MAC, and SBA. Sputum specimens from inhalational anthrax patients should be plated on chocolate agar, SBA, and MAC. Cultures will show isolated *B. anthracis* colonies 2–5 mm in diameter, flat or slightly curved, with a wavy border after 15–24 h. *B. anthracis* shows growth on SBA and not on MAC. Colonies of *B. anthracis* plated on SBA will appear as a "beaten egg white" when teased with a loop (basic diagnostic testing). These blood cultures are useful, especially in the differential diagnosis of anthrax, and should be completed before antibiotic treatment is administered.

A motility test is useful because *B. anthracis* is a nonmotile bacterium. Two motility tests available are the wet mount and motility medium variety. In a wet-mount preparation, organisms with Brownian movement or no movement will support the presence of *B. anthracis*. The presence of *B. anthracis* in a motility medium preparation would be a single line of growth along the original inoculum stab (CDC, ASM, APHL, 2002).

Molecular Tests

Acute and convalescent serum samples for serological ELISA testing should be collected for diagnosis. The CDC developed an ELISA for the detection of *B. anthracis* PA using immunoglobulin G (IgG) antibodies in response to the bioterrorist anthrax plot in 2001. ELISA proved extremely useful in the detection of cutaneous and inhalational anthrax (Quinn et al., 2002). The diagnostic sensitivity and diagnostic specificity for this test are 97.6% and 94.2%, respectively.

A more recent study reports another tool that detects anthrax better than ELISA detection of PA (Biagini et al., 2004). The FCMIA can be multiplexed, meaning that numerous analytes can be measured simultaneously. In anthrax cases, anti-PA and anti-LF can be examined in serum samples at the same time. FCMIA is more sensitive, rapid, and reliable than ELISA.

Histopathology

Punch biopsies can confirm cutaneous anthrax if Gram stain and culture results are negative, due to antibiotic treatment (Godyn et al., 2005), and a suspicion of cutaneous anthrax remains. The center of the eschar, the

erythematous region, and the skin margin should all be included in the biopsy when an eschar is present (Godyn et al., 2005).

Radiology

Radiological tests are available to narrow the differential diagnosis for anthrax. A chest radiograph will almost always exhibit a widened mediastinal contour that includes unilateral or bilateral hilar enlargement, as well as prominent periobronchovascular markings and pleural effusions (Frazier et al., 2006). The size of the widened mediastinum contours and pleural effusion can increase rapidly over hours or days. However, findings from the radiograph might be subtle and even appear normal in the early stages of infection (Frazier et al., 2006). Noncontrast CT will exhibit high-density mediastinal and hilar lymphadenopathy, which can rapidly increase in size over days (Friedlander, 1997; Frazier et al., 2006). This indicates both hemorrhage and edema in the mediastinal lymph nodes. In addition, contrast-enhanced CT on mediastinal nodes can display rim enhancement and central hypodensity (Frazier et al., 2006). The role of radiological tests has not been identified in the diagnosis of gastrointestinal/oropharyngeal anthrax (Beatty et al., 2003). Radiographs of patients with gastrointestinal anthrax displayed findings indicative of obstruction; however, further studies need to be completed to determine if radiology can be used to help diagnose gastrointestinal anthrax (Beatty et al., 2003). Patients with anthrax disease can also acquire complications of anthrax meningitis. CT or magnetic resonance imaging shows hemorrhages in the deep gray matter, subarachnoid space, and ventricles (Sejvar et al., 2005).

RISK ASSESSMENT

The risks posed by an intentional outbreak of anthrax cannot be minimized because the potential effects go beyond merely the medical. The primary costs of a biological terrorism-related anthrax event in terms of lives lost, medical resources required to treat affected individuals, and economic burden to decontaminate spores are significant. Certainly, the subsequent psychological and societal costs could be as high or higher, based on the US Federal government experience with the 2001 anthrax letters. "On a collective level, a major epidemic of anthrax can destroy the social order" (Guillemin, 1999).

The distribution of *B. anthracis* endospores in mailings through the US Postal Service in the fall of 2001 served to ignite public awareness concerning anthrax as a weapon of mass destruction. Deliberate contamination of the mail resulted in 22 cases of anthrax (11 inhalational and 11 cutaneous). These mailings led to five deaths among the inhalational anthrax cases and an enormous economic burden associated with decontamination. The media coverage and public fallout unveiled the deficiencies in our current risk assessment of anthrax.

The effects on public order, as well as economic and social effects, should be considered in an assessment of weaponized anthrax risk. Decontamination of the Hart Senate Office Building in Washington, DC, required 7 months at a cost of $23 million. Decontamination of the postal facilities that processed the "anthrax letters" in Brentwood, DC, and Hamilton Township, NJ, required more than 1 year at a cost in excess of $100 million (Fernandez, 2002). The psychological effects of an anthrax attack could exceed the medical or economic effects, especially in the short term. Panic caused by seemingly random outbreaks of symptoms in apparently unrelated persons or events "could degenerate into panic, flight, communications breakdown, general societal dysfunction" (Wein et al., 2003).

A comprehensive risk assessment is dependent, in part, on determining the health risk posed to the individual by this biological agent. Animal models play a critical role by providing key sources of information for predicting consequences of human exposure because comparable naturally occurring human indices are rare. These animal models have been reviewed in previous sections throughout this chapter. Another method to assess risk is to use an anthrax stimulant. The Defense Research Establishment Suffield in Canada undertook a series of experiments to assess the risk of envelopes filled with anthrax spores (Kournikakis et al., 2001). In their experiments, envelopes containing spores of the nonpathogenic *Bacillus globigii* were opened in a mock mail room inside an aerosol test chamber to estimate aerosol release from the envelopes. Investigators showed that dispersal by passive letter opening was far more effective than initially thought. A lethal dose (LD) could be inhaled within seconds of opening the content (Kournikakis et al., 2001). Not only would the mail handler opening the envelope receive an LD of between 500 and 3,000 LD_{50}s but also other workers in the room would inhale lethal numbers of spores.

In silico models of anthrax release can be used to bridge the gap to help predict consequences of human exposure in the event of an outbreak or biological attack scenario. Several modeling studies have been published (Wein et al., 2003) in attempts to assess anthrax risk. The model of Wein et al. (2003) assumes a point-release of 1 kg of spores at a height of 100 m over a city of 10 million inhabitants. The effects on mortality of different antibiotic treatment strategies in an urbanized anthrax release are examined. This *in silico* biological weapon attack results in more than 100,000 deaths, given that both symptomatic and asymptomatic persons receive antibiotics and the model incorporates availability and distribution parameters. In the base case, an approximation of

current treatment and distribution strategies, deaths are estimated at more than 1% (Wein et al., 2003). The model predicts significantly higher death rates (≥7 times) in cases with less aggressive distribution or administration being modeled (Webb, 2003).

One of the greatest fears of a scenario involving anthrax is the application of genetic engineering to enhance its pathogenicity. Several studies have demonstrated the ability to generate antibiotic-resistant anthrax (Pomerantsev et al., 1993). In addition to developing multiple antibiotic-resistant strains of *B. anthracis*, biotechnology offers a highly likely scenario that the very nature of *B. anthracis* could be altered. It is possible that *B. anthracis* can be engineered to produce novel toxin activity (Sirard et al., 1997a,b). Other approaches may focus on the modification of PA such that it remains functional but immunologically distinct from the PA used in current vaccines.

TREATMENT

Overview

Treatment of any form of anthrax infection is generally the same: aggressive antibiotics and supportive care. Rapid definitive diagnosis of anthrax is critical for effective treatment, although in cases in which anthrax is suspected before confirmation, antibiotic therapy should not be withheld pending test results. Postexposure treatment should be administered in cases of putative exposure to anthrax spores to prevent systemic disease. Antibiotics and supportive management are the mainstays of treatment to prevent septic shock, fluid and electrolyte imbalance, and dyspnea associated with systemic anthrax disease. Initial administration of ciprofloxacin or doxycyclin is recommended (USFDA, 2001; CDC, 2001a–c). A duration of 60 days has been recommended for prophylaxis, although the most efficacious duration has not been determined (Brook, 2002).

Intravenous ciprofloxacin or doxycycline is recommended for treatment of anthrax, usually as part of a cocktail of antibiotics (CDC, 2001a,b). Multiple antibiotics are usually indicated in anthrax cases with signs of septicemia, extensive edema, or for cases with cutaneous lesions in the head and neck (Brook, 2002). Penicillin may be included in the antibiotic cocktail but is not recommended as a stand-alone therapy due to *B. anthracis* β-lactamase production. *B. anthracis* can express β-lactamase variants, penicillinases and cephalosporinases, which would undermine a lone-penicillin therapy (Lightfoot et al., 1990). *B. anthracis* has shown *in vitro* resistance to cephaloporins and trimethoprim-sulfamethoxazole (Inglesby et al., 2002). Corticosteroid therapy may help treat edema from head and neck lesions or prevent airway obstruction. Table 29.3 contains therapeutic guidelines based on CDC recommendations (CDC, 2001a–c) for pharmacologic management of *B. anthracis* infection.

Inhalational, Oral, and Gastrointestinal Anthrax

At the time of writing, the recommended initial therapy for inhalational, oral, and gastrointestinal anthrax in adults is 400 mg of ciprofloxacin every 12 h or 100 mg of doxycycline every 12 h, administered intravenously (Inglesby et al., 2002). In addition to these treatments, one or two additional antimicrobials should be administered (list mentioned) (Inglesby et al., 2002). These guidelines should be followed until the condition of the patient improves. The treatment should then be switched to either 500 mg of ciprofloxacin twice per day or 100 mg of doxycycline twice per day (Inglesby et al., 2002). This stage of treatment should begin intravenously and switch to oral dosing when appropriate. Treatment should continue for 60 days. Oral amoxicillin can be used as an alternative in adults and children for completion of therapy only after clinical improvement (CDC, 2001a,b; Inglesby et al., 2002). Pediatric guidelines for medical management of anthrax are provided in Table 29.3, in addition to recommendations for pregnant women and immunocompromised patients.

Cutaneous Anthrax

The recommended initial therapy for adults with cutaneous anthrax is either 500 mg of ciprofloxacin orally administered twice per day, or 100 mg of doxycycline orally administrated twice per day (Inglesby et al., 2002). The duration of therapy is suggested to be 60 days (CDC, 2001a,b), although previous guidelines recommend 7–10 days (Inglesby et al., 2002). Intravenous therapy and a multidrug regimen approach are recommended for patients with signs of extensive edema or head/neck lesions (CDC, 2001c). The guidelines for pharmacologic management of cutaneous anthrax in pediatric patients and pregnant women are also provided (Table 29.3). With early treatment, systemic disease does not occur, and laboratory cultures will yield negative results for *B. anthracis*; however, treatment will not curtail formation of the black eschar and progression of the edematous skin ulcer (CDC, 2001c).

Bacteremia

Bacteremia often occurs with anthrax infection, especially with gastrointestinal/oropharyngeal and inhalational anthrax. A multidrug approach consisting of ciprofloxacin or doxycycline, along with one or two other antimicrobials, is suggested when bacteremia is suspected (CDC, 2001c). Other drugs that are

TABLE 29.3 Anthrax Therapeutic Guidelines[a]

		Adults		Children		Pregnant Women	
Therapy		**Initial Therapy[b] (Intravenous Dosing)**	**Oral Dosing**	**Initial Therapy (Intravenous)**	**Oral Dosing**	**Initial Therapy**	**Duration**
Recommended first line treatment—inhalational, GI, and oropharyngeal anthrax[c]	Ciprofloxacin (use in combination with one or two additional antimicrobials listed below)	Intravenous treatment initially (400 mg q 12 h) in combination with one or two additional antimicrobials[d]	Switch to oral dosing when appropriate (500 mg PO BID)	Intravenous treatment initially (10–15 mg/kg q 12 h)[e] in combination with one or two additional antimicrobials	Switch to oral when appropriate (500 mg PO BID)	Same for adults	
		Duration: Continue for 60 days total (i.v. and p.o. combined)[f]					
	Doxycycline[g] (use in combination with one or two additional antimicrobials listed below)	Intravenous treatment initially (100 mg q 12 h) in combination with one or two additional antimicrobials[d]	Switch to oral when appropriate (100 mg PO BID)	Intravenous treatment initially >8 years and >45 kg (100 mg q 12 h) >8 years and <45 kg (2.2 mg/kg q 12 h) <8 years (2.2 mg/kg q 12 h)	Switch to oral therapy when appropriate >8 years and >45 kg (100 mg PO BID) >8 years and <45 kg (2.2 mg/kg PO BID) <8 years (2.2 mg/kg PO BID)		
		Duration: Continue for 60 days total (i.v. and p.o. combined)[f]					

	Adults		Children		Pregnant Women		
Therapy	**Initial Therapy (Oral)[h]**	**Duration**	**Initial Therapy (Oral)**	**Duration**	**Initial Therapy**	**Duration**	
Recommended first line treatment—cutaneous anthrax[c]	Ciprofloxacin	500 mg BID	60 days[f]	10–15 mg/kg q 12 h (not to exceed 1 g/day)	60 days[f]	500 mg BID	60 days[f]
	Doxycycline[g]	100 mg BID		>8 years and >45 kg (100 mg q 12 h) >8 years and <45 kg (2.2 mg/kg q 12 h) <8 years (2.2 mg/kg q 12 h)		100 mg BID	
Alternative option in cutaneous anthrax[i]	Amoxicillin		500 mg PO TID		80 mg/kg/day divided every 8 h		

[a] *Therapeutic treatment protocol for immunocompromised persons is the same for immunocompromised adults and children.*
[b] *Steroids may be considered in as adjunct therapy for patients with severe edema and for meningitis. (Dexamethosone: Adults (0.75–0.90 mg/kg/day orally, i.v., or i.m. in divided doses every 6 h); Children (0.25–0.50 mg/kg every 6 h)) (Prednisone: Adults (1–2 mg/kg or 5–60 mg orally/day); Children (0.5–2 mg/kg/day).)*
[c] *Ciprofloxacin or doxycycline are first line therapeutics for all forms of anthrax.*
[d] *Other agents with in vitro activity include rifampin, vamcomycin, penicillin, ampicillin, chloramphenicol, imipenem, clindamycin, and clarithromycin. Because of concerns for beta-lactamases in B. anthracis, penicillin and ampicillin should not be used alone. Consultation with an infectious disease specialist is advised.*
[e] *In children, ciprofloxacin dosage should not exceed 1 g/day.*
[f] *Due to the potential for spores to persist after an aerosol exposure, antimicrobial therapy should be continued for 60 days.*
[g] *If meningitis is suspected, doxycycline may be less optimal due to poor CNS penetration.*
[h] *Cutaneous anthrax with signs of systemic involvement, extensive edema, or lesions of the head or neck require intravenous therapy, and a multidrug therapeutic approach.*
[i] *Amoxicillin is an alternative option in adults and children for completion of therapy only after clinical improvement.*

recommended for use with ciprofloxacin or doxycycline include rifampin, vancomycin, imipenem, chloramphenicol, penicillin/ampicillin, clindamycin, and clarithromycin (CDC, 2001c).

Anthrax Meningitis

There is limited clinical experience for treating patients with anthrax meningitis (Sejvar et al., 2005). However,

the recommended therapy is also a multidrug treatment, including the use of a fluoroquinolone and two additional drugs with excellent CNS penetration. The fluoroquinolone that is recommended for use is ciprofloxacin. The ciprofloxacin level in CSF is 26–50% of serum levels. Other fluoroquinolones include levofloxacin, gatifloxacin, moxfloxacin, and ofloxacin. Doxycycline is not recommended as a first-line agent for anthrax meningitis. Although doxycycline has a low minimum inhibitory concentration (MIC) (0.03 mg/L), it has low CNS penetration and a lower percentage of serum levels in the CSF than ciprofloxacin (10–26%). These drugs have been shown to work against *B. anthracis*; however, these drugs have not been observed in humans and have not been tested in animal models. Drugs with significant CNS penetration include ampicillin, meropenem, rifampicin, or vancomycin. Although penicillin is a beta-lactam, like ampicilin and meropenem, it is not suggested for use against anthrax meningitis for the reasons stated. Similar to the treatment suggestions, the recommended duration of therapy is 60 days. Even with the effectiveness of antibiotic treatment, there is no guarantee that anthrax will be diagnosed in time to initiate therapy. Therefore, anthrax represents a significant risk to the public because it is lethal, potent, and induces public anxiety.

Vaccines

Although vaccines are promising, further research needs to be conducted with the goal of updating vaccine technology. Conclusive demonstration of seroconversion against an anthrax infection was demonstrated in animals previously immunized with an active anti-PA vaccine and later administered postexposure antibiotic therapy (Friedlander et al., 1993). Therefore, other therapies, such as injection of anthrax-specific monoclonal antibodies (Maynard et al., 2002), may lead to a promising treatment after exposure to *B. anthracis*. Both of the current anthrax vaccines, *anthrax vaccine adsorbed* (AVA) and a newer one based on recombinant PA (rPA), offer long-lasting protection against an aerosol challenge of *B. anthracis* in rhesus macaques. Efficacy of both vaccines is primarily attributable to the PA. The AVA vaccine offers similar protection from challenge in rabbits; however, the duration of immunity is unknown (Phipps et al., 2004). Various negative side effects have been reported after administration of the current anthrax vaccine to humans, and it would be beneficial to develop a vaccine that has fewer side effects before instituting a massive vaccination plan. Recent reports suggest that the addition of CpG oligonucleotides may offer an improved response. These CpG motifs interact with various receptors on B cells and dendritic cells, which improve antigen presentation and upregulate pro-inflammatory cytokine expression. After co-administration of CpG oligonucleotides with the AVA vaccine, this combination triggered a fast and higher-titer immune response in comparison with the vaccine alone (Klinman et al., 2004). These findings suggest a potential for using oligonucleotides as an adjuvant in a postexposure vaccine.

Other studies have used an adenoviral delivery system to invoke a protective immune response in mice (Tan et al., 2003). Immunization with this form of vaccine demonstrated a rapid anti-PA antibody response at a higher level than that of the current vaccine. Further, this method of delivery offered a longer protection time in comparison with that of the rPA vaccine. However, current public fears of using a virus as an immunization vector, coupled with the lack of similar findings with other model systems, make it imperative to further research this potential area of vaccine technology.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The fear of *B. anthracis* has only been heightened in recent years as a result of the terrorist events in 2001 and the increase in acts of terrorism worldwide. There are few infectious agents that are more feared or notorious than *B. anthracis*, the causative agent of the zoonotic disease anthrax. *B. anthracis* would be an ideal weapon for a terrorist attack not only because of the disease pathology, but also because this organism can sustain desiccation and survive long-term outside of its host as a dormant spore. Moreover, infection of humans by inhalation of spores has been reported. Thus, *B. anthracis* disseminated in an aerosol could be a feasible means for targeting and infecting a large population.

The processes of *B. anthracis* infection and pathogenesis have been studied in various animal models. During the initial phases of infection, regardless of the route of infection (i.e., inhalational, dermal, or oral), the host animal may display general symptoms that are characteristic of numerous infections or disease states, making a positive diagnosis difficult. Pathogenesis involves initial uptake of dormant spores by phagocytes. Germination takes place soon afterwards either at the site of inoculation or later after transport to the lymph nodes. It is the vegetative cell that moves through the circulatory system and lymphatics to infect other target organs. Lungs and gastrointestinal tract are major targets for vegetative bacilli. Later the spleen, brain, liver, and almost every other organ in the body can be colonized. In systemic anthrax, there is overwhelming septicemia. Although septicemia is important, lethality from *B. anthracis* is believed to be mediated by the actions of anthrax toxins (LT and ET). The toxins are thought to be primarily responsible for causing immune system suppression, necrosis of critical cells, vascular leakage, hemorrhage, shock, and death.

There are numerous gaps in our understanding of anthrax. Although the general pathogenesis of *B. anthracis* has been studied in a variety of animals, a number of important questions still remain unanswered. Although spore uptake has been demonstrated in macrophages and dendritic cells, the molecular interactions involving uptake, spore germination, and escape require additional research. Further *in vitro* and *in vivo* studies will help to elucidate these mechanisms. Understanding of the molecular mechanisms of TLR signaling and its relationship to LT-mediated apoptosis may lead to new therapies. The inhibition of TLR-mediated signaling of apoptosis may inhibit macrophage cell death and assist in controlling the pathogen.

Several studies have shown bacilli in many organs of the body after various routes of exposure. However, the time course of transit to target organs and the targeting order of these organs may be helpful in further defining therapeutic windows of opportunity. Finally, the molecular mechanisms of toxicity and death caused by LT and ET remain unclear. There is no direct evidence that proves the importance of macrophage apoptosis in the course of disease progression. Recent reports suggest that LT may induce apoptosis of endothelial cells, which suggest that macrophages are not the sole cell type affected by LT (Kirby, 2004). In addition, reports that demonstrate LT reduces cytokine expression (Pellizzari et al., 1999; Moayeri and Leppla, 2004) suggest that death may not be due to an inflammatory reaction.

Several animal model studies have focused on the immune response to infection and also to vaccination. Most studies identify antibody titer, but some also show cytokine production or possible mechanisms of bacterial evasion. A focus on immune response studies will help to further define *B. anthracis* pathogenesis and provide insight into the design of future vaccines and therapeutics. Most countermeasure studies have been conducted on vaccines, because antibiotic studies have typically been performed *in vitro*. Primary concerns are the length of required treatment, efficient prophylaxis methods, and less complicated dosing regimens for vaccines. In a theoretical bioterrorist attack scenario, it will be essential to distribute effective treatment and prophylaxis to infected or potentially exposed persons in an expedient manner.

The choice of an appropriate animal model system that most closely mimics the human response to *B. anthracis* infection is crucial to the development of improved treatments and more effective vaccines. Such development relies on testing in appropriate animal models in which disease, infection, and progression mimic that seen in humans. The importance of selecting an appropriate animal model for human anthrax is magnified by the potential of a genetically engineered *B. anthracis* bacterium made more virulent than its predecessor. The addition of antibiotic resistance genes would render prophylactic treatment against infection by modified organisms difficult, if not futile. Further, the inclusion of genes that encode toxins not naturally found in *B. anthracis* may assist the bacterium in escaping host immunosurveillance. Therefore, defining the pathogenesis of *B. anthracis* and designing appropriate countermeasures is a critical necessity for treatment of those exposed in occupational settings, national security, and the global community. Whether medical systems would be able to provide treatment early enough to prevent widespread disease in the event of a bioterrorist attack involving anthrax remains questionable.

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CHAPTER

30

Onchidal and Fasciculins

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INTRODUCTION

Onchidal and fasciculins are natural toxins that produce their toxicity in mammalian systems primarily by virtue of acetylcholinesterase (AChE) inhibition. AChE hydrolyzes acetylcholine (ACh) and inactivates AChE, thereby regulating the concentration of the transmitter at the synapse. Termination of activation normally depends on dissociation of ACh from the receptor and its subsequent diffusion and hydrolysis, except in diseases where ACh levels are limited or under AChE inhibition, conditions that increase the duration of receptor activation (Silver, 1963).

The toxins that inhibit the AChE are called *anticholinesterase (anti-ChE)* agents. They cause accumulation of ACh in the vicinity of cholinergic nerve terminals and thus are potentially capable of producing effects equivalent to excessive stimulation of cholinergic receptors throughout the central nervous system (CNS) and peripheral nervous system (Long, 1963). Nevertheless, several members of this class of compounds are widely used as therapeutic agents; others that cross the blood–brain barrier (BBB) have been approved or are in clinical trial for the treatment of Alzheimer's disease. The treatment approaches in the neurodegenerative pathology of Alzheimer's disease continue to be primarily symptomatic, with the therapeutic strategies based on the cholinergic hypothesis, and specifically on AChE inhibition (Lane et al., 2004).

AChE can be classified in several ways. Mechanistically, it is a serine hydrolase. Its catalytic site contains a catalytic triad—serine, histidine and an acidic residue—as do the catalytic sites of the serine proteases (such as trypsin), several blood clotting factors, and others. However, the acidic group in AChE is a glutamate, whereas in most other cases, it is an aspartate residue. The nucleophilic nature of the carboxylate is transferred through the imidazole ring of histidine to the hydroxyl group of serine, allowing it to displace the choline moiety from the substrate, forming an acetyl-enzyme intermediate. A subsequent hydrolysis step frees the acetate group. Understanding of the catalytic properties of the protein has assisted in our understanding of its inhibition by organophosphate (OP) and carbamate (CM) inhibitors. However, several questions remain to be answered regarding AChE catalysis, such as the mechanism behind the extremely fast turnover rate of the enzyme (Fair et al., 1994). Despite the fact that the substrate has to navigate a relatively long distance to reach the active site, AChE is one of the fastest-reacting enzymes (Nair et al., 1994). One theory to explain this phenomenon has to do with the unusually strong electric field of AChE. It has been argued that this field assists catalysis by attracting the cationic substrate and expelling the anionic acetate product (Ripoll et al., 1993). Site-directed mutagenesis, however, has indicated that reducing the electric field has no effect on catalysis. However, the same approach has indicated an effect on the rate of association of fasciculin, a peptide that can inhibit AChE (Schafferman et al., 1994).

Naturally occurring irreversible inhibitors of AChE are toxins that are often selective inhibitors of protein function, and this property can often be exploited for a variety of purposes (Pita et al., 2003). For instance, physostigmine (also called eserine), is a naturally occurring alkaloid reversible inhibitor of AChE that has been used in understanding the kinetic mechanism of AChE. Through its action at both the central and peripheral cholinergic receptors, physostigmine reverses anticholinergic activity and ameliorates the coma, delirium, and seizures that accompany severe toxicity. Antidotes for such poisoning have been developed to accelerate enzyme regeneration.

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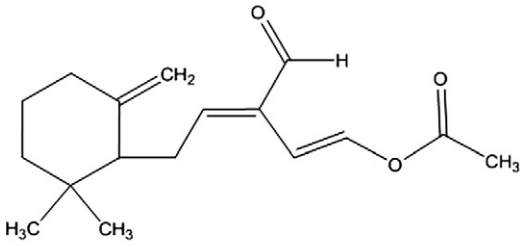
The slow reactivation of AChE is the basis of the use of physostigmine and pyridostigmine (a physostigmine derivative) as a prophylactic in anticipation of the use of these toxins. It is argued that the psychological stress associated with warfare impaired the integrity of the BBB, allowing the peripheral-acting inhibitor of AChE to penetrate the brain, where it activated AChE transcription (Kaufer et al., 1998). For further details on toxins/toxicants and the BBB, see Chapter 49.



Onchidal

FIGURE 30.1 The chemical structure of onchidal.

BACKGROUND

This chapter covers natural agents that prolong the existence of ACh after it is released from cholinergic nerve terminals. Natural toxins are chemical agents of biological origin (including chemical agents and proteins), and can be produced by all types of organisms; such is the case for onchidal and fasciculins. Although a chemical and a protein, respectively, onchidal and fasciculins share the same toxic effect of inhibiting AChE, which is concentrated in synaptic regions and is responsible for the rapid catalysis of the hydrolysis of ACh. As such, the natural toxins onchidal and fasciculins can produce disorders of neuromuscular transmission that are clinically categorized as either presynaptic or postsynaptic; some toxins simultaneously affect both sites. Chemical agents associated with neuromuscular transmission syndromes include the fasciculins, crotoxin, taipoxin, tubocurarine, and OP compounds (Anadón and Martínez-Larrañaga, 1985).

These aforementioned natural anti-ChE agents can be developed for a different purpose, including extensive application as toxic agents (i.e., potential chemical weapons). One of the first interesting structures with cholinergic properties isolated from a marine source is onchidal. This compound was first isolated from the mollusk *Onchidella binneyi* and has an acetate ester similar to ACh. Upon isolation, onchidal was discovered as being an active, site-directed, and irreversible inhibitor of AChE (Abramson et al., 1989).

Neurotoxins from snake venoms have proved to be valuable tools for the understanding of synaptic transmission mechanisms. Likewise, the powerful inhibitory action of fasciculins against mammalian AChE makes them potentially useful for pharmacological and neurochemical research. Studies of their biochemical and electrophysiological effects on the CNS and biochemical characterization are now being carried out.

Natural toxins can be extremely potent, and many of them are effective at far lower dosages than conventional chemical agents. Natural toxins, as compounds of biological origin, are often classed as biological agents, but they are not infectious and are more similar to chemicals with respect to their military potential for tactical use; therefore, they should be considered to be chemical agents. The Chemical Weapons Convention (CWC 2003) (available at www.fas.harvard.edu/~hsp/cwc/cwcbyart.html) also includes natural toxins as chemical agents, specifically including the onchidal and fasciculin toxins in its control regime, along with other highly toxic chemicals.

Onchidal

Onchidal is a toxic component of a poisonous marine opisthobranch mollusk. Like other opisthobranchs, the Onchidiacea family of mollusks does not have the protection of a hard external shell as most mollusks do. They rely instead on the production of a defensive secretion. When the animal is disturbed, it emits a viscous fluid from specialized glands. In two species of Onchidella (*Onchidella floridanun* and *Onchidella borealis*), this defensive secretion has been shown to act as a deterrent to potential predators, including fish and crabs. Chemically, it is a simple lipophilic acetate ester (Figure 30.1).

Onchidal has been identified as the major lipid-soluble component of the defensive secretion of *O. binneyi*, and it has been proposed as the compound responsible for the chemical protection of *Onchidella* species. *O. binneyi* is an opisthobranch mollusk that inhabits the rocky intertidal zone near the area of Baja California, Mexico. The defensive secretion was obtained in the field by squeezing the mollusk and collecting the mucus discharge in capillary tubes. Large quantities of this material could be obtained after the extraction of intact animals with acetone (Ireland and Faulkner, 1978). However, the distribution of onchidal in different species of *Onchidella* was not reported and, apart from inhibiting the growth of *Staphylococcus aureus* (the IC_{50}value was between 0.21 and 0.63 µg/mL), which implies that onchidal is a potent inhibitor of Gram-positive microorganisms, no biological activity of onchidal was described. Additional studies demonstrated that onchidal is contained in several different species of *Onchidella* and that, once purified, it is toxic to fish (Abramson et al., 1989). Onchidal can be found in four of the eight known species of *Onchidella*

TABLE 30.1 Concentration of Onchidal in Different Species of *Onchidella* (Abramson et al., 1989)

Organism	Collection Site	Onchidal Concentration (μg/Animal)
O. binneyi	Baja California, Mexico	230
O. borealis	Central California, USA	33
O. nigricans	New Zealand	18
O. patelloides	Australia	42

TABLE 30.2 Types of Fasciculin Identified

Fasciculins Characterized	From the Mamba Snake Venoms (Elapidae Family) (*Dendroapsis* genus)
Fas1	*D. angusticeps* (green mamba)
Fas2 (formally F_7 toxin)	*D. angusticeps* (green mamba)
Fas3 (formally Toxin C)	*D. polylepis* (black mamba)
Fas4	*D. viridis* (Western green mamba)

collected from different countries (Table 30.1). In addition, the Abramson study found onchidal to be toxic to goldfish. Although goldfish are not potential predators of *Onchidella*, these results demonstrate that onchidal has a distribution and a biological activity consistent with its proposed role in the chemical defense of *Onchidella*.

Fasciculin

The venom of the mamba snake (*Dendroaspis angusticeps*, *Dendroaspis polylepis*, *Dendroaspis viridis*, *Dendroaspis jamesoni*) contains a mixture of neurotoxic compounds, including postsynaptic cholinoreceptor α–neurotoxins, dendrotoxins, fasciculins, and muscarinic toxins (Hawgood and Bon, 1991). Effects at the NMJ include AChE inhibition by fasciculins and increased presynaptic release of ACh by dendrotoxins (polypeptides that facilitate ACh release in response to nerve stimulation); together with the high ACh content of mamba toxin (6–24 mg/g), these effects are synergistic and enhance neurotoxicity and lethality. Moreover, the venom may contain other components that have a synergistic action with dendrotoxin.

Toxins that facilitate neuromuscular transmission are a characteristic component of mamba venom. The four known fasciculins bind to a peripheral regulatory anionic site of AChE in a noncompetitive and irreversible manner (Hawgood and Bon, 1991). The dendrotoxins comprise the second group of facilitatory neurotoxins and are present in most mamba venom (with the exception of *D. jamesoni*); they inhibit voltage-dependent K^+ channels in motor nerve terminals and facilitate ACh release at the NMJ. Postsynaptic toxins present in mamba venom bind to and block nicotinic acetylcholine receptors (nAChRs). The muscarinic toxins present in mamba venom are small proteins (7 kDa) that selectively bind to muscarinic cholinergic receptors and may constitute up to 1% of the venom protein (Adem and Karlsson, 1985; Jerusalinsky and Harvey, 1994). About 12 muscarinic toxins have been isolated. M1 toxin binds noncompetitively and with high affinity to the M_1 muscarinic receptor subtype. MTx1 and MTx2 show high affinity for both muscarinic M_1 and M_3 receptors; little is known about the receptor selectivity of MTx3 and MTx4. Dpα and Dpβ are also muscarinic agonists, displaying similar affinity for both the M_1 and M_2 receptor subtypes. The last two agonists, DpMTx and DvMTX, are selective muscarinic agonists present in the venom of some mamba species; these agonists also show affinity for the M_1 muscarinic receptor subtype.

The fasciculins are a family of closely related ~6,750-Da peptides isolated from the venom of mambas (genus *Dendroaspis*), and are named after the long-lasting muscle fasciculations they produce in mice (Rodriguez-Ithurralde et al., 1983). They are potent and selective inhibitors of AChE. Fasciculins are 61-residue-long polypeptides. They share a three-looped structural motif with other toxins, such as α-neurotoxins, cytotoxins, and muscarinic toxins, directed to diverse specific targets. Four fasciculins are known (Table 30.2), which differ only by 1–3 residues and show selective and potent anti-AChE activity: Fas1 and Fas2 from the venom of *D. angusticeps* (the Eastern green mamba) contains 61-amino-acid residues, including eight half-cystines (Rodriguez-Ithurralde et al., 1983), ToxC from the venom of *D. polylepis polylepis* (the black mamba) (Joubert and Taljaard, 1978), and Fas3, which was isolated from a particular batch of *D. viridis* (the Western green mamba) venom and found to have the same primary structure as ToxC (Marchot et al., 1993). No fasciculin has been found in other *D. viridis* venoms (Marchot et al., 1993), or in *D. jamesoni* (Jameson's mamba).

Angusticeps-type toxins constitute a group of toxins typical for mamba venoms. They consist of 59–61 amino acids residues and four disulfides, and show sequence homology with short-chain postsynaptic toxins and cardiotoxins, but they are immunochemically distinct from these toxins. Eight toxins of this type have been sequenced and structurally classified into four subgroups (Joubert and Taljaard, 1978). Pharmacological studies on these *angusticeps*-type toxins have revealed that only toxins F_7 and C, which belong to subgroup I, exhibit a potent inhibition of cholinesterase (ChE) of various tissues, except for that of chick skeletal muscle. Both F_7 and C stimulate mouse and toad skeletal muscles by their anti-ChE activity, as well as possibly by facilitating ACh release from nerve terminals (Lee et al., 1985).

Fasciculins account for 3–6% of the venom protein, and the relative proportions between Fas1 and Fas2 is about one-third (Cerveñanský et al., 1994).

The structural similarity between onchidal (an acetate ester) and ACh suggested that the toxicity of onchidal could result from inhibition of either nAChRs or AChE. Although onchidal (1.0 mM) did not prevent the binding of ^{125}I-α-bungarotoxin to nAChRs, it inhibited AChE in a progressive, apparently irreversible manner. The apparent affinity of onchidal for the initial reversible binding to AChE (K_d) was approximately 300 μM, and the apparent rate constant for the subsequent irreversible inhibition of enzyme activity (K_{inact}) was approximately 0.1 min^{-1}.

Fasciculins are a family of closely related peptides that are isolated from the venom of mambas and exert their toxic action by inhibiting AChE. The crystal structure of Fas2 from green mamba (*D. angusticeps*) snake venom was first resolved in 1992 (Le Du et al., 1992). The three-dimensional structure of Fas1, obtained from the US National Library of Medicine, National Center for Biotechnology Information, Molecular Modeling Database 3-D Structure Database (MMDB), is illustrated in Figure 30.2.

Fasciculins belong to the structural family of three-fingered toxins from Elapidae snake venoms, which include the α-neurotoxins that block the nAChR and the cardiotoxins that interact with cell membranes. The features unique to the known primary and tertiary structures of the fasciculin molecule were analyzed by Harald et al. (1995). Loop I contains an arginine at position 11, which is found only in the fasciculins and could form a pivotal anchoring point to AChE. Loop II contains five cationic residues near its tip, which are partly charge-compensated by anionic side chains in loop III.

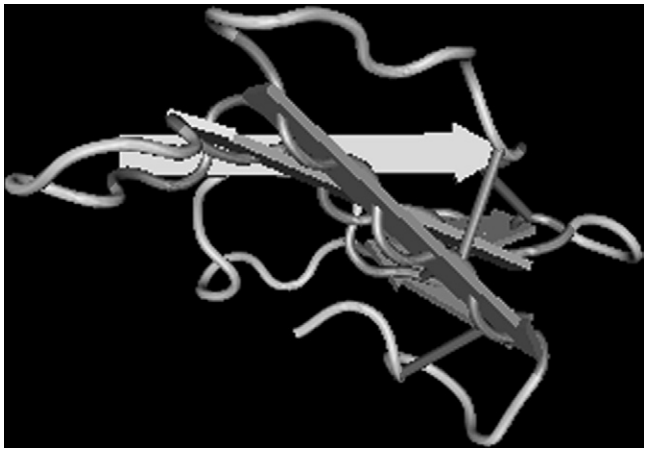


FIGURE 30.2 The 3-D protein structure of Fas1 derived from green mamba (*D. angusticeps*) snake venom. *Source: Image obtained from the public domain at the US National Library of Medicine, National Center for Biotechnology Information, MMDB.*

By contrast, the other three-fingered toxins show full charge compensation within loop II. The interaction of fasciculin with the recognition site on AChE was investigated by estimating a precollision orientation, followed by the determination of the buried surface area of the most probable complexes formed, the electrostatic field contours, and the detailed topography of the interaction surface. This approach has led to testable models for the orientation and site of bound fasciculin.

MECHANISM OF ACTION AND BIOLOGICAL EFFECTS

Onchidal

Onchidal belongs to the group of natural nonprotein neurotoxins and is an irreversible inhibitor of the AChE enzyme, with a novel mechanism of action. It has been suggested, however, that its toxicity could be a consequence of the inhibition of either nAChRs or the AChE enzyme.

Incubation of AChE with onchidal resulted in the production of acetate, demonstrating that onchidal was a substrate for AChE, and approximately 3,250 mol of onchidal were hydrolyzed/mol of enzyme irreversibly inhibited. OP and CM inhibitors of AChE have partition ratios (mol of toxin hydrolyzed/mol of enzyme irreversible inhibited) that approach unit. Therefore, the relatively high partition ratio for onchidal suggests that the mechanism of inhibition utilized by onchidal may be distinctly different from other irreversible inhibitors (Walsh, 1984). The rate of hydrolysis of onchidal (K_{cat}) was 325 min^{-1}; this value is relatively slow, suggesting that onchidal is not a very good substrate. The ability of AChE to hydrolyze onchidal raised the question of whether inhibition of enzyme activity resulted from onchidal itself, or from a product of the enzymatic hydrolysis of onchidal. Enzyme kinetics revealed that onchidal was unable to completely inhibit higher concentrations of AChE. From the experiments performed by Abramson et al. (1989), onchidal was in molar excess and completely hydrolyzed. Thus, irreversible inhibition of enzyme activity resulted either from onchidal itself, or from a reactive intermediate produced during the hydrolysis of onchidal (Walsh, 1984).

In another investigation, irreversible inhibition of enzyme activity was prevented by co-incubation with reversible agents that either sterically block (edrophonium and decamethonium) or allosterically modify (propidium) the ACh site (Barnett and Rosenberry, 1977). Enzyme activity was not regenerated by incubation with oxime reactivators; therefore, the mechanism of irreversible inhibition does not appear to involve acylation of the active site serine.

Because onchidal is an acetate ester similar to ACh, and because cholinergic neurotransmission is often the site of action of natural products involved in chemical defense, Abramson et al. (1989) investigated the ability of onchidal to inhibit AChE and the nAChR. Although onchidal did not prevent the binding of ^{125}I-α-bungarotoxin to nAChRs, it was shown to be an active site-directed irreversible inhibitor AChE. The structure and pharmacology of onchidal suggest that inhibition of AChE results from a novel covalent reaction between onchidal and an amino acid within the ACh binding site. The onchidal could potentially be exploited in the design of a new class of natural anti-ChE agents and in the identification of amino acids that contribute to the binding and hydrolysis of ACh.

Fasciculin

Various toxins in snake venoms exhibit a high degree of specificity in the cholinergic nervous system. The α-neurotoxins from the Elapidae family interact with the agonist binding site on the nicotinic receptor. α-Bungarotoxin is selective for the muscle receptor and interacts with only certain neuronal receptors, such as those containing α7 through α9 subunits. Neuronal bungarotoxin shows a wider range of inhibition of neuronal receptors. A second group of toxins, the fasciculins, inhibits AChE. A third group of toxins, termed the *muscarinic toxins* (MT_1–MT_4), are partial agonists and antagonists for the muscarinic receptor. Venoms from the Viperaridae family of snakes and the fish-hunting cone snails also have relatively selective toxins for nicotinic receptors.

Other reversible inhibitors, such as propidium and fasciculin, bind to the peripheral anionic site on AChE. This site resides at the lip of the gorge and is defined by tryptophan 286 and tyrosines 72 and 124 (Taylor, 2001).

A large number of organic compounds reversible or irreversibly inhibits AChE (Long, 1963), which bind either to the esteratic or the anionic subsite of AChE catalytic site or to the peripheral site of the enzymes. Most of them are synthetic substances, sometimes with insecticidal properties. Few natural inhibitors of AChE are known and, to date, fasciculins are the only known proteinic AChE inhibitors. They have been shown to display a powerful inhibitory activity toward mammalian AChE. Iodination of Fas3 provided a fully active and specific probe of fasciculin-binding sites on rat brain AChE (Marchot et al., 1993). These authors demonstrate that fasciculins bind on a peripheral site of AChE, distinct from the catalytic site and, at least partly, common with the sites on which some cationic inhibitors and the substrate in excess bind; since phosphorylation of the catalytic serine (esteratic subsite) by [1,3-^{3}H]diisopropyl fluorophosphate can still occur on the Fas3. In the AChE complex, the structural modification induced by fasciculins may affect the anionic subsite of the AChE catalytic site.

Cholinesterases have a very different sensitivity toward fasciculins. AChEs from rat brain, human erythrocytes, and electroplax of electric eel (*Electrophorus electricus*) are inhibited by fasciculins with a K*i* of about 10^{-11} M and pseudocholinesterases as human serum ChE are inhibited by fasciculins with a K*i* of about 0.5 μM. A second group of enzymes is partially (10–30%) inhibited by low concentrations (<0.5 nM) of fasciculin. Increasing the concentration of fasciculins to a toxic level of about 1 nM inactivates the enzymes to 90–110% of their initial activity. AChEs from guinea pig ileum, ventricle, and uterus behave similarly. A third group consists of enzymes insensitive to fasciculin; AChEs from chick *biventer cervicis* muscle and brain and from insects, heads of *Musca domestica* (common housefly) and cobra (*Naja naja*) venom. The biochemical mechanism of fasciculins involves displacement of propidium from its binding site on AChE. Since propidium is a probe from a peripheral anionic site, it is concluded that fasciculins also bind to the same site. The different sensitivity of ChE to fasciculin should depend on the nature of their peripheral sites. Fasciculins are basic proteins of 61 amino acid residues and 4 disulfides, highly homologous to short α-neurotoxins and cardiotoxins. Indeed, a large number of AChE inhibitors are cations (e.g., neostigmine, physostigmine, and propidium).

The binding between fasciculin and AChE is strong, as indicated by a K*i* of about 10^{-11} M. This should result from interaction of several amino acid residues in the toxin with the enzyme. A modification of one of these residues should not abolish the activity, but it should significantly decrease it. But the decrease in activity can also depend on structural perturbations caused by the modification. Chemical modification and structural data suggest that Lys32 and Lys51 have a functional role (Cerveñanský et al., 1994). This author acetylated the amino groups of Fas2 with acetic anhydride. The monoacetyl derivatives of the ε-amino acids (Lys25, Lys32, Lys51 and Lys58) retained between 28% and 43% of the initial activity, and that of the α-amino group retained 72%. Acetylation of Lys25 that has the most reactive amino group decreased the activity by 65%, apparently without producing structural perturbation since the circular dichroism spectrum was not affected. The three-dimensional structure shows a cationic cluster formed by Lys32, Lys51, Arg24, and Arg28. A comparison of 175 sequences of homologous toxins shows that Lys32 is unique for fasciculin. Acetylation of lysine residues in the cluster had a large effect and reduced the activity by 72% (Lys32) and 57% (Lys51).

Fasciculins inhibit AChE from mammals, electric fish, and some snake venoms with K*i* values in the picomolar

to nanomolar range; in contrast, the AChEs of avian, insect, and some other snake venoms are relatively resistant, and high micromolar concentrations are required to inhibit mammalian butyrylcholinesterases (Marchot et al., 1993). Dissociation constants of Fas1 and Fas3 are twofold and 60-fold lower, respectively, than that of Fas2 for synaptosomal rat brain.

An examination of fasciculin association with several mutant forms of recombinant deoxyribonucleic acid (DNA)–derivated AChE from mice shows that it interacts with a cluster of residues near the rim of the gorge on the enzymes; the aromatic residues, Trp286, Tyr72, and Tyr124, have the most marked influence on fasciculin binding, whereas Asp74, a charged residue in the vicinity of the binding site that affects the binding of low-molecular-weight inhibitors, has little influence on fasciculin binding. The three aromatic residues are unique to the susceptible AChE and, along with Asp74, constitute part of the peripheral anionic site. Fasciculin falls into the family of three-loop toxins that include the receptor blocking α-toxins and cardiotoxins. A binding site has evolved on fasciculin to be highly specific for the peripheral site on AChE. Acetylthiocholine affects rates of fasciculin binding at concentrations that cause substrate inhibition. In the case of the mutant ChE, where rates of fasciculin dissociation are more rapid, steady-state kinetic parameters also show ACh-fasciculin competition to be consistent with occupation at a peripheral or substrate inhibition site rather than the active center (Radic et al., 1994).

Fasciculin inhibition of AChE is prevented by chemical modification of the enzyme at a peripheral site (Durán et al., 1994). The specific interaction of Fas2 with peripheral sites present in *E. electricus* AChE (K*i*; 0.04 nM fasciculin) was investigated by chemical modification with *N*,*N*-dimethyl-2-phenylaziridium (DPA) in the presence of active or peripheral anionic site protective agents. An enzyme was obtained that compared to the native AChE and was 10^6 times less sensitive to Fas2. This enzyme was fully inhibited by edrophonium and tacrine and was 25–170 times less sensitive to several peripheral site ligands. It seems Fas2 binding to an AChE peripheral site partially overlaps the site of other peripheral site ligands, including ACh.

EXPERIMENTAL AND HUMAN TOXICITY

Administration of Fas1 and Fas2 to mice at doses of 1–3 mg/kg and 0.05–2.0 mg/kg, respectively after intraperitoneal (i.p.) injection caused severe, generalized, and long-lasting fasciculations (5–7 h), followed by gradual recovery to normal behavior. *In vitro* preincubation with fasciculins at concentrations of 0.01 μg/mL inhibited brain and muscle AChE up to 80%. Histochemical assay for AChE showed an almost complete disappearance of the black-brown precipitate at the neuromuscular endplate after *in vitro* incubation with fasciculins. Fasciculins represent a new type of AChE inhibitors exerting muscle fasciculations through a powerful inhibition of enzyme activity at the neuromuscular endplate, interfering with the normal hydrolysis of ACh molecules. Fasciculins have also been demonstrated to be powerful inhibitors of brain AChE (Rodriguez-Ithurralde et al., 1983).

The cause of death due to toxin F_7, an *angusticeps*-type toxin isolated from the venom of *D. angusticeps*, was studied in anesthetized mice (Lee et al., 1986). The carotid arterial blood pressure, electrocardiography, and the respiratory movements were recorded. Within a few minutes after intravenous (i.v.) injection of F_7 (1 mg/kg), both the rate and amplitude of the respiratory movements decreased, and respiratory arrest took place within 15 min in most cases. Before respiratory arrest, marked bradycardia with various types of arrhythmia and oscillation of blood pressure were observed. Artificial ventilation could abolish these cardiovascular changes and maintain the blood pressure for a long period. Toxin F_7 caused a transient and slight increase of arterial blood pressure, which could be prevented by hexamethonium. Intracisternal application of toxin F_7 (1 mg/kg) caused a long-lasting hypertension and bradycardia and the respiratory arrest time was significantly longer than after i.v. injection. A large dose (50 mg/kg, i.p.) of atropine, but not smaller doses (5–10 mg/kg), protected mice against respiratory failure induced by toxin F_7.

In rats, the phrenic nerve discharge was prolonged during respiratory depression. Since the toxin F_7 has a potent anti-ChE activity, it is concluded that the respiratory failure induced by toxin F_7 is peripheral in origin; this was chiefly, if not entirely, due to its anti-ChE activity.

Strydom (1976) performed the purification of *D. polylepis polylepis* venom and found 12 low-molecular-weight proteins, of which 11 have subcutaneous LD_{50} values of less than 40 μg/g in the mouse. Clinically, mamba bites may not provoke a major local reaction. If neurotoxins are injected by the bite, clinical symptoms appear within minutes to hours. Clinical signs of impairment of neuromuscular transmission (ptosis, ophthalmoplegia, bulbar symptoms, or generalized weakness) dictate administration of antivenom (Ludolfph, 2000). The venom of Elapidae (coral snakes) is known to be a potential neurotoxin and may cause paresthesias, weakness, cranial nerve dysfunction, confusion, fasciculations, and lethargy. Often mild local findings, diplopia, ptosis, and dysarthria are common early symptoms. Patients die because of respiratory paralysis. In these cases, early and aggressive airway management is vital. Symptoms may be delayed by 8–12 h (Cameron, 2006).

COMPUTATIONAL TOXICOLOGY ASSESSMENT

Toxicity databases and computer-assisted (*in silico*) computational predictive toxicology modeling are now becoming increasingly important to risk assessors. This is especially true when risk assessment of a chemical using experimental animal toxicological studies is unclear because the data are equivocal or even absent (Bailey et al., 2005; Arvidson et al., 2008; Valerio, 2009). Furthermore, a law passed by the European Union called Registration, Evaluation, Authorization, and restriction of Chemicals (REACH), entered into force on June 1, 2007. The goal of the regulation was to identify and more effectively avoid the risks that the toxic properties of chemicals pose to humans and the environment (Lahl and Gundert-Remy, 2008). Under REACH, firms that manufacture or import more than 1 ton of a chemical substance per year in the European Union are required to assess the chemical's potential toxicological or environmental adverse effects, and to register this information in a central database (Saiakhov and Klopman, 2008). REACH has had an impact on the widespread use of computational predictive modeling because it rules that no animal test should be used if it can be replaced with other techniques, such as reliable computer-based predictions (Lahl and Gundert-Remy, 2008; Saiakhov and Klopman, 2008). Therefore, the use of computer-assisted computational predictive toxicology software has attracted considerable attention worldwide because it may not only reduce, but eliminate the need for animal testing under certain circumstances. Since the acute and chronic toxic potential of onchidal in experimental animal studies are rather limited, strategies using *in silico* assessment of its toxic potential could be envisioned. Computational-based strategies with pharmaceuticals and natural product toxins have already been developed for use in safety evaluation and risk assessment at US government agencies (Bailey et al., 2005; Valerio et al., 2007; Demchuk et al., 2008; Valerio, 2009; Arvidson et al., 2010; Valerio and Cross, 2012; Choi et al., 2013; Valencia et al., 2013; Valerio et al., 2013; Valerio, 2013). These strategies can be considered for an *in silico* risk assessment of onchidal. One approach is to combine different predictive models for the same toxicological endpoint to determine if a consensus prediction regarding the effect is possible. Another approach is to combine computational software that employs human rule-based approaches with software that utilizes statistical algorithm-based predictions using quantitative structure-activity relationship (QSAR) models for the toxic potential of the compound of interest. Consistent with this notion, the current version of (M7) guidance from the International Conference of Harmonisation (ICH) on hazard assessment of DNA-reactive mutagenic pharmaceutical impurities recommends, in the absence of data, the use of a statistical QSAR model and human rule-based structure-activity relationship (SAR) approach to assess if there is a structural concern that an impurity identified in drug substances or drug products is mutagenic. According to ICH M7, this analysis from the *in silico* assessment of the molecular structure of impurities should be focused on predicting the outcome of bacterial mutagenicity (i.e., Ames assay). If the outcome from a QSAR and SAR structural assessment with expert interpretation is negative, then the M7 guidance indicates that the assessment is sufficient to conclude there is no concern and to treat the impurities as nonmutagenic. If the outcome from the complementary *in silico* QSAR/SAR assessments is positive, then the impurity would need to be controlled to safe levels or qualified according to regulatory protocols (ICH S2(R1) and OECD 471). Thus, as evident from the ICH M7 guidance, the use of *in silico* toxicology methods has taken a real practical role and utility as a tool in facilitating regulatory decisions in a risk assessment setting to ensure the safety and quality of pharmaceutical products. There are a variety of computational toxicology predictive software and data mining databases available at either no cost or commercially for a fee (Richard et al., 2008; Marchant et al., 2008; Saiakhov and Klopman, 2008; Shi et al., 2008; Mostrag-Szlichtyng et al., 2010; Choi et al., 2013; Valencia et al., 2013). However, the information obtained from a predictive and data mining *in silico* assessment must be used judiciously in order to serve a true purpose in risk assessment and management regarding the safety of a chemical (Arvidson et al., 2010; Valerio, 2012).

In order to assess the potential toxicological effects of onchidal from a predictive standpoint, the author subjected the two-dimensional molecular structure of onchidal (1) to an *in silico* QSAR computational analysis. Details regarding the approach of the software, including procedures and model building methods, have been described in recent publications (e.g., Choi et al., 2013; Valencia et al., 2013). The *in silico* analysis of the molecular structure of onchidal produced predictive information on nonclinical toxicities. The computational QSAR models included bacterial mutagenicity (*Salmonella typhymurium* mutagenicity (Ames) assay), and phospholipidosis (Table 30.3).

These computational prediction results show that onchidal is not flagged to be of concern for bacterial mutagenicity, but it might be expected to induce phospholipidosis in animals despite not possessing a traditional cationic amphiphilic molecular structure (Choi et al., 2013). Predictions were also made using the QSAR approach regarding clinical cardiac adverse events related to proarrhythmia. The *in silico* predictions are presented in Table 30.4. The predictions show that onchidal's molecular structure is associated with QT

TABLE 30.3 The Molecular Structure of Onchidal Analyzed by a Statistical QSAR Method

Salmonella t. reverse mutation assay (Ames test)	0.225	0.227	0.189	–
Phospholipidosis	0.650	0.875	0.851	+

The data shown in the table indicate onchidal is predicted negative in the Ames test and positive to induce phospholipidosis in animals.

TABLE 30.4 Predictions of the Onchidal Molecule Using Symmetry QSAR Models for QT/QTc Prolongation and Torsade de Pointes

End-Point	Score (Probability)	Std. Dist. AD+	Std. Dist. AD−	Prediction Result
QT/QTc prolongation	0.932	0.315	1.136	+
Torsade de Pointes	0.389	0.195	0.177	–

The QSAR models were built using molecular descriptors M0102 in a risk-based approach (Valerio et al., 2013). A strong positive prediction for QT prolongation resulted from the *in silico* analysis, but onchidal was not predicted by the QSAR model to induce the potentially fatal cardiac arrhythmia Torsade de Pointes.

prolongers in the training set of the QSAR model, but it is not predicted to be a risk for torsade de pointes, a potentially fatal cardiac arrhythmia. Onchidal has anticholinergic effects (Anadón and Martínez-Larrañaga, 1985). Tachycardia as a result of anticholinergic has been suggested to increase or lead to arrhythmias (e.g., with low-potency antiphsychotics; Huffman and Stern, 2003), and this might explain the positive prediction of onchidal as a QT prolonger. However, the negative prediction for torsade de pointes mitigates this theoretical risk.

Overall, these *in silico*/computational toxicology models, as well as many others, have been developed at regulatory agencies, to help inform regulatory science. These models represent structure-based assessments using statistical QSARs to predict with an intended precision the toxicological and adverse human effects of many classes of organic substances (Valerio, 2009; Valerio et al., 2013). As with all methods, these models have some limitations, and in this regard, the main consideration with predictive modeling is the quality of the data used to build it. Advanced efforts have been focused on data integration techniques to combine empirical data with theoretical predictions (Nigsch et al., 2011; Valerio, 2012, 2013). Clearly, building *in silico* tools directed at understanding human adverse effects can be helpful for assessing safety parameters that are difficult to determine in animals or acquire from well-controlled human studies. Thus, if properly validated, described, interpretable and made available for broad consumption, the *in silico* approach should have a bright future, especially in the area of chemical risk assessment (Valerio, 2013). Programs in advancing regulatory science and modernizing toxicology with predictive models are underway (Valerio, 2011; Valerio and Choudhuri, 2012). We anticipate that as capabilities expand, the use of new informatics tools, standards, and predictive technologies via these enabling methods will help build groundbreaking efforts to transform the way that regulated products are developed and evaluated, including therapies for the toxic effects of at-risk substances such as onchidal and fasciculins.

TREATMENT

Antidotal therapy for the toxic effects of ChE inhibitors, such as onchidal and fasciculins used as potential chemical warfare agents, is directed toward blocking the effects of excessive ACh stimulation and reactivating the inhibited enzyme. Atropine in sufficient dosages effectively antagonizes the actions at muscarinic receptor sites (Taylor, 2001). Atropine is used in adults at doses of 1–2 mg i.v. and in pediatrics at a dose of 0.05 mg/kg i.v.; doubling this dose every 5 min (DeLisle, 2006). Larger doses are required to get appreciable concentrations of atropine into the CNS. Atropine is virtually without effect against peripheral neuromuscular compromise. The aforementioned action of the anti-ChE agents, as well as all other peripheral effects, probably can be reversed by reactivators of ChE such as pralidoxime (2-PAM) in the treatment of onchidal and fasciculin poisonings. The usual dosages are as follows:

- In adults, 1–2 g i.v. over 15–30 min, may repeat in 1 h if necessary or start a drip at 500 mg/h.
- In pediatrics, 25 mg/kg i.v. over 15–30 min, follow with continuous infusion of 10–20 mg/kg i.v. (DeLisle, 2006).

On the other hand, the antivenom is indicated for all confirmed eastern coral snake bites (western, no antivenom) (Cameron, 2006).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The natural toxins onchidal and fasciculins behave as anti-ChE agents. Onchidal is an active, site-directed irreversible inhibitor of AChE, and fasciculins are proteinic AChE inhibitors that bind to a peripheral regulatory anionic site of AChE in a noncompetitive, irreversible manner.

There is limited information about the toxicity, toxicokinetics, and toxicological properties of onchidal, and additional data are needed to make a health-effect-based

risk assessment of the natural compound. Although fasciculins are much better known, data on toxicological properties and toxicokinetics will be of interest and useful for risk assessments. This is the case even though it is generally accepted that the toxicity of this proteinic poison occurs at very low doses.

Onchidal and fasciculins are interesting natural compounds, and it is difficult to predict their toxicity. As demonstrated in this chapter, predictive *in silico* approaches using computational models can be used to help assess both the nonclinical and clinical adverse effects of chemical agents. The quality of computational toxicology assessments relates to the quality of data used to derive predictions and appropriate expert interpretation of the data. It is possible to make predictions for human clinical adverse effects using computational models built upon human clinical trial data. Because the *in silico* approach is tailored toward filling data gaps, the paucity of human data on onchidal and fasciculins makes a clear case for the use of *in silico* predictive technologies. The future of computational assessments seems to be in enabling techniques that would facilitate integrating theoretical predictions with empirical evidence in order to strengthen contemporary decision analysis. A major challenge will be to predict the military potential and overall human impact of these natural toxins since their biochemical affinity for enzyme inhibition depend upon the amount and duration of human exposure, and data about human exposure are lacking.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

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CHAPTER

31

Cyanobacterial (Blue-Green Algae) Toxins

Deon van der Merwe

INTRODUCTION

Cyanobacteria, commonly referred to as blue-green algae, are photosynthesizing prokaryotic bacteria. They are important primary producers in most freshwater and marine environments that are close enough to the surface to receive sunlight. Cyanobacteria perform photosynthesis through the green pigment, chlorophyll-a. They often produce accessory photosynthetic blue pigments, called phycobilins or phycocyanins. Red to brown accessory photosynthetic pigments, called phycoerythrins, may also be produced. Colony colors vary depending on the relative pigment levels and range between shades of blue and green and, less commonly, brown and red (Adhikary, 1996). Cyanobacteria may be benthic or pelagic and are morphologically diverse. Five morphologically distinct sections have been recognized: unicellular colonies with binary fission, unicellular colonies with multiple fission, multicellular colonies, multicellular colonies with differentiated cells, and branched multicellular colonies with differentiated cells (Schirrmeister et al., 2011). Colonies are often large enough to be seen with the naked eye and, when numerous, may change the color of the water or form scums at the water surface. Dense proliferations of cyanobacteria are referred to as algal blooms. When blooms have the potential to cause harmful effects, they are called harmful algal blooms (HABs). The incidence of HABs is increasing in most regions and is usually linked to excess nutrient inflow into surface water from agricultural and industrial sources (Paerl et al., 2001; de Figueiredo et al., 2004; Hudnell, 2010; Gkelis and Zaoutsos, 2014).

Several genera of cyanobacteria are able to produce toxins (Briand et al., 2003). The production of toxins is influenced by genetic and environmental factors. Cyanobacteria known to have the genetic potential to produce toxins do not always do so or may not produce toxins at concentrations that are high enough to result in a significant poisoning risk. Certain environmental factors, including nutrient concentrations, water temperature, and pH, may play a role in triggering toxin production. The critical parameter values that trigger toxin production are, however, not fully known or predictable. Toxin production is generally more common during warm weather and abundant sunlight but can occur at any time of the year (Downing et al., 2001; Kanoshina et al., 2003; Graham et al., 2004; Dodds et al., 2009). Toxin production during HABs can be extremely high. For example, a HAB formed by microcystin-LR-producing *Microcystis aeruginosa* in a Kansas lake in 2011, produced toxin concentrations in the water of 126,000 ng/mL (van der Merwe et al., 2012). Such concentrations are highly significant considering that hepatotoxic effects in humans are possible after ingestion of 100 mL of water containing 20 ng/mL microcystin-LR (WHO, 2003). The spontaneous production of high concentrations of toxins in a natural environment, although it is somewhat sporadic, could make them accessible to harvesting for use as chemical weapons, requiring only simple equipment and low levels of technical expertise. Deriving toxins from cyanobacteria produced in bioreactors is also possible but requires a greater level of equipment investment and technical expertise. Only one cyanotoxin, the neurotoxin known as saxitoxin or paralytic shellfish poison (PSP), is listed as a Schedule 1 substance in the Chemical Weapons Convention. Other cyanotoxins may also pose credible threats based on their potency, environmental persistence, and widespread production in surface waters where they may be easily accessible.

The toxins produced by freshwater cyanobacteria can be classified based on their toxic effects, their molecular

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structure, or their origins. A classification based on toxic effects is used in this discussion.

HEPATOTOXINS

Microcystins and Nodularins

Introduction

Microcystins are cyclic heptapeptides produced by several genera, including *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Oscillatoria*, and *Anabaenopsis*. The frequency of microcystin-producing HABs appears to be increasing in most regions in association with the expansion of intensive agriculture, industrial development, and urbanization. Because of their widespread and frequent occurrence, their high potency, and environmental persistence, microcystins are a significant public health risk. Nodularins are mainly produced by *Nodularia spumigena* in brackish waters. They are similar to microcystins in structure and mechanism of toxicity and, for the purposes of this discussion, are considered equivalent to microcystins (Namikoshi et al., 1994; Chen et al., 2013). Microcystin concentrations are generally correlated with cell density, although the correlation is not consistent and high cell densities without toxin production are possible. People are most often exposed when swimming, skiing, or boating in contaminated waters. Other routes of exposure include drinking water, contaminated foods or nutritional supplements, and, in at least one lethal and tragic incident, contaminated water used in dialysis (Azevedo et al., 2002).

Chemistry

Microcystins have a cyclic heptapeptide structure (Figure 31.1). More than 80 naturally occurring structural variants have been described (Welker and Von Dohren, 2006). Six amino acids, including four nonprotein amino acids and two protein amino acids, form a ring structure. One nonprotein amino acid, referred to as ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca;4,6-dienoic acid), forms a side chain. The ADDA side chain is consistent between microcystin variants and can be used to quantify microcystins independent of the variant type. The two protein amino acids, at positions 2 and 4 in the ring structure, contribute most to the structural variability. Microcystin variants are named based on the protein amino acids at the variable positions. One of the most common variants, for example, contains leucine and arginine at the protein amino acid positions and is referred to as microcystin-LR.

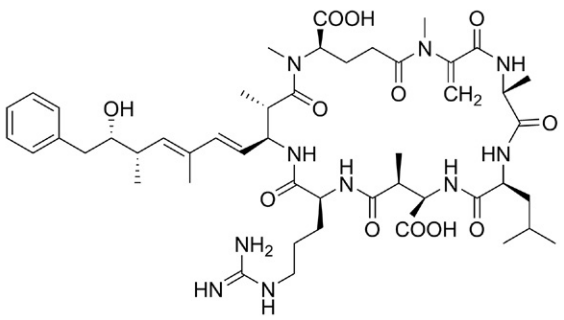

FIGURE 31.1 Microcystin chemical structure.

Microcystins are not produced by cyanobacterial cells and therefore are strongly associated with cyanobacterial cells as long as the cells remain intact. However, microcystins may be released into the surrounding water when cyanobacterial cells disintegrate. Microcystins are stable, with a typical environmental half-life of 10 weeks. The rate of breakdown is increased under direct sunlight, at high environmental temperatures (>40ºC), and extremely low pH (<1) or high pH (>9) (Tsuji et al., 1994; Harada et al., 1996). Microcystin concentrations may be reduced by boiling or heating in a microwave oven (Gutierrez-Praena et al., 2013). However, the concentration of free microcystins in muscle tissue from exposed fish may increase after boiling because of the release of phosphatase-bound microcystins (Zhang et al., 2010).

Toxic Effects

Most of the available toxicological data on microcystins have been based on microcystin-LR. Other microcystin variants appear to be similar to microcystin-LR in their toxicological effects, but they differ in potency. Other factors that need to be taken into account when interpreting available data are that much of the data have been derived from experiments in rodents and rabbits using intraperitoneal injection as the route of exposure, and the extracts used in studies may consist of complex, crude mixtures of components derived from toxic algal blooms, or more or less purified and characterized components (Zhao et al., 2008, 2009). Extrapolation between routes of exposure and between the toxicities of different complex mixtures can make the comparison of data from different studies challenging.

The clinical effects of microcystin poisoning depend on the route of exposure, the level of exposure, and the mixture of components involved in the exposure. In typical exposures of mammals to toxic blooms, low to mild exposure levels are associated with irritant effects, resulting in inflammatory responses in the skin, respiratory system, and gastrointestinal system. Higher exposure levels, particularly oral exposures, result in liver damage and, if the liver damage is severe, liver failure (Briand et al., 2003). The earliest detectable signs of liver damage include increased concentrations of liver enzymes in the blood and liver swelling. Early symptoms typically appear within minutes to hours of exposure and include inappetance, depression, and vomiting,

followed by diarrhea, which may become extreme and hemorrhagic. Inappetance and depression become progressively worse. The final stages of lethal poisoning may be associated with variable central nervous system (CNS) dysfunction, recumbency, and coma (DeVries et al., 1993; van der Merwe et al., 2012). Exposure to microcystins may be a contributing factor to the development of hepatocellular cancer and colorectal cancer in humans (Meneely and Elliot, 2013).

An important difference between exposures in aquatic organisms and terrestrial organisms is that exposures in aquatic environments often involve exposures to sub-lethal doses over days or weeks. In fish, exposures to microcystins cause cellular damage, particularly liver damage, similar to the effects seen in mammals (Tencalla and Dietrich, 1997). Microcystins also inhibit fish embryo development (Gotz et al., 2000), and toxic effects in fish embryos may be seen at relatively low microcystin concentrations (Pietsch et al., 2001). Mature fish are generally more resistant to microcystin toxicosis compared with juvenile fish and mammals (Malbrouck and Kestemont, 2006). Although fish may have the ability to avoid areas of accumulation of toxic algae, sub-lethal liver damage in fish is associated with algal blooms because of the accumulation of microcystins in fish food items such as mussels, snails, and zooplankton (Liu et al., 2002; Malbrouck and Kestemont, 2006). Fish are also susceptible to decreased water oxygen levels associated with the decay of algal scum (Ibelings and Havens, 2008), and this effect may play a role in fish kills associated with microcystin-producing algal blooms.

Mechanism of Action

After ingestion, microcystins are released from cyanobacterial cells and are absorbed into the portal circulation from the small intestine via bile acid transporters in the intestinal wall. Microcystins are then accumulated in hepatocytes via similar bile acid transporters on hepatocyte membranes (Hooser et al., 1991). Microcystins irreversibly inhibit serine/threonine protein phosphatases 1 and 2A (Yoshizawa et al., 1990). Microcystin-LR may also bind to AP synthase, leading to hepatocyte apoptosis (Mikhailov et al., 2003).

Protein phosphatases are ubiquitous. They are found in all tissues and across species as diverse as mammals, plants, and bacteria, and they play a critical role in the regulation of multiple cellular metabolic pathways. Protein phosphatases reverse the active state of kinases through the hydrolytic removal of the phosphoryl group from kinases. The protein phosphatases inhibited by microcystins have broad substrate specificity and play roles in the regulation of a wide range of cellular functions. Protein phosphatase 2A is highly conserved and is a major downregulator of active protein kinases in eukaryotic cells. Toxic effects in hepatocytes and other living cells are therefore multifaceted and include disruption of the cytoskeleton, DNA damage, apoptosis related to mitochondrial damage, and oxidative stress attributable to the formation of free oxygen radicals (Zegura et al., 2004; Ding and Nam Ong, 2006).

Chemical Warfare Potential

Microcystins are commonly produced in freshwaters when nutrient levels and weather conditions are suitable for HAB formation. Toxin concentrations can reach extremely high levels (van der Merwe et al., 2012) and, coupled with the persistence of microcystins (Harada et al., 1996), represent an opportunity for the collection and storage of microcystins using widely available equipment and minimal technical skill. Contamination of water and food supplies represents a significant health risk. However, the major impact of such an event will be the cost associated with avoidance of exposure, including testing, water purification, and the procurement of alternative water and food. Contamination of medical supplies represents a particularly dangerous potential use of microcystins, as demonstrated by the lethal consequences of accidental contamination of dialysis water supplies (Azevedo et al., 2002). There are no specific antidotes for microcystin-poisoning.

Cylindrospermopsin

Introduction

Cylindrospermopsin is an alkaloid cyanotoxin produced by several freshwater cyanobacteria genera, including *Cylindrospermopsis*, *Aphanizomenon*, *Anabaena*, *Lyngbya*, *Umezakia*, and *Raphidiopsis* (Guzman-Guillen et al., 2013). It is found worldwide in surface freshwaters (de la Cruz et al., 2013).

Chemistry

Cylindrospermopsin is an alkaloid consisting of a tricyclic guanidine coupled with hydroxymethyluracil (Figure 31.2). It is a zwitterionic, highly water-soluble molecule (Ohtani et al., 1992). There are two known structural variants: deoxycylindrospermopsin (Norris et al., 1999), which is relatively less toxic, and 7-epicylindrospermopsin (Banker et al., 2001), which is relatively

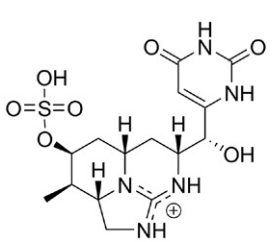

FIGURE 31.2 Cylindrospermopsin chemical structure.

more toxic. Cylindrospermopsin is resistant to high temperatures, sunlight, and pH extremes (Chiswell et al., 1999). Unlike microcystins, cylindrospermopsin is often released from the cells into the surrounding water (Rucker et al., 2007). It bioaccumulates, particularly in organisms low in the food chain such as gastropods, bivalves, and crustaceans (Kinnear et al., 2009).

Toxic Effects

A major outbreak of poisoning in humans, including 148 cases, occurred in 1979, associated with a reservoir on Palm Island, Queensland, Australia (Griffiths and Saker, 2003). The reservoir produced a dense bloom of cylindrospermopsin-producing *Cylindrospermopsis raciborskii*. People living close to the reservoir and using the water were affected with a syndrome that included liver and kidney damage, as well as severe gastroenteritis. Symptoms included hemorrhagic diarrhea, vomiting, fever, hepatomegaly, dehydration, electrolyte imbalances, acidosis, and hypovolemic shock. However, the potential role that a copper sulfate water treatment soon before the start of the poisoning outbreak could have played in the disease process remained uncertain. Intraperitoneal injection of extracts from *C. raciborskii* collected from the reservoir produced liver and kidney damage in mice (Hawkins et al., 1985).

Cylindrospermopsin was isolated from water used in dialysis in Brazil that caused liver failure in dialysis patients. However, the role of cylindrospermopsin in the disease process was not clear because the water was also contaminated with toxic concentrations of microcystin (Azevedo et al., 2002).

C. raciborskii can cause mild skin irritation in some individuals, but the role of cylindrospermopsin in the reaction, if any, is not clear (Pilotto et al., 2004).

Pure cylindrospermopsin injected into tilapia caused progressive tissue damage over a period of 5 days in the liver, kidney, heart, and gills (Gutierrez-Praena et al., 2013). Liver and kidney damage are consistently caused in laboratory rodents after exposure to acutely toxic doses of cylindrospermopsin-containing extracts. Typical liver pathology includes lipid infiltration and necrosis, mostly in the periacinar region (Shaw et al., 2000).

Mechanism of Action

The main target tissue of purified cylindrospermopsin toxicosis is the liver, but several other tissues are also affected, including the kidneys, thymus, and heart (Terao et al., 1994). Cylindrospermopsin is a potent inhibitor of protein synthesis, but the exact mechanism of action has not been elucidated. Four sequential phases of hepatocyte damage were identified by time-series analysis, including protein synthesis inhibition, membrane proliferation, lipid infiltration, and necrosis. Cylindrospermopsin has been shown to cause DNA fragmentation *in vitro*, and metabolic activation by a P-450 enzyme appears to be necessary for this effect to occur (Bazin et al., 2010). Kidney pathology includes necrosis of the proximal tubules and protein accumulation in distal tubules (Falconer et al., 1999).

Studies using crude extracts report higher potency and a wider range of effects compared with studies using purified cylindrospermopsin, indicating the components other than cylindrospermopsin contribute to the toxic effects (Shaw et al., 2000; Seifert et al., 2007).

Chemical Warfare Potential

The mechanisms of toxicity of cylindrospermopsin are not yet fully understood, but ample evidence of its potential impact on human health exists, based on clinical cases, *in vitro* studies, and animal model studies (Poniedziałek et al., 2012). Its relatively infrequent natural occurrence, compared with more common cyanotoxins such as microcystis, make it generally less accessible. The primary threats include contamination of drinking water supplies and food supplies.

NEUROTOXINS

Anatoxin-a

Introduction

Anatoxin-a is a potent, fast-acting neurotoxin. It occurs worldwide in freshwaters and is produced by several genera of cyanobacteria, including *Anabaena*, *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Raphidiopsis*, *Arthrospira*, *Cylindrospermum*, *Phormidium*, *Nostoc*, and *Oscillatoria* (Osswald et al., 2007). Exposures occur mainly through consumption of contaminated drinking water but have also occurred from recreational use of lakes and through contaminated dietary supplements (Rogers et al., 2005).

Chemistry

Anatoxin-a is a bicyclic amine alkaloid (Figure 31.3). It contains a homotropane scaffold that is derived from glutamic acid. It is chiral, with two symmetric centers (Wonnacott and Gallagher, 2006). Only the (+)-anatoxin-a enantiomeric form is naturally produced, and the pK_a of this form, at 9.4, indicates that it is mostly in the protonated form under typical environmental pH conditions

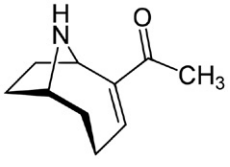

FIGURE 31.3 Anatoxin-a chemical structure.

(Valentine et al., 1991). Anatoxin-a is stable under sterile conditions but is susceptible to microbial biodegradation (Rapala et al., 1994). Breakdown is also accelerated by high temperature, UV light, and alkaline conditions (Kaminski et al., 2013; Stevens and Krieger, 1991). The half-life in a reservoir was reported to be 5 days under typical environmental pH conditions (Smith and Sutton, 1993). A structural analog, called homoanatoxin-a or methylene-anatoxin-a, has been isolated from *Oscillatoria formosa* (Skulberg et al., 1992). Small quantities of anatoxin-a are produced synthetically for use in acetylcholine (ACh) receptor research (Aronstam and Witkop, 1981).

Toxic Effects

Anatoxin-a was originally called very fast death factor because of its rapid lethal effects within 2–7 min in laboratory mice after intraperitoneal injection of cell culture extracts from anatoxin-a producing algal blooms (Carmichael and Gorham, 1978). Acute deaths after exposure to anatoxin-a have been recorded in multiple species, including dogs (Edwards et al., 1992), cattle (Carmichael and Gorham, 1978), and wildlife (Carmichael, 1981). There are species differences in susceptibility to anatoxin-a. Mallard ducks, for example, are more sensitive compared with ring-necked pheasants (Carmichael and Biggs, 1978). Anatoxin-a is rapidly absorbed from the gastrointestinal tract, as indicated by the rapidity of clinical effects after oral exposure. Clinical effects of poisoning may appear within minutes to hours after exposure and may include loss of muscle coordination, muscle tremors and fasciculations, convulsions, and respiratory distress. The principal lethal effect is respiratory failure after loss of control over respiratory muscles (Osswald et al., 2007).

Anatoxin-a was implicated in the death of a 17-year-old boy who swallowed water from a lake containing a bloom of *Anabaena flos-aquae*. The boy died 2 days after exposure. Terminal clinical signs included convulsions, shock, and heart failure. However, uncertainty regarding the identity of the toxin and the unusual clinical course compared with confirmed cases in animals have cast some doubt on the diagnosis of anatoxin-a poisoning in this case (Carmichael et al., 2004).

Mechanism of Action

Anatoxin-a appears to be a pure neurotoxin, with no characteristic blood chemistry changes and no gross or histological lesions associated with poisoning (Rogers et al., 2005).

It is an agonist of peripheral and central ACh receptors, with a 100-fold selectivity for nicotinic receptors over muscarinic receptors (Aronstam and Witkop, 1981). It binds to the ACh receptor at the same position as ACh, causing sodium/potassium ion channels to open and inducing a depolarizing blockade. Anatoxin-a is more potent than ACh or nicotine (Swanson et al., 1986), with a high nicotinic receptor affinity (Aronstam and Witkop, 1981). It also has the ability to modulate dopamine release through agonistic binding to $\alpha4\beta_2$-receptors and α_7-receptors (Sharples et al., 2000). Binding of anatoxin-a to the nicotinic ACh receptors at neuromuscular junctions results in uncontrolled action potential propagation that manifests clinically as uncoordinated muscle contraction, muscle fatigue, and paralysis (Stevens and Krieger, 1991). Cholinesterase does not break-down anatoxin-a, leading to persistent muscle stimulation (Spivak et al., 1980; Wonnacott and Gallagher, 2006). Stimulation of nicotinic receptors in the cardiovascular system causes increased heart rate and blood pressure (Siren and Feurstein, 1990). Stimulation of presynaptic nicotinic receptors in the CNS by anatoxin-a may also cause the release of neurotransmitters such as dopamine, which could further increase the susceptibility of postsynaptic receptors to overstimulation (Wonnacott and Gallagher, 2006). CNS receptors are, however, less sensitive to anatoxin-a compared with peripheral receptors (Aracava et al., 1987).

Chemical Warfare Potential

Anatoxin-a is potentially dangerous as a chemical warfare agent because of its high potency, fast action, and occasional availability of highly lethal concentrations of toxin during natural cyanobacterial blooms. However, it is susceptible to rapid bacterial breakdown under nonsterile storage conditions, making its intentional use challenging without relatively sophisticated extraction and storage procedures.

Anatoxin-a(s)

Introduction

Anatoxin-a(s) is a natural organophosphate analogue produced by cyanobacteria in the genus *Anabaena* (Molica et al., 2005; Patocka et al., 2011). The "(s)" in the name "anatoxin-a(s)" refers to salivation, which is a characteristic sign of poisoning observed in laboratory rodents after exposure (Mahmood and Carmichael, 1986; Carmichael et al., 1990).

Chemistry

Anatoxin-a(s) is a cyclic *N*-hydroxyguanine with a phosphate ester moiety (Figure 31.4) similar to typical

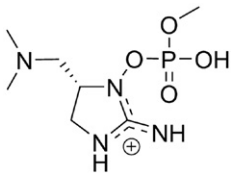

FIGURE 31.4 Anatoxin-a(s) chemical structure.

synthetic organophosphate insecticides and chemical warfare nerve agents. No structural variants have been described. It is susceptible to rapid degradation in alkaline conditions (Matsunaga et al., 1989).

Toxic Effects

Clinical signs are indistinguishable from organophosphate poisoning. Muscarinic signs include salivation, lacrimation, urinary incontinence, and defecation. Nicotinic signs include muscle tremors and fasciculations, convulsions, and respiratory failure (Mahmood and Carmichael, 1986). The muscarinic effects of anatoxin-a(s) can be suppressed by atropine (Cook et al., 1990), but it is relatively resistant to oxime reactivation, compared with typical organophosphate insecticides, because of the formation of an enzyme-adduct (Hyde and Carmichael, 1991).

Mechanism of Action

Anatoxin-a(s) is a noncompetitive inhibitor of acetylcholinesterase (AChE). Similar to other organophosphate poisons, anatoxin-a(s) is activated via oxidative metabolism. AChE is necessary for the inactivation of ACh at nicotinic and muscarinic receptor sites. Inhibition of the enzyme therefore causes ACh level to build-up at receptor sites, triggering excessive nicotinic and muscarinic receptor stimulation, resulting in persistent postsynaptic membrane depolarization (Cook et al., 1990). In this regard, the mechanism of action is similar to that of anatoxin-a, except that it is less selective for nicotinic effects. Anatoxin-a(s) has a high potency, with an LD_{50} of 20–50 μg/kg body weight when administered by intraperitoneal injection in mice (Mahmood and Carmichael, 1986). For further details, readers are referred to Patocka et al. (2011).

Chemical Warfare Potential

Anatoxin-a(s), similar to anatoxin-a, is a potential threat but will require relatively sophisticated procedures and equipment to implement.

Saxitoxins

Introduction

Saxitoxins are produced in freshwater and marine environments. In marine environments, they are often referred to as PSPs. Most human saxitoxin toxicoses have been associated with the ingestion of marine shellfish, which accumulate saxitoxins produced by marine dinoflagellates (Cusick and Sayler, 2013). In freshwaters, saxitoxins are produced by cyanobacteria in the genera *Anabaena*, *Aphanizomenon*, *Planktothrix*, *Cylindrospermopsis*, *Lyngbya*, and *Scytonema* (Smith et al., 2012; Wiese et al., 2012). Saxitoxins can accumulate in freshwater fish such as tilapia (Galvao et al., 2009).

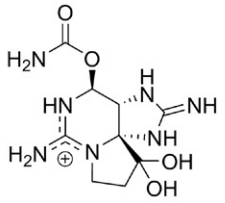

FIGURE 31.5 Saxitoxin chemical structure.

Chemistry

Saxitoxins are nonvolatile, tricyclic, perhydropurine alkaloids (Figure 31.5) (Schantz et al., 1975). Various structural substitutions produce at least 57 analogues. Activity is mediated through positively charged guanidinium groups (Wiese et al., 2012). Saxitoxins are heat-stable, particularly in slightly acidic environments, and are highly water-soluble. They are tasteless and odorless and are not destroyed by normal food preparation methods (Trevino, 1998). Methods of synthetic saxitoxin production have been published (Akimoto et al., 2013).

Toxic Effects

The clinical presentation of saxitoxin-poisoning varies depending on the level of exposure. High levels of exposure are usually related to ingestion of toxin-accumulating shellfish or fish, in association with toxin-producing dinoflagellate blooms (marine environments) or cyanobacterial blooms (freshwater environments). The lag time between exposure and the appearance of clinical signs is highly variable and can range from minutes to as long as 72 h (Montebruno, 1993). At relatively low exposure levels, moderate paresthesia, often described as a tingling sensation, are experienced around the mouth and extremities. Larger exposures lead to a spreading numbness of the mouth, throat, and extremities. High exposure levels may cause acute muscle paralysis and respiratory failure (Garcia et al., 2004).

Mechanism of Action

Saxitoxins are selective, reversible, voltage-gated sodium channel blockers (Huot et al., 1989; Tarnawa et al., 2007; Walker et al., 2013). Saxitoxins cross the blood–brain barrier, and sodium channel blockade in the CNS contributes to its paralytic effects (Borison and McCarthy, 1977). Saxitoxins are extremely potent, with mouse LD_{50} values of 263 μg/kg (oral), 10 μg/kg (intraperitoneal), and 3.4 μg/kg (intravenous) (Wiberg and Stephenson, 1960).

Chemical Warfare Potential

Saxitoxins are the only cyanotoxins listed in the Chemical Weapons Convention Schedule 1, where it is referred to as Agent TZ. Saxitoxins are also listed in the

War Weapons List of the German War Weapons Control Act. Reasons for the relatively high level of concern regarding saxitoxins include that they are potent and stable, they can be produced synthetically, and they have been shown to be suitable for incorporation into ammunition. A factor that complicates the regulatory control of saxitoxins is the ongoing need for its availability as an analytical standard in paralytic shellfish poisoning monitoring and risk management programs, and its use in medical research on voltage-gated sodium channels. Because of these needs, saxitoxins are subjected to lower levels of restriction under the Chemical Weapons Convention when small quantities (5 mg or less) are to be used for diagnostic or medical purposes.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Some cyanotoxins, particularly microcystins, can reach extremely high concentrations during HABs and could be collected and placed in storage with minimal equipment and technical sophistication. Use of such material to intentionally contaminate water, food, and medical supplies represents a significant health and economic risk. Other cyanotoxins, such as cylindrospermopsin, anatoxin-a, and anatoxin-a(s) are potentially dangerous because of their high potency and occasional availability during HABs but are less likely to be used intentionally because of unpredictable and sporadic availability and the need for complex extraction and storage procedures. Saxitoxins are an exception because they are listed in the Chemical Weapons Convention as a Schedule 1 compound. The listing is justified relative to other cyanotoxins based on its high potency, stability, availability of synthetic production methods, and suitability for incorporation into ammunition.

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CHAPTER

32

Radiation and Health Effects

Kausik Ray and Melissa Stick

INTRODUCTION

Exposure to ionizing radiation occurs throughout our lives, with beneficial and detrimental consequences. The majority of ionizing radiation exposure occurs as a result of natural sources; however, industrial, medical, and consumer sources also account for exposure (http://www.world-nuclear.org/info/Safety-and-Security/Radiation-and-Health/Nuclear-Radiation-and-Health-Effects/). Ionizing radiation cannot be detected without the use of specialized devices; therefore, the effects of exposure to humans have been extensively explored in large, retrospective population studies. These studies were conducted after large populations were exposed to harmful levels of radiation (for example, survivors of the Hiroshima and Nagasaki atomic bomb explosion and workers and residents around Chernobyl). In individuals from Hiroshima and Nagasaki, the major health effect observed at the lowest doses of exposure has been cancer, while heart, digestive, and other systemic complications have been noted at higher doses of exposure (Harley, 2001, 2008).

For many years, deliberate or accidental releases of ionizing radiation have been universally feared. Alternatively, ionizing radiation has been used in controlled, defined amounts as radiation therapy for certain tumors in both pet animals and man. Acute radiation toxicity responses, often known as *acute radiation syndrome (ARS)*, occur with high amounts of radiation and become apparent within 24 h after exposure. Because ARS can present with hematopoietic, gastrointestinal, and neurological/vascular symptoms, history is an important criterion in determining whether the radiation is related to the cause of a particular complication or adverse effect. Chronic radiation exposure may appear months to years after the exposure; complications may be associated with the bone marrow, kidneys, liver, or central nervous system (CNS) (Center for Drug Evaluation, 2005). The presentation of these symptoms assumes whole-body exposure and is not valid if the entire body has not been exposed. The speed with which symptoms manifest, as well as the difference in sensitivity of organs, is related to the absorbed dose of radiation, with greater doses resulting in a shorter period of time before the onset of symptoms.

Natural resources account for most radiation exposure. Attempts to determine if there are subtle effects from background irradiation have only begun to yield information about these widely dispersed effects. Advent of new techniques for such estimation has increased the sensitivity and reliability of detection. The objectives of this chapter are to describe common types of ionizing radiation, discuss radiation toxicity in humans, and provide information on the possible benefits and risks associated with ionizing radiation.

HISTORICAL PERSPECTIVE

Human understanding of ionizing radiation began with the pioneering work of individuals like Wilhelm Conrad Roentgen, Henri Becquerel, and Marie and Pierre Curie just before the turn of the twentieth century. In 1895, Roentgen observed that the external effects of an electric discharge tube covered with black paper caused a dye outside the tube to fluoresce. Although the term *Roentgen rays* is used in many countries, the more common term for these electromagnetic waves is *x-rays*, the name given to them by Roentgen himself. A year later, an Austrian surgeon named Leopold Freund demonstrated that this invisible energy source could be used therapeutically to treat a hairy mole. In the same year, Becquerel discovered radioactivity when a photographic plate protected from visible light was exposed to a uranium salt. In 1898, Marie and Pierre Curie published their findings of the isolation of a new compound that they

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suggested be named *polonium* after Marie Curie's homeland of Poland. They introduced the term *radioactivity* to describe the properties of the newly discovered element. A few months later, they published a paper about another radioactive element they had discovered, which they named *radium*. These early discoveries encouraged additional research on the uses of radiation.

BASIC RADIATION CONCEPTS

The nucleus of an atom contains one or more protons and (with the exception of hydrogen) neutrons, while any associated electrons, frequently equal in number to the protons, move around the nucleus in specific orbitals. Each element is defined by the number of protons in the nucleus, its atomic number (Z); for instance, $Z = 1$ for hydrogen, $Z = 6$ for carbon, and $Z = 8$ for oxygen. Although the number of protons defines the element, the number of neutrons defines the different isotopes of that element. For example, hydrogen has no neutrons in its nucleus, but there are two additional isotopes of hydrogen: deuterium, with one neutron, and tritium, with two neutrons. The atomic mass of an isotope is determined by the total number of nucleons (protons and neutrons); electrons with a mass that is 1/1,838th that of a proton or neutron contribute little to the total atomic mass. The shorthands for hydrogen, deuterium, and tritium are ^{1}H, ^{2}H, and ^{3}H (or H-1, H-2, and H-3), respectively. Most isotopes are stable (that is, the number of protons and neutrons remains unchanged indefinitely), but some isotopes have unstable nuclei, making them radioactive. For example, hydrogen and deuterium are stable isotopes, while tritium is a radioactive isotope that is commonly used in biomedical research.

Radioactive isotopes go through a process of radioactive decay that involves emitting ionizing radiation in the form of subatomic particles, electromagnetic radiation, or both. The source of radiation is the nucleus, and the decay continues until the nucleus is converted to a stable isotope. Tritium decays by emitting only one particle, becoming a stable isotope of helium (He-3, as opposed to the more common form of helium, He-4), while uranium (U-238) emits a total of 14 particles before becoming a stable isotope of lead (Pb-206). There are four naturally occurring radioactive families on Earth. Two of these have almost disappeared because their half-lives are relatively short in comparison to the age of the Earth (Table 32.1), leaving only the thorium and the uranium–radium series.

Each decay event on the way to becoming a stable isotope is known as a *disintegration*, and the rate of decay is given in disintegrations per minute (dpm) or disintegrations per second (measured in becquerels, abbreviated Bq); see Table 32.2 for a list of units and magnitudes.

TABLE 32.1 Naturally Occurring Radioactive Families

Series	Half-Life of Parent (Years)	Parent Radioisotope	Stable Decay Product
Thorium	1.41×10^{10}	Th-232	Pb-208
Uranium–radium	4.47×10^{9}	U-238	Pb-206
Uranium–actinium	7.04×10^{8}	U-235	Pb-207
Neptunium	2.14×10^{6}	Np-237	Bi-209

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The particles emitted during radioactive decay include alpha and beta particles and neutrons. In addition to ionizing particles, radioactive decay can also produce a form of ionizing shortwave electromagnetic radiation known as *gamma rays*. Important information about the steps involved in the decay of a radioactive isotope is contained in its decay scheme. An example for iodine (I-131), an important radioisotope with regard to nuclear power plant accidents, is given in Figure 32.1.

The four main types of irradiation are alpha particles; beta particles, which can be electrons (negatively charged) or positrons (positively charged); gamma rays; and x-rays. An atom can decay by the loss of mass (protons and neutrons), or the loss of charged electrons (beta particles or positrons). Gamma irradiation occurs when excess energy is released from the nucleus, usually after an alpha, beta, or positron transition. Ionizing radiation can also originate from outside the atomic nucleus. Examples include x-rays and extreme ultraviolet radiation (EUV). Historical observation demonstrated that radioisotopes emitted three unique types of radiation, which were differentiated based on the way they behaved when subjected to a magnetic field. Two types of radiation followed a curved path in a magnetic field, but in opposite directions, indicating that they had opposite charges. The positively charged radiation is called *alpha rays*, while the negatively charge radiation is referred to as *beta rays.* The third type of radiation, not deflected by a magnetic field, is referred to as *gamma rays*. Other atomic particles qualifying as ionizing radiation, but not discussed in this chapter, include a variety of particles generated in particle accelerators and particles, other than alpha and beta particles, which are produced in nuclear fission and fusion reactions.

Alpha and Beta Particles

In 1903, Ernest Rutherford discovered that alpha particles were equivalent to a helium nucleus (2 protons and 2 neutrons) and contained an overall charge of +2. Alpha particles are also given off by decaying radionuclides,

TABLE 32.2 Abbreviations for Units and Orders of Magnitude

Abbreviation	Parameter
UNITS	
Bq	becquerel
C	coulomb
erg	erg (10^{-7} J)
eV	electron volt
g	gram
Gy	gray
J	joule
m	meters
R	roentgen
rad	radiation absorbed dose
rem	roentgen equivalent in man (or mammals)
sec	seconds
Sv	sievert
MAGNITUDES	
a	atto (10^{-18})
f	femto (10^{-15})
p	pico (10^{-12})
n	nano (10^{-9})
Î¼	micro (10^{-6})
m	milli (10^{-3})
c	centi (10^{-2})
k	kilo (10^{3})
M	mega or million (10^{6})
G	giga (10^{9})
T	tera (10^{12})
P	peta (10^{15})
E	exa (10^{18})

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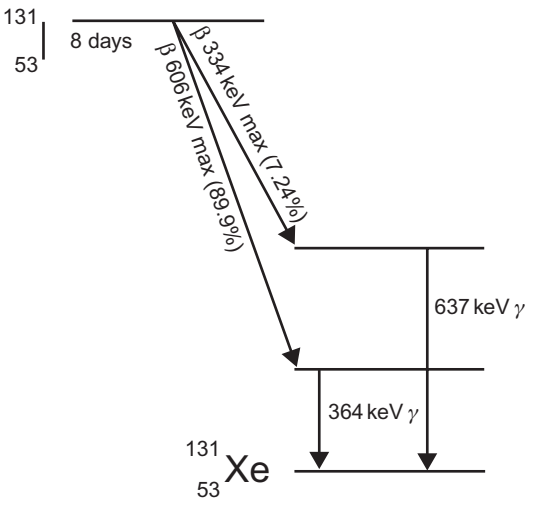

FIGURE 32.1 Decay scheme for ^{131}I, showing beta particle emissions that lead to the formation of a stable isotope of xenon (Xe-131). The decay occurs mostly in two ways: 7.24% of the time, a beta particle with maximum energy of 334 keV is emitted with subsequent emission of a 637-keV gamma ray; and 89.9% of the time, the decay occurs by the emission of a beta particle with a maximum energy of 606 keV and the subsequent emission of a 364-keV gamma ray. *Source: This image has been reprinted from Rebois and Ray, 2012. Copyright 2012, with permission from Elsevier.*

such as radium, and are present as a natural source of radiation. For example, $^{226}Radium_{86}$ decomposes to $^{222}Radon_{84}$ and an alpha particle ($^{2}He_{2}^{++}$) + energy (5.2 MeV). Most alpha particles are in the energy range of 4–8 MeV. Henri Becquerel was credited with the discovery of the beta particle in 1900, and he showed that beta particles were identical to electrons, which were discovered by Sir Joseph J. Thomson in 1897. Beta particles are emitted when a neutron is converted to a proton plus an electron, and the electron is lost. Unlike the discrete energy emissions from the decay of alpha particles, beta particles are emitted along a spectrum of energies because energies are shared between positive and negative electrons. A positron is emitted when a proton becomes a neutron and decays by beta emission or an electron is captured. Unlike the other particles (electron, proton, and neutron), positrons were detected as particles in cosmic rays by C. D. Anderson in 1932, were not found in "ordinary matter," and were formed only during interconversion of free elementary particles. Beta particles have the mass (9.1×10^{-31} kg) and the negative charge (1.6×10^{-19} coulombs) of an electron. A positron contains the same mass as a beta particle and has a +1 electric charge (whereas an electron has a −1 charge), but are equal in magnitude to an electron and may also be referred to as a *beta particle.*

The kinetic energy imparted to nonrelativistic alpha and beta particles when they are ejected from the nucleus is given by the following formula:

$$E = \tfrac{1}{2}mv^2,$$

where E is the energy of the particle in joules, m is its mass in kilograms, and v its velocity in m/s. The unit of energy for this equation is the joule (J), but the energy of ionizing particles is usually given in electron volts (eV), where 1 eV is the amount of energy imparted to an electron when it is accelerated by a potential difference of 1 volt. The factor for converting joules (kg m^2/s^2) to eV is:

$$1\text{eV} = 1\text{V} \times 1.6 \times 10^{-19}\text{C} = 1.6 \times 10^{-19}\text{J}.$$

Ionizing particles have energies in the thousands or millions of electron volts (keV or MeV, respectively):

$$1\text{KeV} = 1\text{eV} \times 10^3 \text{ and } 1\text{MeV} = 1\text{eV} \times 10^6.$$

Alpha particles emitted by a particular radioisotope all have the same energy. For example, U-238 emits an alpha particle with an initial energy of 4.19 MeV, but the energies of alpha particles from different isotopes vary. The energy of alpha particles ranges from 3 to 7 MeV, while beta particles have energies ranging from 20 keV to a few MeV. Beta particles from the same isotopic source vary in their energy. The value given in decay schemes represents the maximum energy (Figure 32.1). As a rule of thumb, the average energy is one-third of the maximum. The additional energy lost when lower-energy beta particles are emitted is contained in a neutrino that is also emitted.

Gamma Rays (Photon) Emission

Gamma ray emission is mostly secondary to alpha, beta, or positron emission or electron capture. When all of the energy is not used by the emission, the nucleus is in an excited state with energy available for emission. This excitation energy is emitted as a photon when the particle is emitted. In many cases, the photon will not be emitted; rather, it binds to an electron, which is ejected as a monoenergetic particle that equals the photon energy minus the binding energy. This latter process is called *internal conversion* (Harley, 2001, 2008). The wavelength (λ) of electromagnetic radiation varies from more than 10^8 m in length to less than 10^{-16} m. The energy of electromagnetic waves is given by the following equation:

$$E = hc/\lambda,$$

where h is Planck's constant (6.626×10^{-34} J.s or 4.136×10^{-15} eV.s) and c is the speed of light (3.0×10^8 m/s). Only electromagnetic waves with λ of less than approximately 10^{-8} m have sufficient energy to qualify as ionizing radiation. Electromagnetic radiation with λ between 1.2×10^{-7} and 10^{-8} m are referred to as *EUV rays*, while x-rays generally range from 10^{-8} to 10^{-11} m, and gamma rays are any electromagnetic radiation with λ of less than 10^{-11} m (Table 32.2). Plugging these values into the preceding equation gives a range of energy of 0.01–0.12 keV for EUV, 0.12–120 keV for x-rays, and greater than 120 keV for gamma rays. Electromagnetic radiation interacts with matter in three ways: (i) the photoelectric effect, (ii) Compton scattering, and (iii) pair formation. The photoelectric effect results in the complete absorption of a gamma photon's energy by an electron in the inner K and L shells of an atom. The electron escapes with energy (Ee) approximately equal to that of the photon (Eγ) minus the binding energy of the electron (Eb).

TABLE 32.3 Comparison of Biodosimetry Methods for Ionizing Radiation Exposure

Assay/Biomarkers	Detection Limit (Gy)	Postexposure Duration	Advantages/Disadvantages
Physical measurement/ESR	0.1	Indefinite (years)	No chemical analysis/large equipment required
Blood counts	0.5–1	Weeks, months	Simple, rapid
Tooth, enamel, fingernails	0.1	Weeks, months	Dose estimated even after years/low detection limit
Cytogenetic biomarkers			
Dicentric (unstable)	0.5–1	Several years	Radiation-specific/technical expertise
Micronuclei	0.1–0.3	1 year	Fast, simple/specific technique
PCC	0.1–0.5	Days, weeks	Cheap, fast, simple
FISH (stable)	0.1–0.25	Years	Rapid/technical expertise
SOMATIC CELL BIOMARKER			
GPA mutation	1–2	Indefinite (years)	Fast, simple, detected after years
HPRT mutation	1–2	Transient (<1 year)	Simple/longer length of time
Nucleic acid biomarkers (DNA, RNA)	0.2–1	2 days or more	Reliable markers/specific techniques
Protein Biomarkers	1–2	Within a day or more	Reliable/lacks amplification
Metabolite biomarkers	0.2–1	Within 24 h or more	Within 24 h or more
Microbiome biomarker	0.2–1	Undetermined	Noninvasive/instruments needed

ESR, electron-spin resonance; PCC, premature chromosome condensation.

Eb varies with the atomic number (Z) but is generally much less than $E\gamma$, so the energy carried away by the electron is close to that of the incident gamma photon. Gamma photons can also be scattered by an encounter with an electron. This is known as *Compton scattering*, and it results in the deflected gamma photon continuing in a new direction, with reduced energy, while the electron carries off the balance of the energy in another direction. Pair formation occurs when a gamma photon is converted into an electron–positron pair. The energy represented by an electron (or positron) with resting mass m_o (9.1×10^{-31} kg) is given by $m_o c^2$. Since $c = 3 \times 10^8$ m/s and 1 MeV = 1.6×10^{-13} J (kg m^2/s^2),

$$m_o c^2 = 0.51 \text{ MeV}.$$

Because both an electron and a positron are formed, the photon's energy must be at least 1.02 MeV. Any energy in excess of this amount is shared by the particles as kinetic energy. Positrons are unstable and, after coming to rest, will quickly recombine with resting electrons, producing a pair of gamma photons each of 0.51 MeV moving in opposite directions.

X-Rays

When a beam of electrons hits a heavy metal target, their rapid deceleration by positively charged atomic nuclei within the target causes some of the electron energy to be converted to electromagnetic radiation in the form of x-rays. Radiation produced in this way is also known as *bremsstrahlung* (which means "braking" in German). The beam of electrons originates at the negatively charged cathode within an evacuated tube and is accelerated toward the metal target (the anode) by a voltage difference between the two electrodes. The energy of the x-rays depends upon the magnitude of this voltage difference. X-rays generated by voltage differences of 100–300 kV have enough energy to ionize molecules in the target tissue by Compton scattering. The electrons that are stripped from molecules are known as *secondary electrons*, and if they have sufficient energy to produce additional ionizations, then they are known as *delta rays*. X-rays produced with high voltages have important diagnostic and therapeutic applications. The energy of x-rays produced by x-ray tubes is limited by the potential difference that can be applied to the electrodes. The practical limit for conventional x-ray tubes is 300 kV. By definition, the electrons produced by this voltage would have a maximum energy of 300 keV and are capable of producing x-rays with the same maximum energy. Only about 1% of the electrons striking the anode generate x-rays, and their average energy is one-third of the maximum. The energy of the remainder of the electrons is converted to heat. Generating x-rays with energies on the order of 10 MeV requires a linear accelerator. An x-ray with energy of 10 MeV would correspond to a wavelength on the order of 10^{-3} m or 0.0001 nm. This overlaps extensively with gamma rays (Table 32.1). What distinguishes gamma rays from x-rays is not wavelength, but the source of the radiation. Gamma rays originate in the nucleus of an atom, while x-rays arise from sources outside the nucleus. X-ray tube voltages between 25 and 50 kV are used to generate "soft" x-rays (Table 32.1), which are absorbed completely after producing a single ionization by the photoelectric effect described previously. Soft x-rays are important for medical diagnostic procedures such as mammography because they produce better contrast between tissues that do not differ greatly in electron density, making it easier to distinguish between tumor tissue and normal tissue.

INTERACTION OF RADIATION WITH MATTER

The interactions of radiation with matter generate ionized and excited atoms and molecules in the irradiated material and result in changes in its physical, chemical, and biological behavior. Kinetic energy transfer from alpha, beta, or gamma photons to atoms and molecules of matter leads to ionization or excitation of these atoms and molecules. Ionizing radiation loses energy by producing ion pairs (an electron and a positively charged atom). Radiation energy with about 33.85 eV is needed to produce an ion pair (ionization) by ejecting one or more orbital electrons from an atom. Ion pairs themselves can interact with surrounding matter, producing more ion pairs or secondary ionization. If the energy transfer is not sufficient to cause ionization, excitation occurs, and electrons in an excited state may break or form molecular bonds or can revert to the original energy level by emitting electromagnetic radiation.

Alpha Particles

An alpha particle, with a mass equal to 7,300 times that of an electron, loses its energy over a relatively short distance. Since 33.85 eV is required to produce an electron pair, an alpha particle (typically 5 MeV of energy) can produce approximately 7,400 electron pairs within 1 micron (μm) of the decay. Penetration by alpha particles can easily be stopped by a sheet of paper (100 μm thick) or the protective (dead) layers of skin. Most damage caused by alpha particles occurs when they are ingested or inhaled because they end up in close proximity to living cells. The effects of ingested or inhaled alpha particles will be discussed in greater detail later in this chapter.

The rate at which an electron transfers energy to a material is known as *linear energy transfer (LET).* LET is used to describe the local concentration of energy in human tissue resulting from ionization by charged particles passing through human tissue. The actual relationship of the efficiency in producing damage to LET values (keV per μm-keV/μm) depends on the biological effect considered. For a biological effect, there is a LET value that produces an optimum energy concentration within the tissue. Radiation with lower LET values does not produce an adequate concentration of energy, but radiation with higher LET values tends to deposit more energy than is needed to produce the effect. Inhaled or ingested alpha particles that do not escape the body lose all their energy over a relatively short distance. This energy is deposited in the tissue over the space of a few millimeters, resulting in a high LET. In addition to tissue damage, cellular changes, including deoxyribonucleic acid (DNA) strand breakage, are also associated with high LET activity; this topic will be discussed further later in the chapter.

Beta Particles

Beta particles with small mass, high velocity, and a single negative charge can travel through matter farther than alpha particles before producing ionization; therefore, their energy loss in matter is calculated differently. Even low-energy beta emitters travel near the speed of light and must be corrected for mass increase by the term 0.511 (1 − particle velocity^2/light velocity^2) + 0.511. When traveling through matter, electrons of beta particles interact with the negative charge of orbital electrons, ejecting them from their orbits, thus producing ion pairs or causing excitation. When approaching the nucleus of the atom within the target, the velocity of beta particle increases and then decreases as it moves away due to electrostatic interaction (positively charged protons in the nucleus). This type of emission, referred to as *bremstrahlung radiation*, increases with the atom number of the target atom, increasing electrostatic attraction. Products with a low Z value (Z = number of protons in the nucleus), such as aluminum or plexiglass, can serve as a shield and protect from high-energy beta emitters. Beta particles of 1 MeV will penetrate to about 0.5 cm in water for soft tissue and have much less LET than alpha particles. Because damage to tissue is related to LET, exposure to beta particles results in less damage than exposure to alpha particles.

Gamma and X-Rays

Gamma and x-rays are both electromagnetic radiation but differ in their origin. As stated earlier, gamma rays originate in the nucleus of an atom, while x-rays arise from sources outside the nucleus. Because gamma rays possess more energy that alpha and beta particles, they are able to penetrate much farther into tissue and deposit their energy over larger distances. If lead shielding is used, the density would be much greater, and penetration beyond the lead shield would be much less. If energy emitted is sufficiently low, penetration of the rays is negligible. While lead shielding confers protection, some fraction of energetic gamma-emitters (e.g., ^{60}Cobalt) irradiation can penetrate even lead, and added earth shielding is required. Radiation leakage, which means the penetration of radiation, is usually measured to determine the amount of external radiation that is still present in spite of the presence of a source and shielding. If therapeutic gamma radiation is used instead of x-irradiation, radiation leakage may become as high as 1–10% of the source strength depending on the amount of shielding. Because both gamma rays and x-rays have low LET, travel at a low rate along their path, emerge from the body and continue on their way, x-rays can be used to generate images on a photographic plate.

ABSORBED DOSE

Total Dose and Dose Rate

The term *absorbed dose* (total ionizing dose) describes the amount of radiation absorbed by an object or person. The units for absorbed dose are gray (Gy) and rad. Absorbed dose is a function of the mass and density of the media. Sometimes absorbed dose is called *kerma* (kinetic energy released in matter). Because exposure and dose are often used interchangeably, dose is often confused with exposure level. The absorbed dose depends not only on the radiation incident, but also on the absorbing material; a soft x-ray beam may deposit a dose four times greater in bone than in air, and none at all in a vacuum.

If the dose is delivered quickly in a single exposure at a high dose rate, there is almost no time to repair radiation damage, and the damage/unit of dose absorbed is high. Alternatively, if the total dose is delivered slowly, at a lower dose rate, or in divided dose fractions, over the life span of the subject, there is more time to repair radiation damage and the damage/unit of dose is much lower. For example, if multiple fractionated doses are delivered per hour, the damage/unit of total dose decreases significantly. In this situation, the radiation therapist can deliver higher doses to the area, but less total rem/h from the source or x-radiation machine.

Dose Equivalent and Cancer Risk

Dose equivalent (or effective dose) takes into consideration the amount of radiation absorbed and the medical effects of the type of radiation. Alpha particles, with

large mass, produce intense ionization tracks per unit distance relative to beta particles, whereas beta particles produce more intense ionization than gamma rays. As stated earlier, LET measures this transfer of energy per unit distance traveled by the emission from the radiation particle. In situations where dose is considered to be proportional to response, that dose must be normalized for LET. For a particular end point (e.g., cell death in mouse fibroblasts), the relative biological effectiveness (RBE), which is equal to the dose of radiation under study relative to the dose of gamma rays needed to achieve the same effect, is calculated. Radiation with high LET will be expected to have an RBE of greater than 1. For beta and gamma radiation, the dose equivalent (1 rem or 1,000 mrem) is the same as 1 rad of the absorbed dose, but is larger than the absorbed dose for alpha and neutron radiation, as these types of radiation are more damaging to the human body.

Dose equivalent (or effective dose) is calculated as follows: Dose equivalent = (Absorbed dose) × (weighting factor or RBE). Recommended weighting factors are approximately 1 for x-rays, gamma rays, beta particles, and electrons, and 20 for conventional neutrons (>0.1–2 MeV), protons, alpha particles and charged particles of unknown energy. As an example, the weighting factor or RBE of plutonium-238 (^{238}Pu) is between 10 and 30; thus, the roentgen equivalent in humans (rem) is 10–30 roentgens in air (rad). Similarly, the dose equivalent following alpha emission is larger than the absorbed dose, which is confined to an immensely small area in human tissue because alpha irradiation travels only a short distance in tissue and has a high LET.

The term *effective dose* allows the comparison of cancer and genetic risks from different partial-body and whole-body doses to be integrated for the purposes of estimating a whole-body response. Complicating the matter, different organs vary in their sensitivity to the effects of radiation. This is taken into account by using a tissue-weighing factor that varies for different organs. Some tissues, like gonads, are more sensitive than others, such as bone marrow, colon, stomach, and lung. Similarly, bone marrow, colon, stomach, and lung are more sensitive than bladder, breast, liver, esophagus, and thyroid. Skin and bone surface are the least sensitive. Other tissues not specified are taken to have a weighting factor of 0.05 as a conservative estimate (Harley, 2001, 2008). Effective dose is calculated by multiplying the tissue-weighting factor in protracted exposures (exposures over significant periods of time) by the dose equivalent.

Committed Dose Equivalent

The *committed dose equivalent* is the dose of radiation that a specific organ or tissue receives from an intake of radioactive material over a specified period of time. Once radioactivity is placed internally in the body, it becomes an internal emitter. Internal emitters can be used therapeutically (actually implanted in a tumor) or result from accidental exposure via ingestion or inhalation or penetration through skin. Usually, internal emitters cannot be removed simply or easily, and an irreversible committed dose equivalent is said to exist. For radionuclides with a half-life of 0–3 months, the dose equivalent or effective dose is equal to the annual dose of the year of intake. After 12 months of exposure, a radionuclide with a half-life of 3 months will have undergone the passage of four half-lives; the fraction of dose delivered will be $(1 - ½^4)$ (or approximately 94% of the total dose) for all time. Thus, 94% of the possible dose will have been delivered, and only 6% will remain to be delivered. Radionuclides with a shorter half-life deliver almost 100% of the dose within a year of exposure [half-life of 1 month $(1 - ½^{12})$, or approximately 99.98% of the total dose for all time]; therefore, any effort to remove an internal emitter will reduce the internal dose only minimally. Alternatively, if the half-life of the radionuclide is 1, 10, or even 5,000 years, considerably less radioactive decay will occur, and much more of the total dose will remain to be delivered: $(1 - ½)$, or approximately 50%, $(1 - ½^{1/10})$, greater than 50%, and $(1 - ½^{1/5,000})$, significantly greater than 50%, respectively, at the end of 1 year. So, for elements with longer half-lives, it is more advantageous to remove the particulates than to allow the natural process of decay and the risks associated with it to continue. It is a good idea to perform such analyses with each radionuclide to see how much of the dose remains to be delivered; this way, the effort associated with removing the remaining dose can be weighed against the risks of not removing the remaining dose.

Negligible Individual Risk Level (Negligible Dose)

The concept of linear, nonthreshold cancer induction from ionizing radiation is an accepted fact, and this allows calculation of cancer risk regardless of how small or large the dose may be. Calculating very low risk enables us to calculate a risk below the ability to detect a response. In 1987, the National Council for Radiation Protection (NCRP) defined the *negligible individual risk level (NIRL)* as the level of average annual excess risk of fatal health effects attributable to irradiation below which efforts to reduce radiation exposure to the individual is unwarranted. The NCRP emphasized that this level should not be confused with *acceptable risk level*, a level of significance or a standard. The NCRP recommended a level of about half the natural background radiation level, which is approximately 2 mrem; the final recommended NIRL level is 1 mrem or 0.01 mSv. This level is now called the *negligible individual dose (NID)* level.

CONSEQUENCES OF RADIATION-INDUCED DNA DAMAGE

Chromosomal Aberrations and Radiation-Induced Genomic Instability

The cytogenetic effects of x-rays were first documented in plants irradiated during the G1 cell cycle phase and included the production of dicentrics, centric rings, and deletions (Sax, 1940). The current view is that the majority of radiation-induced chromosome aberrations are produced by mispairing of DNA double-strand breaks (DSBs), possibly due to damage to multiple DNA sites and complex exchanges of DNA lesions. While repair of DSBs is performed by nonhomologous end-joining and homologous recombination mechanisms in mammalian cells, in some cases, the pairs of DSBs required for the formation of chromosomal aberrations are produced by one or more electron tracks from the single photon and in others by two or more tracks of different photons (Charlton et al., 1989). The prevailing view is that chromosomal aberrations of all types result from the interactions of multiple pairs of DNA lesions, and that these lesions can be induced by a single track or by combinations of two or more radiation tracks. Ionizing radiation can induce a wide range of unique DNA lesions, both double-stranded and single-stranded breaks can be expressed as chromosomal aberrations (Price and D'Andrea, 2013). DNA DSBs are particularly lethal, and a complex cascade of signal transduction pathways respond to the radiation-induced cell damage in an attempt to restore the integrity of the damaged DNA molecules (Kavanagh et al., 2013). Based on the complexity of DNA damage (such as defects in sister chromatid exchanges, chromosome aberrations, and micronucleation), DNA lesions can also lead to cell cycle arrest. Many cytogenetic assays are now available to measure chromosomal aberrations along with cell death markers, but these tests are slow in detecting the circulating markers and are not always useful for predictive, prognostic, and diagnostic purposes. There is increasing interest in developing automated, noninvasive DNA and chromosome-based tests for radiation exposure (Amundson et al., 2001; Varga et al., 2004; Martin et al., 2007; Pinto et al., 2010a,b).

Chromosomal changes are the best-described phenomenon associated with radiation-induced instability. Radiation-induced genomic instability is observed at delayed times after irradiation and is manifest in the progeny of exposed cells many generations after the initial insult. Among the instabilities described in a number of reviews are chromosomal alterations, micronucleus formation, gene mutations, gene amplifications, and changes in ploidy (Seizer and Hebar, 2012). Multiple molecular pathways have been shown to initiate and promote instability; the contributions of these different pathways also depend on the genetic background of the target cells or organisms (Klarer and McGregor, 2011; Jacquet, 2012; Vijg and Suh, 2013; Mukherjee et al., 2014).

In previous studies, delayed responses associated with radiation exposure have included the loss of reproductive potential and cell death among the clonal progeny of irradiated cells (Puck and Marcus, 1956; Trott and Hug, 1970; Seymour et al., 1986) and chromosomal instability in skin fibroblast cultures obtained from mouse fetuses derived from irradiated zygotes (Pampfer and Streffer, 1989). More definitive studies of chromosomal instability were obtained using an *in vitro* clonogenic assay to obtain clonal bone marrow cell populations derived from irradiated hematopoietic stem cell populations from both murine and human bone marrow (Kadhim et al., 1992, 1994). In these studies, cytogenetic analysis revealed nonclonal aberrations with a high frequency of *de novo* chromatid-type aberrations. Comparable ongoing chromosomal instability in bone marrow has been demonstrated *in vivo* (Watson et al., 1996, 2001; Zyuzikov et al., 2011). While some investigators describe chromosomal gaps or breaks as the primary observed aberration, it is unlikely these contribute to the instability process since gaps have no known phenotype and breaks are generally lethal events. Of more significance are gross chromosomal rearrangements, particularly chromosomal duplications, partial trisomies, or both, which appear to involve the amplification and recombination of large chromosomal regions by a currently unknown mechanism (Marder and Morgan, 1993; Limoli et al., 2000; Savage, 2004).

Radiation-Induced Bystander Effect

The term *bystander effect* describes the ability of cells affected by irradiation to convey manifestations of damage to other cells not directly targeted for irradiation. An irradiated cell can send out a signal and induce a response in a cell whose nucleus was not directly hit by radiation. This amplification can cause similar radiation-induced effects and may result in genetic damage or instability. Some radiation-induced bystander effects can be detrimental, while others are not, depending on the type of cell producing the bystander signal after irradiation and the type of cells receiving these signals. The first indications of radiation-induced bystander effects were reported in 1992, when in two independent studies of α-particle irradiations, cytogenetic responses were noted in more cells than were actually irradiated (Kadhim et al., 1992; Nagasawa and Little, 1992). In these studies, low fluencies of α-particles were used, resulting in a situation where many cells were not actually "hit" by a particle; however, the number of cells expressing responses was greater than the number of cells actually

irradiated. The phenomenon of more cells than were actually irradiated expressing responses has been confirmed by a number of investigators (Deshpande et al., 1996; Azzam et al., 1998; Nagasawa and Little, 1999; Zhou et al., 2000; Prise et al., 2002). In unrelated studies, significant decreases in cloning efficiency were observed in nonirradiated cells exposed to medium from irradiated cell cultures (Mothersill et al., 1997; Mothersill and Seymour, 1998). Radiation-induced bystander effects have also been demonstrated in the coculture of irradiated and nonirradiated cells (Pinto et al., 2006, 2010a,b). These indirect effects of radiation may result in genetic damage or instability and may be lethal to nonirradiated cells. Although the majority of bystander studies have focused on end points associated with genomic damage, the effects are not necessarily always detrimental. Favorable bystander effects on cells include enhanced cell differentiation (Belyakov et al., 2002), increased cell proliferation (Iyer et al., 2000; Gerashchenko and Howell, 2004, 2005), and a radioprotective adaptive response.

Our understanding of these nontargeted bystander effects is still in its infancy. Because much of the data to date has been obtained from *in vitro* studies, the significance of these indirect effects on human health has yet to be elucidated. It would seem prudent to consider the implications of nontargeted delayed effects of radiation exposure when considering models of radiation carcinogenesis, particularly at low doses.

HUMAN EXPOSURES AND RADIATION TOXICITY

Different doses of irradiation produce a variety of radiation-induced toxicity in humans and as stated before, different organs vary in their sensitivity to the effects of radiation (Figure 32.2). Biological effects of radiation are typically divided into two categories. The first category consists of exposure to high doses of radiation over short periods of time producing acute or short-term effects. The second category represents exposure to low doses of radiation over an extended period of time, producing chronic or long-term effects. High doses tend to kill cells, while low doses tend to damage or change

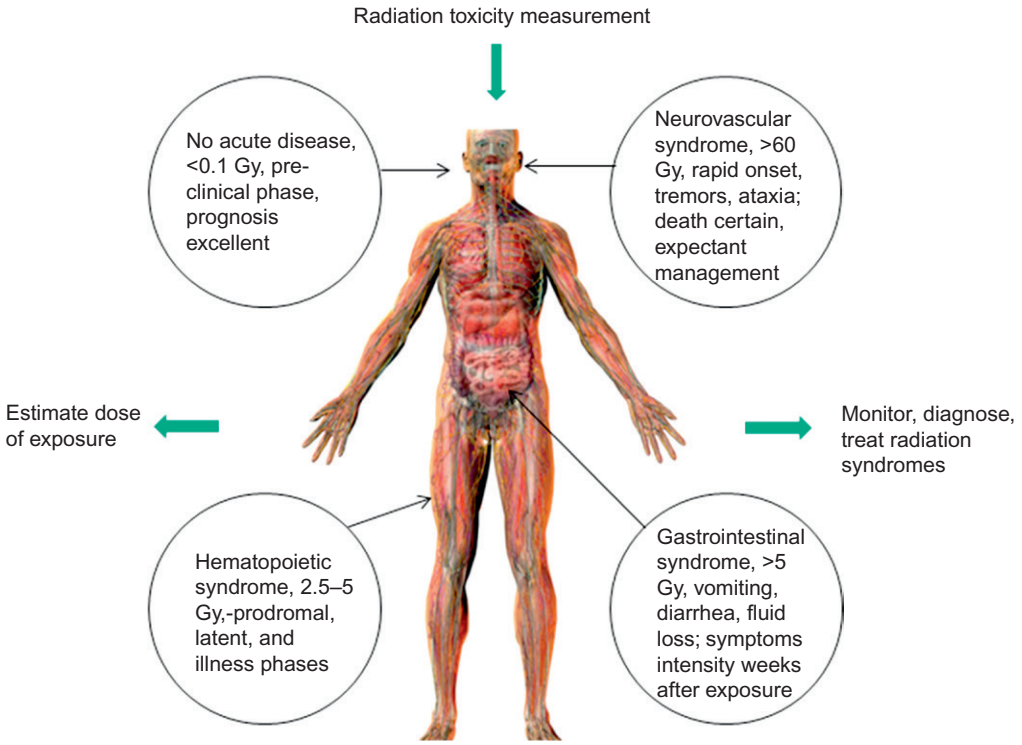

FIGURE 32.2 Radiation dose ranges and health impacts in humans after total body exposure. Health effects are categorized by time of appearance after irradiation as *early* and *late effects*. Two major variables needed to predict outcomes in humans are the amount of radiation dose and its distribution in time; that is, dose rate and fractionation. Health impacts of uniform irradiation of the whole body include three ARSs—the hematopoietic (bone marrow), GI, and neurovascular syndromes, with thresholds of about 2, 5, and 50 Gy. When total body exposure occurs, a series of events takes place. Within a few days to weeks, the prodromal symptoms occur, which vary based on the dose magnitude and length of time of the exposure. In total body exposures of about 100 Gy, cerebrovascular syndrome results in rapid death as a result of blood leaking through vessels in the brain. GI syndrome results in days and weeks after 5–10 Gy of exposure due to the destruction of the epithelial lining of the GI tract. Hematopoietic syndrome results days to weeks after total body radiation exposure ranging from 2.5–5 Gy. That syndrome leads to bone marrow depletion and a reduction in circulating red blood cells, white blood cells, and platelets.

cells. High doses affect many cells, can cause tissue/organ damage, and ultimately yield ARS. Even normally radio-resistant cells, such as those in the brain, cannot withstand the cell destruction capability of very high doses of radiation. The initial signs and symptoms of the ARS are nausea, vomiting, fatigue, and loss of appetite. Below 0.1 Gy, these symptoms, which are no different than those produced by a common viral infection, may be the only outward indication of radiation exposure. Depending on the level of the dose above 1 Gy, the presentation of one of three radiation syndromes (neurovascular, gastrointestinal, and hematopoietic) may begin.

Radiation levels greater than 60 Gy produce CNS changes, also known as *neurovascular syndrome*, characterized by agitation, apathy, disorientation, disturbed equilibrium, vomiting, opisthotonus, convulsions, prostration, coma, and death usually occurs within 24–48 h after exposure (NCRP, 1989). Prior to 1970, the human brain was thought to be radio-resistant to lower doses of radiation, with acute CNS syndrome occurring after a single dose of greater than 30 Gy and white matter necrosis occurring at fractionated doses greater than 60 Gy. During the 1980s–1990s, late radiation-induced brain injury, characterized by vascular abnormalities, demyelination, and ultimately white matter necrosis, was recognized as a dose-limiting morbidity presenting more than 6 months after irradiation (Schultheiss et al., 1995). Extensive preclinical studies in rodents revealed dose-dependent changes in these histopathological lesions (Calvo and Hanks, 1988). Classically, late radiation-induced brain injury was viewed as solely attributable to a reduction in the proliferative capacity of glial or vascular endothelial cells in rodents (van den Maazen, et al., 1993). Of importance, these late effects were viewed as progressive and irreversible. Although white matter necrosis is uncommon with modern-day radiation therapy technologies, there has been a growing belief that patients receiving fractionated partial or whole-brain irradiation can develop significant cognitive impairment more than 6 months after irradiation, even in the absence of detectable anatomic abnormalities (Fike, 2011; Greene-Schloesser and Robbins, 2012).

Gastrointestinal (GI) syndrome can result after acute exposure to 10 Gy or less. The radiation exposure causes destruction of the epithelial lining of the GI tract, and GI syndrome is characterized by lethargy, diarrhea, dehydration, degeneration of bowel epithelium, and death in 10–14 days (NCRP, 1989). The other syndrome associated with acute exposure is hematopoietic syndrome, which may present days to weeks after total body radiation exposure ranging from 2.5 to 5 Gy. The hematopoietic syndrome is characterized by granulocytopenia, thrombocytopenia, hemorrhage, infection, and electrolyte imbalance. Even lower doses (1–5 Gy) can cause hematopoietic syndrome, which results in what is commonly termed *bone marrow death.* Death resulting from hematopoietic syndrome is usually associated with higher dose exposure and typically occurs within 2–3 months after exposure; at lower doses (1–3 Gy), survival may be much longer (Fliedner and Graessle, 2008).

HUMAN POPULATION AND HEALTH RISKS

Radium Exposures

In the mid- to late-1920s, many young women in the United States who were watch-dial painters accidentally ingested radium through the practice of "pointing" (touching) their paintbrush tips to their lips while painting the hands and faces of clocks and watches with radium paint. The cohort studies of cancer risk among radium watch-dial painters were initially carried out at the Massachusetts Institute of Technology and the Argonne National Laboratory (Stebbings et al., 1984; Stehney, 1994). The survival times of women radium dial workers first exposed before 1930 showed definite life-shortening, radium-induced malignancies. Effects that emerged later were osteogenic sarcoma and head carcinoma, primarily in the sinuses.

Radium exists in several different forms depending on the parent radioactive element. Ra-226, the most common form, is created during the decay of uranium (specifically U-238), an element with a half-life of approximately 4.5×10^9 years. In contrast, Ra-228 has a half-life of 6.7 years. Once taken into the human body, radium acts somewhat similar to calcium. It is incorporated on the surface of the bone into the bone matrix. Raabe et al. (1980) modeled dose and risk and defined the level at which bone cancer will not appear from radium exposure to be 0.04 Gy (4 rad/day), or a total dose of 0.8 Gy (80 rad). A multivariate analyses of the dose–response relationship of the two radium isotopes (Ra-226 and Ra-228) found in the body of a dial painter determined that both isotopes of radium contributed significantly and independently to the rate of mortality from bone sarcomas. The ability of Ra-228 to induce bone sarcomas, per microcurie (μCi) of intake, is two and a half times greater than Ra-226. The term *initial systemic intake* may be used to define the risk of the induction of a bone sarcoma in a dial painter. It is the sum of the activity of Ra-226 (in μCi) that entered the body, plus two and a half times the activity of Ra-228 (in μCi) that entered the body. In another study, British dial painters who were exposed to lower doses of radium and did not ingest radium by brush pointing showed no evidence of bone sarcomas (Baverstock and Papworth, 1985). Bone sarcomas also were observed in patients with tuberculosis, ankylosing spondylitis (AS), and other diseases in

which high doses of ^{224}radium exposure were part of the treatment (Nekolla et al., 2000).

Atomic Bomb Survivors

The greatest number of war-related acute-radiation syndrome deaths occurred when nuclear bombs were detonated over Hiroshima and Nagasaki in August 1945. Within 1–2 km of the epicenter, people received radiation doses as high as several Sv. At distances greater than 2.5 km, irradiation was not significantly above the background. Risk estimates are important because they are used for occupational exposure guidelines. Based on more than 60 years of collected data and a follow-up study of atomic bomb survivors, the Life Span Study (LSS) investigated the radiation effects on human health (Sakata et al., 2012). The LSS cohort consisted of 93,741 atomic bomb survivors and another 26,580 age and sex-matched subjects who were not in either city at the time of the bombing. Radiation doses were computed based on individual location and shielding status at the time of the bombings. Radiation exposure significantly increases the risks of death (22% at 1 Gy), cancer incidence (47% at 1 Gy), death due to leukemia (310% at 1 Gy), and incidence of several noncancer diseases (e.g., thyroid nodules, chronic liver disease and cirrhosis, uterine myoma, and hypertension). Significant effects on maturity (e.g., growth reduction and early menopause) were also observed, as was an increased incidence and mortality of diseases other than cancer. Another similar long-term follow-up study of atomic bomb survivors has provided reliable information on health risks for the survivors and was helpful in establishing radiation protection standards for workers and the public (Fujiwara et al., 2008; Grant et al., 2012; Semmens et al., 2013).

Children Treated with X-Irradiation for Ringworm of the Scalp (*Tinea Capitis*)

Between the 1940s and the 1950s, more than 2,200 children between the ages of 1 and 15 years received x-ray treatment for ringworm of the scalp at New York University Medical Center. When compared to 1,400 control children with essentially the same disease, but treated with other methods, a striking temporal pattern of radiation-induced skin cancer was observed (Shore et al., 1984). No excess risk of skin cancer was noted 20 years postirradiation, but thereafter, the excess risk became pronounced. The risk increased with time, and the excess rates for 20–24, 25–29, and 30–35 years postirradiation were 79, 200, and 420/10^5 person-years (PY) at risk, respectively. The risk for basal cell carcinoma of the skin was highest, and females had a greater mortality rate than males. Also noted was that the risk of skin cancer occurring on the face increased, and lightness of skin complexion was an important risk factor. This suggests that ultraviolet (UV) ray exposure or sensitivity, along with exposure to ionizing radiation, may have a cumulative effect in defining skin cancer risks in these children (Roy et al., 1984; Shore et al., 1984).

Chernobyl, Three Mile Island, and Fukushima Dai-ichi Nuclear Reactor Accidents

The Chernobyl nuclear accident in 1986 resulted in the largest radiation exposure in recent history. The radioactive materials released contained high levels of radioactive iodine (particularly, ^{131}I, with a half-life of 8 days), an element that accumulates in the thyroid gland as a component of thyroid hormone. Following the explosion, people were exposed to deadly radioactive materials estimated to be 100 times greater than that associated with the detonation of the atomic bomb over Hiroshima. In Belarus, thyroid cancer in children under 18 increased from an incidence of 0.03–0.05 cases per 100,000 (1986–1988 data) to more than 10 times that level (5–8 cases per 100,000) in the period 1993–2002. Increases in thyroid cancer also were noted in Ukraine, with rates going from 0.02 per 100,000 (1986–1988 data) to 5–10 times that level (1–2.2 per 100,000) over the period from 1993–2002 (Reiners et al., 2013). There is little doubt that Chernobyl radiation exposure caused thyroid cancer among children in the affected area.

It is worth mentioning that potassium iodide (KI) is a compound that blocks the uptake of ^{131}I. If KI had been available and use of the contaminated milk been discontinued in favor of other sources, it is doubtful that large numbers of thyroid cancers would have occurred after the Chernobyl disaster.

The human health consequences of the accident at the Three Mile Island Unit 2 (TMI-2) nuclear reactor in Pennsylvania in 1979 were minimal. The small radioactive releases at Three Mile Island have had no detectable health effects on plant workers or the public, and a recent study determined that the actual release had negligible effects on the physical health of individuals or the environment (World Nuclear Association report, January 2012).

While it is still too early to determine the long-term health consequences of the Fukushima Dai-ichi accident in 2011, France's Institute for Radiological Protection and Nuclear Safety (IRSN) estimated the maximum external doses to people living around the plant were unlikely to exceed 30 mSv/yr in the first year. People living in Fukushima prefecture are expected to be exposed to around 10 mSv over their entire lifetimes, while for those living farther away, the dose would be 0.2 mSv per year. The findings from several international reports to date (Becker, 2011, 2013; Sugimoto et al., 2014), including a health risk assessment report from the World Health Organization (WHO) in 2011 (http://apps.who.int/iris/

bitstream/10665/78218/1/9789241505130_eng.pdf), concluded that long-term health risks for those individuals with the greatest exposure are possible.

Patients Irradiated with X-Rays for AS

AS patients diagnosed between 1935 and 1957 in the United Kingdom were exposed to x-rays to relieve and/or cure them. Patients received a total body dose of 2.64 Gy during several courses of treatment over a 5-year period; vertebrae received the highest dose. Cancer mortality was significantly higher compared to the expected national rates, and significant increases in leukemia, non-Hodgkin's lymphoma, multiple myeloma, and cancers of the esophagus, colon, lungs, pancreas, bones, bladder, kidney, and prostate, relative to a control population, were observed 6–20 years after exposure. A linear dose–response model for all cancers except leukemia gave a higher relative risk in the period 5–24.9 years after first radiation treatment, but decreased significantly in the period more than 25 years after the first treatment (Weiss et al., 1994).

Miners Exposed to Radon

An increased risk of lung cancer has been demonstrated in miners exposed to radon, including uranium, tin, silver, and coal miners and those mining other substances in radon-contaminated mines. Most cancers from radon are produced by radon daughter decay products (polonium, 3 isotopes; bismuth, 1 isotope; and lead, 3 isotopes) adhering to the delicate cell lining and passageways leading to the lungs. The increase in the excessive relative risks (ERRs) of lung cancers is approximately linear to the exposure, as estimated by cumulative working level months (WLMs) and ranges from 0.002 to 0.08 per WLM of 170 h of exposure. 1 WLM is about 200 picocurie (pCi) per liter of air in a home and 300 pCi/L in an underground mine. Excess lung cancer risk diminishes with time from exposure, and some data show the excess risk may disappear completely 30–40 years after a single exposure (Roscoe et al., 1989; Smigel, 1989). While cigarette smoking is the most common cause of lung cancer and radon represents a far smaller risk for this disease, it is important to note that radon is the second-leading cause of lung cancer in the United States. Scientists estimate that radon exposure is responsible for about 21,000 lung cancer deaths per year in the United States (NCI, 2004; EPA, 2007).

Natural Radioactivity and Background Radiation

Occupational, accidental, and wartime experiences have provided the basis for the estimation of risk to humans following radiation exposure; however, cosmic, cosmogenic, inhaled, and in-body radiation account for total body effective doses of 3 Bq per year. Thus, both natural and artificial sources of ionizing radiation contribute to human exposure and consequently pose a possible risk to human health. Much of this is unavoidable (e.g., natural background radiation), but as the use of radiation increases, so do the potential health risk and the public's concerns (Morgan and Bair, 2013). One such example of exposure to natural radioactivity is exposure to indoor radon gas, which usually is not sufficiently high enough to produce clinical signs. Two main sources for radon in the home are the soil and water supply. Research has shown that the risk of lung cancer from breathing radon in the air is much larger than the risk of stomach cancer from swallowing water with radon in it. If the average indoor radon concentration in a house exceeds 4 pCi/L, a passive system should be activated, including a vent fan to help reduce indoor radon levels. It is interesting to note that indoor air pollution is responsible for most of our total effective radon and total effective radiation dose from natural radioactivity and background radiation. Radon levels are specific to each indoor dwelling; therefore, the average indoor radon concentration level may vary considerably. This variation has led to measuring radon concentration in houses to ensure safety. As stated earlier, radon gas decays into radioactive particles that can get trapped in the lungs during breathing. As they break down, these particles release small bursts of energy, which can damage lung tissue and lead to lung cancer. Not everyone exposed to elevated levels of radon will develop lung cancer, and the amount of time between exposure and the onset of the disease may be many years (Truta et al., 2014). Like other environmental pollutants, there is some uncertainty about the magnitude of radon health risks; however, we know more about the risks of radon because this information is based on studies of miners exposed to radon.

RADIATION HORMESIS

Hormesis is defined as a phenomenon in which a harmful substance gives stimulating and beneficial effects to living organisms when the quantity of the harmful substance is small (Sakai, 2006). Although radiation has been thought to be harmful no matter how low the dose exposure, accumulating evidence suggests that hormesis is a real phenomenon and that bioprotective functions including antioxidant capacity, DNA repair, apoptosis, and immune responses are induced by low doses of radiation (Sakai, 2006; Sanders, 2010; Hamada et al., 2011).

Experimental data support the concept of radiation hormesis, and several review articles provide detailed evidence of this (Ren, 2006; Hoffman and Stempsev,

2008; Liu, 2010; Morgan and Bair, 2013). The effect of low-dose irradiation on the immune system was investigated in mice (Ren et al., 2006). When a 0.2-Gy (20-rad) dose of x-irradiation was administered every other day for a total of four times, the number of lymphocytes yielded by the liver, spleen, and thymus decreased over the first 10 days, but increased above the level of the control mice by day 28. In the same study, the population of natural killer (NK) T cells dramatically increased, especially in the liver, where primordial lymphocytes were present. Functional and phenotypic activation of these cells occurred at the recovery stage, raising the possibility that an initial activation of macrophages by low-dose irradiation mediated the innate immune system (Ren et al., 2006). While data suggested that the low-dose radiation preexposure could induce adaptive responses or bioprotective responses, how low doses of radiation similar to background levels influence human health is still a matter of debate. A number of studies compared natural radiation levels and cancer risk with cancer risk in high-background radiation areas (HBRAs) (Tao et al., 2000; Hendry et al., 2009; Jaworowski, 2010). Interestingly, data from HBRA studies in China, India, Brazil, and the United States showed a decrease in the incidence of cancer and mortality (Wei et al., 1997; Tao, 2000; Sanders, 2010) in these high-risk areas. Future research is needed to assess this issue more completely.

BIOMONITORING AND BIOMARKERS OF RADIATION

The possibility of a radiation disaster from a nuclear detonation or accident has existed for over 45 years. In recent years, national security experts have expressed increasing concern about the threats of radiological and nuclear terrorism. The scenarios by which overt dispersion of radioactive materials, attacks on nuclear power plants, or the detonation of stolen or improvised nuclear weapons might be deployed have been discussed in some detail (Graham et al., 2008). Thousands of victims might not initially show signs and symptoms of radiation toxicity, even if exposed to substantial doses of radiation, or individuals could display signs and symptoms of radiation toxicity despite minimal evidence of exposure. In addition, due to person-to-person variation in response to a given radiation dose, estimates of radiation exposure dose alone will not necessarily predict the extent of radiation injury to organs and tissues.

The U.S. Homeland Security Council and the Office of Science and Technology Policy created the Weapons of Mass Destruction Medical Countermeasure Subcommittee to oversee the research and development of improved countermeasures. This subcommittee listed the development of biomarkers and devices for biodosimetry as one of the highest-priority areas of research (Pellmar and Rockwell, 2005). *Biodosimetry* can be defined as individual dose evaluation based on biological end points induced by ionizing radiation (so-called biomarkers).

There is a critical need for rapid, accurate, and sensitive assays and techniques and diagnostic platforms that can confirm exposure and predict acute and delayed radiation injury to different organs and tissues in victims of radiation incidents. Table 32.3 lists some of the common methods of biodosimetry comparison for radiation exposure. Knowledge about dose levels in radiation protection is an important step in risk assessment; however, in most cases of real or suspected accidental exposures to ionizing radiation, physical dosimetry cannot be performed for retrospective estimates. Because the relationship between biological end points (such as vomiting and diarrhea) and the absorbed dose is not always straightforward, a precise correlation between symptoms and absorbed dose is rarely achieved. In such situations, biodosimetry has been proposed as an alternative for investigation (Ainsbury et al., 2011). The scoring of unstable chromosomal-type aberrations (such as dicentrics, rings, and micronuclei) in mitogen-stimulated peripheral blood is the most extensively used biodosimetry assay to detect radiation exposure. The dicentric assay is the gold standard in biodosimetry since the presence of dicentrics is generally considered to be specific to radiation exposure; however, the scoring of micronuclei is easier and faster than that of dicentrics for dose assessment (Blakely et al., 2001). While biodosimetry techniques and devices can be useful in determining whether radiation exposure occurred, these methods cannot assess interindividual differences to radiation sensitivity, which are influenced by an individual's genotypic composition and heterogeneity in the dose of radiation received. Furthermore, these techniques and devices do not predict the severity of injury sustained by specific organs and tissues and thus do not allow for the prompt organ- and tissue-directed medical treatment. There is a critical need to develop radiation-specific biomarkers and devices to predict acute and delayed damage to specific organs and tissues. Such developments are underway and should facilitate precise and timely medical intervention, reduce morbidity, and save lives.

Protein biomarker-based dosimetry multiparameter approaches are able to provide dose assessment over extended time ranges. The levels of a number of proteins are up- or down-regulated, and enzymatic modifications occur as a consequence to irradiation. Such changes can be identified in cells, tissues, urine, and blood samples using a range of biochemical methods. High-throughput, antibody-based assays have also been developed to identify and quantify radiation-responsive protein biomarkers (Partridge et al., 2010). Proteomic

approaches to detect global changes in protein expression and composition are promising for the discovery of clinical biomarkers of radiation, but they warrant further study.

Another diagnostic instrument is a metabolomics biomarker, which is a noninvasive tool that studies small-molecular-weight molecules and metabolites. Radiation metabolomics seeks to define patterns of metabolic changes that are associated with exposure to radiation (Coy et al., 2011). An interesting approach is underway that utilizes information from microbiomes. The human microbiome consists of a population of more than 100 trillion microorganisms that live in the mouth, nasal cavities, GI tract, urogenital tract, skin, and other places in the human body. Several studies in the 1950s and 1960s, prompted by the development of atomic bombs, attempted to identify specific bacteria in the feces that were sensitive to radiation. Along with animal studies, a recent study provided some evidence of a decrease in *Escherichia coli* in the feces of cancer patients in the first days after undergoing radiotherapy (Packey and Ciorba, 2010). In this study, a PhyloChip assay developed by Second Genome, Inc., identified changes in the levels of 212 bacteria. The microbial changes seen after exposure persisted for 3 weeks and also showed significant increases in the number of the proteobacteria. If similar microbial signatures are identified in radiation-exposed patients, this assay may become a noninvasive and cost-effective way to triage and treat a large number of the population with potential radiation exposure.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Radiation has existed for more than 3 billion years. Radiation is of concern because it cannot be seen, and although exposure is frequently not painful, it can cause significant physiological damage. Ionizing radiation has both positive and negative aspects; when released by nuclear weapons, it can cause agonizing death, but treatment with it (for certain diseases) can save lives. Appreciation of the potentially devastating effects of radiation arose in the early twentieth century with the plight of the radium dial workers, but widespread fear of radiation was not established until nuclear weapons were used to destroy Hiroshima and Nagasaki in 1945. Today, the choices made with regard to the use of radiation require careful consideration. Nuclear power stations carry immense risk, as shown by the accidents at Three Mile Island, Chernobyl, and Fukushima Dai-ichi and the future possibility of a radiation disaster from a nuclear detonation or accident remains high. In recent years, national security experts have expressed increasing concern about the threats of radiological and nuclear terrorism. Overt dispersion of radioactive materials, attacks on nuclear power plants, or the detonation of stolen or improvised nuclear weapons are real concerns. During emergencies, there need to be multiple technologies that can be used to monitor radiation exposure levels. The current gold standard for biodosimetry radiation exposure is chromosomal aberration analysis, which involves a team of skilled personnel, expensive equipment, and several days to complete; however, these requirements make the technique not readily applicable for triage in the case of a nuclear detonation. There are complementary, blood-based methodologies that measure the biological processes affected by ionizing radiation. The advantages of these methods are that they yield immediate information and that they measure cellular damage rather than the dose received; however, the disadvantages are that the measurements may not be specific to radiation damage. There are also physically based dosimetry methods that estimate radiation dose by changes to the teeth and nails. With these methods, there is no complex variation by time or response, but the disadvantages include the relatively low sensitivity and with no internal control, the heterogeneity of the reporting signal is always there, and the resolution is low. The most useful markers are those that are most specific and quantitatively predictive of radiation-induced severity. This chapter addressed the need for the development of new biomarkers, including protein products, physiological markers, and metabolites. Research and development on biomarkers to monitor radiation tolerance and advances in the field of radiation markers of tissue toxicity are expected in the next decade.

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CHAPTER

33

Depleted Uranium

George C-T Jiang and Michael Aschner

INTRODUCTION

Uranium (U) is a naturally occurring heavy metal that is radioactive and ubiquitous (ATSDR, 1999; Harley, 1999; Jiang and Aschner, 2006). Depleted uranium (DU) is the by-product of the enrichment process of uranium for its more radioactive isotopes. Enriched uranium (EU) is used for nuclear energy, whereas DU is used in other applications that require a heavy metal, such as aviation counterweights and naval ballasts. Because of its pyrophoric and dense metallic properties, DU is used primarily by the military in armor and ammunitions. DU retains 60% of its natural radioactivity and, as such, there are health hazards associated with exposure to the retained inherent radioactivity, but the radiological risks are less than those of natural uranium. However, the chemical toxicity of DU cannot be overlooked and the possibility that it exerts specific chemical toxicological effects separate from its radiological effects has been a subject of intense investigation.

There has been significant public concern regarding the use of DU by the military. For instance, it has been hypothesized that DU may be a cause of Gulf War Syndrome. The public concern also stems from the lack of awareness regarding the specific physical chemistry and hazards of DU, and the belief that DU is still a form of uranium and therefore radiologically hazardous. These concerns have given rise to the belief that DU may be used as a weapon of mass destruction in the form of a dirty bomb, or as an agent of bioterrorism.

BACKGROUND

Small amounts of uranium are found in rock, soil, air, water, and food, and it is estimated that total annual intake of uranium by human adults approximates 460 μg by ingestion of food and water and 0.6 μg by inhalation (Fisenne et al., 1983; UNSCEAR, 2000a,b; Pietrzak-Flis et al., 2001). Natural uranium is composed of three isotopes, ^{234}U, ^{235}U, and ^{238}U, in the proportions shown in Table 33.1.

DU is formed as a by-product of the enrichment of naturally occurring uranium for its most radioactive isotope ^{235}U. As such, DU contains significantly less ^{235}U and more ^{238}U. EU contains much greater levels of ^{235}U, ranging from 3% to more than 90%, whereas its ^{238}U content is decreased from less than 10–97%. For every 1 kg of uranium enriched to 3% ^{235}U during the enrichment process, approximately 5 kg of DU (as a fluoride, UF_6) is produced (Bem and Bou-Rabee, 2004; Jiang and Aschner, 2006). It is estimated that 700,000 tons of UF_6 are stored in the United States, and that each year the mass of accumulated DU increases by 30,000 tons (Hartmann et al., 2000; Bem and Bou-Rabee, 2004; Jiang and Aschner, 2006).

DU has 40% less radioactivity than natural uranium but may contain trace levels of plutonium, neptunium, americium, technetium, and ^{236}U, which increase the radioactivity by 1% but are insignificant with respect to chemical and radiological toxicity (WHO, 2001; Sztajnkrycer and Otten, 2004). Because of the decreased radioactivity of DU, it is believed that DU is a safer form than natural uranium while maintaining the same chemical properties. As the heaviest occurring element, uranium is extremely dense, and both uranium and DU

TABLE 33.1 Forms of Uranium and Their Respective Isotope Percentages

	Forms of Uranium		
Isotope	**Natural**	**Depleted**	**Enriched**
^{238}U	99.275%	99.8%	<10% to 97%
^{235}U	0.720%	0.2%	3% to >90%
^{234}U	0.0055%	0.001%	0.03%

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are often used in applications that require such dense metals.

This chapter describes DU and its applications in weapons of mass destruction. The DU exposure pathways, pharmacokinetics, health effects, toxicity, and available treatments are also reported.

Civilian Uses of DU

DU in civilian applications is marginal compared with its use in military applications (Cantaluppi and Degetto, 2000; Betti, 2003; Bem and Bou-Rabee, 2004; Jiang and Aschner, 2006). As a heavy metal, DU is used as ballasts in yachts, counterbalances in commercial jets, shielding in radiation therapy, and as containers for transportation of radioactive materials (Mould, 2001; Betti, 2003; Sztajnkrycer and Otten, 2004; Jiang and Aschner, 2006). DU has also been used in glassware and ceramics, and even historically in dentistry as parts of dental porcelain (Betti, 2003). Considering ingestion, inhalation, and dermal exposure as the typical routes of exposure, typical civilian exposure to uranium from food, water, and air is considered minimal under normal circumstances (Fisenne et al., 1988). These exposures are well below the daily tolerable intake levels for soluble uranium (0.5 μg/kg body weight), insoluble uranium (5 μg/kg body weight), and inhaled uranium (1 $\mu g/m^3$ in the respirable fraction), as set forth by both the World Health Organization (WHO) and Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR, 1999; WHO, 2001). Because uranium is present in greater levels in war-ravaged regions, it is believed that uranium may pose a potentially greater exposure danger to civilians in these regions (Bleise et al., 2003; Bem and Bou-Rabee, 2004).

Military Uses of DU

The major use of DU is by the military as an alloy in armor and ammunition. These applications take advantage of the unique metallic properties of DU, specifically the density and pyrophoric properties. Uranium is the heaviest naturally occurring element and is extremely dense. Uranium has a density 1.7-times the density of lead, and rods made of uranium are resistant to deformation (Bem and Bou-Rabee, 2004). Uranium shielding is therefore used in the armor of military armored vehicles, allowing the deflection of enemy projectiles. Furthermore, alloys of uranium that contain 2% molybdenum or 0.75% tungsten have a unique attribute such that they will sharpen themselves on impact with a hard target, which allows for greater penetration of uranium-based projectiles compared with traditional tungsten-based alloys (Jiang and Aschner, 2006). DU is also pyrophoric, such that particles will ignite at relatively low temperatures. Fine particles will burn rapidly at relatively low temperatures (150–175°C), whereas particles will spontaneously burn violently with air hotter than 600°C, releasing heat and uranium oxide aerosols (Harley, 1999; Bem and Bou-Rabee, 2004). These unique properties of uranium make it an excellent material for military applications.

It is estimated that 300 tons of DU were used in the aircraft rounds and tank-fired shells in Kuwait and southern Iraq over an area of 20,000 km^3 during the 1991 Gulf War (Operation Desert Storm) (Bem and Bou-Rabee, 2004). Studies have shown that DU penetrators hitting armored targets convert 17–28% of a projectile's mass into DU aerosols (Harley, 1999; Parkhurst, 2003; Bem and Bou-Rabee, 2004). Of these aerosols, 83% are S-type (S for slow dissolution) oxides, whereas 17% are M-type (M for medium dissolution) oxides, and the respirable fraction (diameter <10 μm) may be 50% of the total mass of the aerosol (Harley, 1999; Jiang and Aschner, 2006). This would mean that of 50 tons of tank-fired and 25 tons of aircraft-fired DU munitions, 10 tons of uranium oxides, would have been released into the air in the form of respirable uranium (Jiang and Aschner, 2006).

The US Department of Defense (DOD) has conducted studies to evaluate distribution of aerosolized DU after destruction of an armored tank to assist in determining DU health risk (Harley, 1999). The concentration of uranium inside a tank can reach hundreds or thousands of mg/m^3 just after an explosion caused by a DU penetrator (Harley, 1999). Because uranium has such a high density, the aerosolized DU particles fall within 10 m of a burning tank. The US DOD estimates that the DU intake of a person in the vicinity of a tank hit by a single 120-mm DU-containing projectile is 0.1-mg uranium, and the maximum intake in traversing a cloud of smoke plume that is 200 m in length is 0.8 μg of uranium (Harley, 1999; Parkhurst, 2003; Bem and Bou-Rabee, 2004). These US DOD studies demonstrate that the risks of uranium inhalation are greatest for crews of damaged tanks and rescue teams (Jiang and Aschner, 2006).

Because the DU particulate matter can be carried by winds and deposited in soil and water, there are concerns that both civilian and military units may be exposed to DU dust, vapors, and aerosols, particularly as military conflicts continue. The concerns regarding the unknown exposure data of these individuals have spurred recent studies of war veterans and civilians living in war-ravaged regions to try to determine if DU has human health and environmental impacts. These studies are often general survey studies that look for any signs of adverse human health effects, either radiological or chemical (Bleise et al., 2003; Danesi et al., 2003a; Durakovic, 2003; Milacic et al., 2004; Hindin et al., 2005; Oeh et al., 2007b; Carvalho and Oliveira, 2010; Alaani et al., 2011, 2012).

EXPOSURE PATHWAYS AND BODY RETENTION OF DU

The chemical toxicity of DU is only an issue if the metal is internalized. The three traditional pathways of exposure are inhalation, ingestion, and dermal contact (Figure 33.1). Typically, in nonmilitary situations, the main routes of natural uranium uptake are by inhalation and ingestion. Because of the use of DU in ammunitions and armor by the military, the more important routes of exposure are inhalation and internalization of the DU (Figure 33.1). This internalization of the DU results from embedding of DU projectile fragments (shrapnel) because of explosions of DU-containing armor and ammunitions. Follow-up studies of 1991 Gulf War veterans with embedded DU shrapnel have attempted to determine if there are adverse health effects associated with internalized DU (McDiarmid et al., 1999, 2000, 2001a,b, 2002, 2004a,b, 2006, 2007, 2009, 2011a,b, 2013; Ejnik et al., 2000, 2005; Hodge et al., 2001; Gwiazda et al., 2004; Brown, 2006; Squibb and McDiarmid, 2006; Cazoulat et al., 2008; Bakhmutsky et al., 2011, 2013; Todorov et al., 2013). Several animal studies have also been completed to mimic the effects of DU fragments in humans (Pellmar et al., 1999a,b; Arfsten et al., 2001, 2005, 2006, 2007, 2009; Hahn et al., 2002; Leggett and Pellmar, 2003; Lestaevel et al., 2005; Linares et al., 2005; Fitsanakis et al., 2006).

Inhalation

Inhalation is a likely route of intake of DU, primarily because of its use in DU munitions, whereas naturally occurring uranium dust particles are only inhaled in very small quantities (ATSDR, 1999; Jiang and Aschner, 2006). Studies of people in New York City show that approximately 1 μg of uranium is inhaled each year by each person (Fisenne and Welford, 1986; Fisenne et al., 1987; ATSDR, 1999; Harley, 1999). Inhalation risk from DU is attributable to the aerosolization after the impact of ammunitions, forming DU oxides and particles. These particles become suspended in the air by the wind or settle into the environment for later resuspension. For example, it was determined that the estimated mean annual concentration of suspended matter in ambient air in Kuwait after the 1991 Gulf War was 200 $\mu g/dm^3$, one of the highest concentrations in the world (Bem and Bou-Rabee, 2004). With the increased scrutiny of DU, several studies have looked at the air concentrations in or near conflict arenas where DU aerosols would likely be present; these studies, performed after conflict, show that uranium levels in the air are not higher than background levels because the DU particles are quickly dispersed (Jia et al., 2005; Carvalho and Oliveira, 2010; Yousefi and Najafi, 2013).

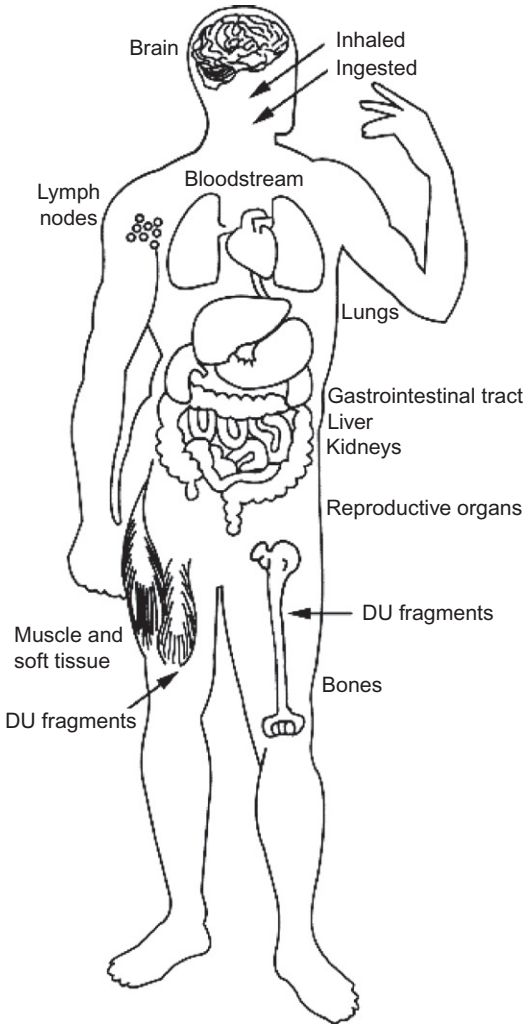

FIGURE 33.1 Routes of DU entry into the body and the corresponding target organs where DU has been shown to accumulate.

The particle size of the aerosolized DU and its correlated physical and chemical properties (e.g., solubility) have a significant impact on how far into the lungs the particles will penetrate, and if they will result in deposition in the respiratory tract (ATSDR, 1999). Only small particles less than 10 μm in diameter will reach and accumulate in the bronchioles and alveoli, whereas larger particles are effectively cleared by mucociliary clearance (Harley, 1999; WHO, 2001; Gwiazda et al., 2004). These respirable particles present a potential health hazard from uranium inhalation, but studies have shown than less than 1% of inhaled uranium actually reaches the kidneys (Spencer et al., 1990; Harley, 1999; WHO, 2001; Gwiazda et al., 2004). These studies have demonstrated that of the uranium that is inhaled, 75% is exhaled and 25% is retained in the respiratory tract and lungs. Of the retained fraction of uranium in the lungs, 80% is removed by bronchial clearance, 15% is deposited in lymph nodes, and 5% actually enters the blood (Spencer et al., 1990; Harley, 1999).

Ingestion

Soil ingestion by children is an important exposure pathway but typically, for humans, ingestion is only an important route of entry if food and drinking water are contaminated by DU (Jiang and Aschner, 2006). The average daily intake of uranium from food and water is estimated to be between 1 and 5 μg/day from uncontaminated areas (ATSDR, 1999; Harley, 1999). In uranium mining areas, ingestion has been found to range between 13 and 18 μg/day (ATSDR, 1999; Harley, 1999). Obviously, these concerns of adverse health effects from DU-contaminated food and drinking water are amplified in battlefield arenas, such as Iraq, Kuwait, the Balkans, and Afghanistan. A number of studies have demonstrated increased uranium levels in the soil in these sites several years after the conflict (Sansone et al., 2001a,b; Danesi et al., 2003a,b; Durante and Pugliese, 2003; Bem and Bou- Rabee, 2004; Di Lella et al., 2004, 2005; Jia et al., 2005, 2006; Oliver et al., 2007, 2008a,b; Zunic et al., 2008; Giovanetti et al., 2010). To date, there have been a number of studies that have attempted to link higher malignancy rates and genotoxic effects in these regions with varying degrees of correlation (Milacic et al., 2004; Obralic et al., 2004; Milacic, 2008; Milacic and Simic, 2009; Darolles et al., 2010; McDiarmid et al., 2011a; Bakhmutsky et al., 2013). Furthermore, bone ash data indicate significant baseline uranium differences across countries (Fisenne et al., 1980, 1983, 1988; Fisenne and Welford, 1986), and there is no clear indication as yet that these different levels of uranium have led to any adverse health consequences (ATSDR, 1999; Harley, 1999).

The solubility of the uranium compound is an important consideration in determining adsorption and distribution because toxicity is related to uptake efficiency of the gastrointestinal (GI) tract. Generally, absorption is increased with the increased solubility of the compound. Uranium oxides (U_3O_8 and UO_2) are relatively insoluble (types M and S; M for medium and S for slow dissolution), whereas uranium trioxide (UO_3) is more soluble (between type M and F; F for fast dissolution). These more insoluble forms are less likely to enter the bloodstream and cause toxicity. However, uranyl nitrate hexahydrate, uranium hexafluoride, or uranyl fluoride are all relatively soluble forms and tend to be absorbed through the GI epithelium. However, unlike other heavy metals, uranium is not efficiently absorbed through the intestinal lumen (Spencer et al., 1990; ATSDR, 1999; Harley, 1999). Studies in humans ingesting uranium nitrate in spiked grapefruit juice demonstrate only 0.5–5% absorption of the original dose (Karpas et al., 1998) (IARC publication). Only 0.2–2.0% of uranium in food and water is absorbed by the GI tract; of the amount that is absorbed, 67% will be filtered out by the kidneys and excreted in the urine within the first 24 h (ATSDR, 1999; Harley, 1999; WHO, 2001).

Dermal Contact and Embedded Fragments

Dermal absorption has not been characterized in human studies, and there have not been any dermal effects reported from uranium miners, millers, or processors (ATSDR, 1999). There have also been no human deaths related to dermal exposure of uranium. Dermal contact is not an important route of exposure because DU does not pass through the skin into the blood unless there are open wounds or embedded fragments (ATSDR, 1999; Harley, 1999; WHO, 2001).

Embedded DU-containing shrapnel, if not removed, is a permanent exposure source within the body and will allow the DU to enter the systemic circulation. The DU Follow-Up Program at the Baltimore Veterans Administration Medical Center has been surveying a small population of approximately 230 soldiers since the 1991 Gulf War, and reports from this program have shown that the uranium leaches into the circulation, resulting in elevated urinary uranium levels compared with those of control patients (McDiarmid et al., 1999, 2000, 2001a,b, 2002, 2004a,b, 2006, 2007, 2009, 2011a,b, 2013; Ejnik et al., 2000, 2005; Hodge et al., 2001; Gwiazda et al., 2004; Squibb et al., 2005, 2012; Brown, 2006; Squibb and McDiarmid, 2006; Cazoulat et al., 2008; Bakhmutsky et al., 2011, 2013; Todorov et al., 2013).

PHARMACOKINETICS

Adsorption

Ingestion

Uranium ingested from food and water consumption ranges from 1 to 5 μg of uranium in uncontaminated regions to 13–18 μg of uranium in uranium mining areas (ATSDR, 1999). The absorption of uranium across the GI tract is related to the solubility of the compound and generally increases with increased solubility. Only a small fraction is absorbed of even the relatively soluble uranium compounds, such as uranyl nitrate hexahydrate, uranium hexafluoride, or uranyl fluoride. For example, studies have shown that humans ingesting uranium nitrate hexahydrate or uranyl nitrate absorb only 0.5–5% of the original dose (Hursh et al., 1969; Karpas et al., 1998). In animal studies with uranyl nitrate and uranium dioxide directly intubated into the GI tract, only 0.77% and 0.11% of the total doses, respectively, were absorbed (Harrison and Stather, 1981). Studies in rats with an intragastrically applied relatively insoluble form of uranium, UO_2 (type S dissolution), showed that uranium could not be detected in liver, kidney, muscle, bone, brain, blood, and urine, and that uranium was not absorbed or retained significantly in the epithelial cells of the intestinal wall (Lang and Raunemaa, 1991).

Inhalation

Typically, uranium is present in limited concentrations in the air, and uranium particle inhalation is minimal (ATSDR, 1999; Harley, 1999). Uranium particle deposition in the respiratory tract is governed by the physical forces that effect particle behavior in the air, as well as the anatomy of the respiratory tract (ATSDR, 1999; Bleise et al., 2003; Chazel et al., 2003; Phalen and Oldham, 2006; Cheng et al., 2009; Valdes, 2009). The anatomy of the lungs is important because this affects the clearance mechanisms available to deal with deposited particles and the degree of actual uranium absorption that will occur. In addition to the aerodynamic diameter (AD) of the particle, the solubility of the inhaled uranium is an important determinant regarding how much uranium will be absorbed (Lang and Raunemaa, 1991; Eidson, 1994).

Most inhaled uranium particles have an AD that does not permit them to be carried deep in the lungs (Igarashi et al., 1987; ATSDR, 1999). Studies have shown that only small uranium particles less than 10 μm in diameter will reach and accumulate in the bronchioles and alveoli, whereas larger particles are effectively cleared by mucociliary clearance (Harley, 1999; WHO, 2001; Gwiazda et al., 2004). These inhaled respirable particles do present a potential health hazard from uranium inhalation, but studies have shown that less than 1% of inhaled uranium actually reaches the kidneys (Morris et al., 1990; Spencer et al., 1990; Lang et al., 1994; Harley, 1999; WHO, 2001; Gwiazda et al., 2004). Studies have demonstrated that of the uranium that is inhaled, 75% is exhaled and 25% is retained in the respiratory tract and lungs. Of the retained fraction of uranium in the lungs, 80% is removed by bronchial clearance, 15% is deposited in lymph nodes, and 5% actually enters the blood (Spencer et al., 1990; Harley, 1999).

Dermal

In animal studies, soluble uranium compounds have been shown to penetrate the skin of rats within 15 min of application (de Rey et al., 1983). The penetration was as high as 7 g/kg body weight for ammonium uranyl tricarbonate, whereas uranyl nitrate hexahydrate ranged from 0.5 to 7 g/kg body weight. No penetration through the skin was observed when uranium dioxide, a more insoluble form, was applied (de Rey et al., 1983). Two days after exposure, dermally absorbed uranium was no longer localized to the epithelium, and rats either had significant weight loss or had died. Other studies have demonstrated that other uranium compounds (uranium tetrafluoride, uranium tetrachloride, and uranium trioxide) are absorbed through the skin of mice, rats, and guinea pigs at a rate of 0.1% of dermally applied uranium, which is a relatively low absorption rate (Orcutt, 1949). These studies show that soluble uranium compounds can be absorbed through the skin. However, the concentrations of uranium applied to the skin were extremely high, and it is unlikely that humans would typically experience such exposures. Dermal absorption has not been characterized in humans (ATSDR, 1999).

Distribution

Once absorbed into the system circulation, uranium undergoes chemical transformations to complex with the blood. Uranium in the trivalent form will oxidize to the hexavalent species to form uranyl ions, which form soluble complexes with bicarbonate, citrate, or proteins in the plasma (Chevari and Likhner, 1968; Stevens et al., 1980; Cooper et al., 1982). The distribution of uranium in the blood is approximately 47% complexed with bicarbonate in plasma, 32% bound to plasma proteins, and 20% bound to erythrocytes (Chevari and Likhner, 1968, 1969).

Uranium is then distributed by the systemic circulation primarily to the bones and kidneys. Of the total absorbed uranium, 85% is associated with bone, where uranium replaces calcium in the hydroxyapatite complex (Donoghue et al., 1972). Of the remaining 15% uranium, more than 90% is associated with the kidneys, accumulating primarily in the proximal tubule (ATSDR, 1999). Uranium also distributes in detectable amounts to the liver, brain, testes, and spleen (Pellmar et al., 1999a; Leggett and Pellmar, 2003; Fitsanakis et al., 2006). The embedded uranium fragments continuously release uranium into the circulation, and the fragment size diminishes with time.

Less soluble uranium compounds (type M and type S dissolution), which are not effectively absorbed, are distributed in bronchial lymph nodes as well as the lung tissue itself (Leach et al., 1970, 1973).

Metabolism and Excretion

Once uranium is in the systemic circulation, it is transported as various complexes in the blood. The predominant form of uranium is as a bicarbonate complex, the stability of which is highly dependent on the pH of the solution (Chevari and Likhner, 1968; ATSDR, 1999). In the kidneys, the bicarbonate complex is filtered at the renal glomerulus and excreted in the urine (Bowman and Foulkes, 1970; Foulkes, 1971; Adams and Spoor, 1974; Blantz, 1975; Blantz et al., 1985; Brady et al., 1989; ATSDR, 1999). Protein-bound uranium will remain in the blood because little protein passes through the glomerulus.

Studies using humans have shown that approximately 66% of an intravenous injection of uranium is eliminated from the plasma within 6 min, whereas 99% of the uranium is eliminated from the plasma 20 h after injection (Luessenhop et al., 1958; ATSDR, 1999; Harley, 1999). Another study has shown that the kidneys excrete

more than 90% of intravenously injected soluble hexavalent uranium salt, with less than 1% excreted in the feces; approximately 70% of the dose is excreted within the first 24 h (Bassett et al., 1948).

Oral Exposure

The average GI uptake of uranium is limited, ranging from 1% to 5% in adult humans (Leggett and Harrison, 1995; ATSDR, 1999). Absorption generally increases with increasing solubility of the compound. It is highest for the soluble uranium compounds; nevertheless, only a small fraction of uranium is absorbed across the GI epithelium. Uranium absorption takes place predominantly in the small intestine, with no absorption from the buccal cavity, stomach, or large intestine (Dublineau et al., 2005). Measured with *ex vivo* techniques, the apparent uranium permeability was shown to be similar in various parts of the small intestine (Dublineau et al., 2005). Uranium transport across the GI epithelium likely occurs via a transcellular pathway. Approximately 90% of the ingested uranium in humans is excreted in the feces without being absorbed, whereas the remainder is excreted in the urine (Wrenn et al., 1985; Spencer et al., 1990; Oeh et al., 2007a,b). Studies using rats show similar excretion patterns to humans, of which the majority of ingested uranium (99%) is eliminated in the feces and 95% of the absorbed uranium is excreted in urine within 1 week of exposure, with a half-life of 2–6 days (Wrenn et al., 1985; La Touche et al., 1987; Dublineau et al., 2005).

Inhalation Exposure

The rate of deposition and clearance of uranium-containing particles from the lung depends on its chemical form and particle size. As previously discussed in the adsorption section, most of the larger uranium particles are transported out of the respiratory system by mucocilliary action or swallowed and eliminated in the feces. Smaller particles with higher solubility are more rapidly absorbed into the systemic circulation, but can be excreted in the urine.

The aerosol by-products of exploded DU munitions are primarily the uranium oxides with varying dissolution rates. Uranium trioxide (UO_3) is soluble like uranyl salts, and systemic absorption accounts for more than 20% of the exposure burden, with 20% of the excreted uranium being in the urine (Morrow, 1964; Morrow et al., 1964, 1972, 1982). UO_3, being soluble, has a fast dissolution rate (type F) and is rapidly removed from the lung (half-life of 4.7 days). Uranium dioxide (UO_2) and triuranium octaoxide (U_3O_8) are relatively insoluble and have slow dissolution rates (type S), resulting in pulmonary clearance rates dominated by particle size and mucocilliary transport out of the lungs. In humans, studies of these insoluble uranium compounds suggest a two-phase clearance process consisting of a short phase with a biological half-time of between 11 and 100 days and a slow phase of clearance with a biological half-time between 120 and 1,500 days (Hodge, 1973; Eidson, 1994; Taylor and Taylor, 1997).

Embedded Fragment Exposure

DU fragments from embedded shrapnel act as reservoirs to continuously release uranium into the circulation (Pellmar et al., 1999a; Leggett and Pellmar, 2003; Arfsten et al., 2006; Fitsanakis et al., 2006; Jiang and Aschner, 2006; Zhu et al., 2009). Significant percutaneous diffusion of soluble uranium through intact skin has been described (de Rey et al., 1983; Lopez et al., 2000; Petitot et al., 2007a,b). In rats, the absorption of uranium via deep wounds was shown to depend on the chemical form of uranium. On intramuscular injection of uranium nitrate, approximately 98% of the uranium was in the blood (Houpert et al., 1999). In contrast, after UO_4, 70% and 85% of instilled uranium was found in the blood after 1 and 3 days, respectively (Houpert et al., 1999).

Surgical removal of fragments attempts to remove as many fragments as possible, but often there will be residual fragments that cannot be removed because of location and/or size. Over time, the fragment sizes will decrease as the uranium leaches from these fragments; the body has no other way to effectively remove such embedded fragments.

MECHANISM OF ACTION

There have been many *in vitro* studies evaluating the toxic effects of uranium in different cell types, many of which demonstrate cytotoxicity. There is no specific mechanism that emerges to explain all the results, but the weight of evidence suggests that DU-induced cytotoxicity may result from oxidative stress and eventual cell death. Uranyl compounds have high affinity for phosphate, carboxyl, and hydroxyl groups, and they easily combine with proteins and nucleotides to form stable complexes (Weir, 2004). DU can cause oxidative DNA damage by catalyzing hydrogen peroxide and ascorbate reactions, resulting in single strand breaks in plasmid DNA *in vitro* (Miller et al., 2002a,b; Yazzie et al., 2003). Evidence for induction of oxidative stress and reactive oxygen species (ROS) by DU has been shown by increases in NO (Abou-Donia et al., 2002), lipid oxidation (Briner and Murray, 2005; Ghosh et al., 2007; Jiang et al., 2007; Periyakaruppan et al., 2007; Lestaevel et al., 2009; Daraie et al., 2012; Shaki et al., 2013), transcriptomic, and proteomic changes (Malard et al., 2005; Prat et al., 2005; Periyakaruppan et al., 2007). Moreover, some studies indicate that heat shock proteins may be involved in the cellular response to DU exposure and acquired resistance to uranium rechallenge (Furuya

et al., 1997; Mizuno et al., 1997; Tolson et al., 2005). These data strongly suggest the possibility that uranium may result in the formation of ROS, leading to cell death. An apoptotic mechanism has even been suggested after a study during which significant apoptotic events were seen in mouse macrophage cells treated with 100 μM DU (Kalinich et al., 2002).

TOXICITY OF DU EXPOSURE

Scientists in the mid nineteenth century first believed that natural uranium had homeopathic properties and could be an effective treatment for diabetes and albuminuria after promising results in animals and humans dosed with uranium (Hodge, 1973; Jiang and Aschner, 2006). However, animal studies in the early twentieth century showed that uranium was not therapeutic, and the misuse of uranium in humans was halted (Hodge, 1973). There is extensive literature regarding the toxicology of uranium, which now includes studies specifically of DU. The following section highlights a number of key *in vitro*, animal, and human studies of DU as they relate to health effects from uranium exposure. Chemical toxicity of uranium only poses a threat if the metal is internalized.

Nephrotoxicity

The kidney is the major target organ for uranium toxicity (ATSDR, 1999; Harley, 1999; Jiang and Aschner, 2006; Aschner and Jiang, 2009). Renal toxicity associated with uranium exposure results as the kidneys work to eliminate internalized uranium, and this has been known for two centuries (Goodman, 1985). In the kidneys, the site of action is the proximal tubule where proton secretion degrades the bicarbonate complex of the uranyl ion. Uranium can then react with apical cell membranes of the tubule epithelium (Goodman, 1985; Harley, 1999). Nephrotoxicity is clearly associated with uranium exposure and has been documented in animal studies at high exposure levels (Leggett, 1989; Harley, 1999; Sztajnkrycer and Otten, 2004). Recent *in vitro* studies of renal cells demonstrated a concentration-dependent uranium toxicity (L'Azou et al., 2002; Carriere et al., 2004; Thiebault et al., 2007). Any observed uranium nephrotoxicity results from acute exposure. Currently, there is no evidence that DU has any long-term effects on renal function, or that long-term renal impairments will develop if no acute effects are seen (ATSDR, 1999; Harley, 1999; Jiang and Aschner, 2006; Aschner and Jiang, 2009). There is even evidence in animals and humans that there may be repair of damaged tubular epithelial tissue (Goodman, 1985; Diamond et al., 1989; Harley, 1999).

Carcinogenicity

Bones

Bones are the secondary target organ of uranium toxicity (ATSDR, 1999). The majority of absorbed uranium is distributed to the bones and, not surprisingly, the carcinogenic potential of uranium in bones has been explored in uranium workers and personnel living in or having served in the war-ravaged regions potentially exposed to DU (Harley, 1999; Abu-Qare and Abou-Donia, 2002; Boice et al., 2003a–c, 2007; Obralic et al., 2004; Storm et al., 2006).

Animal studies to mimic injuries of Gulf War veterans and to assess the chronic effects of internalized DU have utilized rats implanted with DU and control tantalum pellets (de Rey et al., 1984; Miller et al., 1998b; Pellmar et al., 1999a,b; Hahn et al., 2002; Leggett and Pellmar, 2003; Arfsten et al., 2005, 2006, 2007, 2009; Fitsanakis et al., 2006; Zhu et al., 2009). Carcinogenicity of these surgically implanted DU fragments has been evaluated and is related to the size of the fragments—DU fragments of sufficient size can cause localized proliferative reactions and soft tissue sarcomas (Hahn et al., 2002). Urine and serum mutagenicity studies with rats implanted with DU demonstrated enhanced mutagenic activity in the *Salmonella* TA98 strain and the Ames II mixed strain (TA7001–7006) in a dose-dependent manner with excreted urinary concentration (Miller et al., 1998b). There are numerous other studies that demonstrate the deleterious effects of DU on viability, micronuclei, chromosomal instability, and sister chromatid exchanges by human bronchial, bone marrow, and Chinese hamster ovary cells (Kennedy and Saluga, 1970; Kadhim et al., 1992, 1994; Nagasawa and Little, 1992; Lin et al., 1993; Schroder et al., 2003; Stearns et al., 2005; Holmes et al., 2014). However, it is still unclear if the mutagenic and carcinogenic effects are a result of the chemical or radiological effects of DU, but it is believed that these effects are primarily due to the latter. Osteosarcoma risk has clearly been demonstrated to be related to the amount of radiation exposure, and there is a wealth of *in vitro* studies of bone cells and animal models that illustrate this (Miller et al., 1998a, 2001, 2003, 2005; Ibrulj et al., 2004, 2007; Miller and McClain, 2007). However, many scientists believe that the amount of radiation emitted by DU is insufficient to raise a significant risk in humans, but may be more of a concern for children whose bones are growing rapidly (Brugge et al., 2005; Jiang and Aschner, 2006; Aschner and Jiang, 2009).

Lungs

It is often difficult to pinpoint specific cancerous agents using epidemiological studies (Priest, 2001) but, because the lungs are the primary portal of inhaled uranium, numerous studies have examined the health

effects of inhaled uranium. It has been known since the 1940s that the most soluble uranium compounds are the most toxic (Voegtlin and Hodge, 1949a,b). Early animal studies did not reveal significant animal mortality from inhalation unless very soluble forms of uranium were used. However, most of the effects of uranium on the lungs occur because of the insoluble forms of uranium. Researchers have found that the lungs and tracheobronchial lymph nodes are the major sites of uranium accumulation for large particles and relatively insoluble forms of uranium, and fibrotic changes in the lung tissue have been observed that are suggestive of radiation injury (Leach et al., 1970, 1973). These insoluble particles deposited in the lungs have a long residual time and may result in increased risk for cancer (Houpert et al., 1999; Hartmann et al., 2000). Typical health effects seen in uranium inhalation studies are the development of pneumonia and chemically irritated passages, which are considered early signs of lung cancer (Voegtlin and Hodge, 1949a,b, 1953a,b).

The mechanisms associated with lung injury in high-level uranium exposure are not well-understood. Treatment of rat lung epithelial cells with uranyl (VI) acetate was shown to result in increased oxidative stress and decreased cell proliferation, which were attributed to the loss of cellular redox (Periyakaruppan et al., 2007). Effects of uranium on cytokine secretion and on the proteasome–ubiquitin system have also been advanced (Gazin et al., 2004; Malard et al., 2005). Several studies have also established the deleterious effects of DU on viability, micronuclei, chromosomal instability, and sister chromatid exchanges in cells (Lin et al., 1993; Prabhavathi et al., 1995; Miller et al., 1998a, 2001, 2003; Schroder et al., 2003; Ibrulj et al., 2004; Wolf et al., 2004; Krunic et al., 2005; Milacic, 2008; McDiarmid et al., 2011a). These effects are believed to be more attributable to the radiological properties of uranium than to the chemical effects (Mould, 2001; Bolton and Foster, 2002).

Reproductive/Developmental Toxicity

The reproductive effects of DU have recently been studied in various animal models (Paternain et al., 1989; Llobet et al., 1991; Arfsten et al., 2001, 2005, 2006, 2007, 2009; Domingo, 2001; Linares et al., 2005). Uranium has been shown to be a developmental toxicant when administered orally or subcutaneously, resulting in decreased fertility, embryo/fetal toxicity including teratogenicity, and reduced growth of offspring, after uranium exposure during different gestation periods (Domingo et al., 1989a,b; Ortega et al., 1989b; Paternain et al., 1989; Llobet et al., 1991; Bosque et al., 1993; Arfsten et al., 2001, 2002, 2005, 2006, 2007, 2009; Domingo, 2001). It is still unclear what the exact mechanism of action is, although *in vitro* studies in Chinese hamster ovary cells showed DU-induced genotoxicity and cytotoxicity (Lin et al., 1993). In humans, studies have evaluated the reproductive effects in male miners, uranium processors, and Gulf War veterans and show that these individuals have uranium in their semen, but they do not otherwise show any detrimental reproductive effects (Voegtlin and Hodge, 1953a,b; Llobet et al., 1991; McDiarmid et al., 2000, 2006; Arfsten et al., 2001, 2005, 2006, 2009; Domingo, 2001; Abu-Qare and Abou-Donia, 2002; Linares et al., 2005; Squibb and McDiarmid, 2006; Abu-Musa et al., 2008; Todorov et al., 2013).

Neurotoxicity

As concerns mounted regarding the cause of Gulf War syndrome, a growing body of evidence suggested that DU may affect the central nervous system (CNS). Animal studies demonstrated that uranium crosses the blood–brain barrier (BBB) and readily accumulates in the brain (Gilman et al., 1998a–c; Pellmar et al., 1999a,b; Leggett and Pellmar, 2003; Lemercier et al., 2003; Houpert et al., 2004, 2005, 2007; Lestaevel et al., 2005; Monleau et al., 2005; Fitsanakis et al., 2006). Furthermore, studies using rats and some follow-up studies using Gulf War veterans suggested that DU may cause subtle changes in CNS function without any corresponding nephrotoxicity (McDiarmid et al., 1999, 2000, 2001a,b, 2002, 2004a,b, 2006, 2007, 2009, 2011a,b, 2013; Pellmar et al., 1999a,b; McDiarmid, 2001). The public concern regarding the potential neurotoxic effects of DU has spurred recent novel scientific research to extensively evaluate if there are reasons to be concerned about DU exposure and neurotoxicity.

One of the earliest studies evaluating the specific effects of DU on the CNS demonstrated that uranyl nitrate facilitated the release of acetylcholine from the nerve terminals to potentiate muscle contraction in phrenic nerve preparations from mice (Lin et al., 1988). It was then shown that uranium could cross the BBB *in situ* using rat brain perfusions (Lemercier et al., 2003). Significant amounts of uranium accumulated in the brain after only a 2-min perfusion. Studies using rats embedded with DU and/or control tantalum pellets for 1 day and for 6, 12, and 18 months confirmed previous biodistribution data that demonstrated kidneys and bone as the primary target organs, but also identified other sites in the lymphatic, respiratory, reproductive, and central nervous systems (Pellmar et al., 1999a). High-dose (20 pellets) DU implantation resulted in significantly increased uranium levels in the skull (12.5 ng U/g skull tissue) after only 1 day compared with controls (1.41 ng U/g skull tissue) (Pellmar et al., 1999a). By 6 months, there was a significant difference in the amount of uranium in brain samples from rats that received high doses (approximately 5,000 ng U/g tissue) compared with tantalum control

samples (approximately 50 ng U/g tissue) (Pellmar et al., 1999a). Significant differences in brain region distribution of uranium were also found. Differences were also seen in electrophysiological studies in hippocampal slices of rats implanted with DU; the hippocampus is important for learning, memory consolidation, and spatial orientation functions. The 6-month and 12-month high-dose groups exhibited decreased neuronal excitability compared with controls, whereas rats in the 18-month DU treatment groups did not show significant changes in neuronal excitability, perhaps obscured by the aged rats (Pellmar et al., 1999a,b). No significant differences in hippocampal weights of DU-treated animals compared with controls were seen.

Generation of nitric oxide (NO) and evaluation of the central cholinergic system of male Sprague-Dawley rats after uranium exposure have also been studied (Abou-Donia et al., 2002). Intramuscular injection of 0.1 and 1.0 mg/kg for 7 days, followed by a 30-day observational period, resulted in sensorimotor deficits in rat behavior, differential levels of NO, and increased acetylcholinesterase activity in the cortex of animals dosed with 1 mg/kg, suggesting multiple exposures to low doses of uranyl acetate caused prolonged neurobehavioral deficits in rats after the initial exposure has ceased, similar to the exposures of Gulf War veterans (Abou-Donia et al., 2002).

Short-term and long-term differences in brain lipid oxidation and open-field behavioral differences in rats exposed to DU have been shown (Briner and Murray, 2005). After 2 weeks of DU exposure, brain lipid oxidation was increased and correlated with increases in line-crossing and rearing behavior. Although open-field behavior differences remained after 6 months of DU exposure, brain lipid oxidation could no longer be clearly correlated with the behavioral changes (Briner and Murray, 2005). Male rats were also more sensitive to the behavioral effects of DU compared with female rats (Briner and Murray, 2005). The gender differences may warrant further study to allay the fears of the public because the US armed forces deployment to Kosovo, Bosnia, and the Persian Gulf in 2000 were approximately 91% males (DOD, 2002). The results of both of these studies support previous studies that indicate that DU is a toxicant capable of crossing the BBB and producing prolonged behavioral/neurological changes.

Studies have recently addressed the cytotoxicity of uranyl acetate in primary rat cortical neuron cultures. Researchers found no evidence that uranyl acetate at concentrations less than 100 μM caused cytotoxicity (Jiang et al., 2007; Aschner and Jiang, 2009). Furthermore, there was no significant change in the levels of F_2-isoprostanes, biomarkers of oxidative stress, as well as thiol metabolite levels on treatment with uranium. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assays and lactate dehydrogenase activity were also unchanged in neurons treated with uranyl acetate. In complementary studies in the nematode *Caenorhabditis elegans*, researchers found no evidence for neuronal degeneration using green fluorescent protein reporter strains corroborating the neuron culture results (Aschner and Jiang, 2009; Jiang et al., 2009).

With respect to human subjects, Gulf War veterans subjected to friendly fire and embedded with shrapnel from DU projectiles have been followed-up (Hooper et al., 1999; McDiarmid et al., 1999, 2000, 2002; Hodge et al., 2001; Gwiazda et al., 2004; Dorsey et al., 2009; Bakhmutsky et al., 2011, 2013). The fragments are a permanent exposure source inside the body and these veterans have demonstrated consistently higher urine levels of uranium, compared to controls without DU shrapnel, even after years of exposure (Hooper et al., 1999; Ejnik et al., 2000, 2005; McDiarmid et al., 2000, 2001a,b, 2002, 2004a,b, 2006, 2007, 2009, 2011a,b, 2013; Hodge et al., 2001; McDiarmid, 2001; Gwiazda et al., 2004; Squibb et al., 2005, 2012; Squibb and McDiarmid, 2006; Dorsey et al., 2009; Bakhmutsky et al., 2011, 2013; Hines et al., 2013). These Gulf War veterans do not show evidence of kidney damage or dysfunction, but there was an indication that increased uranium exposure may be marginally correlated with decreased neurocognitive performance, as measured by paper and pencil, and automated tests. In more recent follow-up studies, the initially identified neurological disabilities can no longer be detected. Many groups have called for further studies to examine the potential relationship between DU exposure and cognition (Harley, 1999; DOD, 2000; Durakovic, 2001, 2003; Priest, 2001; Society, 2001, 2002; WHO, 2001; Bem and Bou-Rabee, 2004; Sztajnkrycer and Otten, 2004).

TREATMENT

Currently, the treatments for uranium exposure are limited. Chelation therapy is used to prevent acute toxicity of high doses of uranium in systemic circulation, typically resulting from some sort of ingestion. Chelating agents are used to competitively compete for the uranyl ion. Numerous studies have tested the efficacy of different chelating agents, such as gallic acid, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), diethylenetriaminepentaacetic acid, 5-aminosalicylic acid, catechol-3,6-bis(methyleneiminodiacetic acid) (CBMIDA), and ethane-1-hydroxy-1,1-bisphosphonate (EHBP) (Ortega et al., 1989a; Durbin et al., 1997, 2000; Fukuda, 2005; Fukuda et al., 2005, 2006a,b, 2008, 2009a,b). Recent uranium chelation therapy studies have evaluated compounds *in vitro* in cell lines (Liu et al., 2011; Zhang et al., 2011) and to treat dermal contamination (Belhomme-Henry et al., 2014). These chelating agents

can be effective in removing uranium and providing protective effects from acute uranium toxicity with differing efficacy. However, the length of time before initiating chelation therapy for acute uranium intoxication greatly influences the effectiveness of this therapy (Domingo et al., 1990).

For embedded DU fragments, surgical removal is the best option to get rid of as much of the shrapnel as possible. Because shrapnel can be of different sizes and dispersed in multiple locations, it is often difficult to remove every piece. Large fragments and fragments that are easily accessible, such as in soft tissues, are removed when possible to minimize the amount of uranium that will remain in fragments in the body.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

DU is an excellent metallic substrate for radiation shielding and for armor and ammunition by the military due to its density and pyrophoric properties. Furthermore, the unique ability of uranium-based ammunitions to sharpen themselves on impact, allowing for deeper penetration of the ammunitions, also makes DU a better substrate for weapons of mass destruction. As such, it is not surprising that the use of DU in military applications continues to grow, even while alternative metals are explored. This continued increased use has been bolstered by recent scientific studies showing that DU exposure has relatively low adverse health effects, contrary to the public backlash and belief that DU is a harmful chemical and may be the cause of Gulf War syndrome, and by the studies illustrating increased toxicity of metallic alloy alternatives.

There has also been speculation that there is a potential black market use of DU as a weapon for bioterrorism, or as part of a dirty bomb. However, a thorough evaluation of the toxicology of uranium indicates that DU is relatively inert compared with other potential materials and organisms that could be used to make an effective bioterrorism weapon or dirty bomb. The reason why DU may still be considered to be an excellent bioterrorism agent is the public perception that DU is a harmful chemical, even though the toxicology of the chemical and radiological effects are relatively mild.

Recent studies have provided a great deal of new information about DU and have narrowed the gaps in our understanding. For example, we now know that uranium will cross the BBB and accumulate in the brain, and there appear to be minimal functional changes associated with brain uranium accumulation. However, while the evidence suggests that DU is relatively inert, the controversy still remains regarding whether DU may be an agent that causes Gulf War syndrome, even though long-term follow-up studies have shown minimal chemical effects. Although there is not a great demand for pharmacological interventions, potential development of better chelating agents would be beneficial to assist in the treatment of uranium exposures, both acute and chronic.

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SECTION III

TARGET ORGAN TOXICITY

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CHAPTER

34

Chemical Warfare Agents and the Nervous System

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INTRODUCTION

Chemical warfare has a long history. One of the earliest forms of chemical warfare involved the application of natural toxins to the tips of arrowheads and spears, used by early civilizations in Europe, Asia, South America, and Africa and described in Greek and Norse mythology (Mayer, 2008). The various types of chemical warfare agents (CWAs) are now listed as scheduled chemicals (Schedule 1, 2, and 3) in the Chemical Weapons Convention, administered by the Organization for the Prohibition of Chemical Weapons (OPCW). The use of CWAs in modern warfare began in World War I (1917–1919), during which chlorine gas, mustard gas, and phosgene (primarily dermal and pulmonary irritants) were used. Mustard gas was used again during World War II. A number of organophosphorus chemicals, primarily targeting the cholinergic nervous system, were synthesized as potential CWAs during the 1930s and 1940s and are often referred to as "nerve gases," or more appropriately, "nerve agents." There remains widespread concern for threats from CWAs, in particular the organophosphorus nerve agents, reinforced by their military use in the Iran–Iraq War of 1980–1988 and their use by terrorists in Japan in the mid-1990s.

Concern over the use of CWAs has again increased because of recent events in Syria. The Syrian government reportedly possessed a stockpile of over 1,000 tons of chemical agents and precursors including sulfur mustard (2,2′-dichlorethyl sulfide; HD), sarin, and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX). Sarin was employed in a series of well-documented attacks in the city of Damascus in August 2013. There have also been reports of use of chlorine gas in munitions during the Syrian conflict. These concerns are being mitigated by the efforts of an OPCW–United Nations (UN) joint mission in Syria, targeting the removal or destruction of all priority chemicals in Syria which was completed by September 30, 2014.

There is some suggestion that a class of compounds could have been developed as binary CWAs; i.e., *novichoks* (which means "newcomers" in Russian). The OPCW recently concluded that there was insufficient information to comment on the existence or chemical/toxicological properties of novichoks. There is limited information, however, suggesting that their toxicity could be greater than that of the more common CWAs and that their mechanisms of action could be different, potentially rendering traditional treatments ineffective (Moshiri et al., 2012).

Different types of CWAs can affect one or multiple major organ systems. This chapter focuses on the nervous system as a potential target for CWAs. A brief overview of the nervous system, highlighting some of the special features that often contribute to its unique sensitivity to CWAs (and toxicants in general), will be provided. Specific information on the effects of selected CWAs on the nervous system will be discussed as well.

OVERVIEW OF THE NERVOUS SYSTEM

The nervous system can be divided into two major structural divisions: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord, covered by three membranes—the meninges. The outermost layer is the dura mater. The PNS consists of all parts of the nervous system that lie outside the dura mater. The nervous system can also be classified based on functionality into the

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autonomic and somatic nervous systems. The autonomic nervous system, which can be further divided into the sympathetic, parasympathetic, and enteric divisions, regulates visceral functions and various processes that are not under voluntary control, while the somatic nervous system controls voluntary skeletal muscle activity. Maintenance of homeostasis and proper communication within and among these various components of the nervous system is pivotal for physiological coordination and normal functioning of the whole organism.

There are two basic types of cells in the nervous system: neurons (accounting for about 10% of the total number of cells) and glia (the remaining 90% of the cells, including astrocytes, oligodendrocytes, NG2 cells, microglia, satellite cells, and Schwann cells). Neurons are highly specialized cells optimized for the conduction of electrical impulses to other nerve cells and to innervated tissues such as glands and muscles. In all neurons, there exists a soma (cell body), dendrites (smaller branching structures), and an axon (electrical impulse–conducting fiber) that terminates in multiple endings and gives rise to specialized areas (i.e., the presynaptic terminals). The junctional region between the presynaptic terminal and its target (innervated) cell is the synapse, where intercellular communication occurs, mediated by signal molecules (e.g., neurotransmitters) released by the presynaptic terminal. Anatomically, neurons can be classified into three basic types based on extensions of the dendrites and axon: unipolar, bipolar, and multipolar. Functionally, neurons can be characterized into afferent neurons (sensory neurons), efferent neurons (motor neurons), and interneurons. Afferent neurons conduct electrical impulses from tissues to the CNS, efferent neurons carry electrical impulses from the CNS to the tissues, and interneurons transmit information between neurons.

Despite having a markedly higher density in the nervous system, glial cells have historically been considered to provide metabolic and physical support for the neurons. For example, astrocytes aid in the maintenance of homeostatic conditions of the extracellular fluid surrounding neurons by removing excess potassium ions. Microglia participate in phagocytosis following neuronal damage/degeneration. Oligodendrocytes and Schwann cells (found in the CNS and PNS, respectively) form the lipid-rich myelin layers around axons, which effectively enhance conduction speed down the axon and prevent the spread of impulses between juxtaposed axons. It is now recognized, however, that glial cells play many important roles as key regulators of many aspects and processes associated with development, aging, and pathology, such as synaptogenesis, reactive gliosis, immunoreactivity, and homeostasis of neurotransmitters (Allen, 2013; Verkhratsky et al., 2014).

Neurons are excitable cells undergoing dynamic phases of depolarization and repolarization. As with all cells, neurons maintain a resting potential across the plasma membrane, typically −60 to −75 mV relative to the extracellular fluid. When excited, the membrane potential in a neuron exceeds a threshold voltage, which triggers a series of events that result in the initiation and propagation of an action potential along the axon. Each action potential begins with a rapid reversal of voltage from the negative resting potential to a positive potential (depolarization phase) and ends with an almost equally rapid change to a negative potential (repolarization phase). Once initiated, the action potential is self-propagated to the end of the axon in an all-or-none fashion. When an action potential reaches the presynaptic terminal, voltage-gated Ca^{2+} channels open in response to depolarization, resulting in extensive influx of Ca^{2+} ions, which then trigger exocytosis. The synaptic vesicles in the presynaptic terminal fuse with the plasma membrane and release their contents (neurotransmitters and other signals) into the synaptic cleft. Electrical impulses are thus transformed into chemical signals and thereby conducted from one neuron (presynaptic) to another (postsynaptic) through synaptic transmission.

Intercellular communication in the nervous system is typically mediated through synaptic transmission via the release of neurotransmitters and their subsequent binding to specific receptors. The transmitter-receptor interaction then elicits changes in ion channel permeability and/or second messenger formation in the innervated cell. Neurotransmitters can also interact with receptors located on the presynaptic terminal (either *autoreceptors*, which are activated by the same transmitter, or *heteroreceptors*, which are activated by a different transmitter released by a different neuron) to regulate the presynaptic function, often by influencing neurotransmitter release. Termination of synaptic neurotransmission depends upon the removal of neurotransmitter molecules from the synaptic cleft by either enzymatic degradation or by reuptake into the presynaptic terminal.

Classical neurotransmitters in the nervous system can be classified into amino acids (e.g., glutamate, aspartate, gamma-aminobutyric acid (GABA), glycine), monoamines (e.g., catecholamines, serotonin, histamine), and acetylcholine (ACh). Adenosine triphosphate (ATP) and nitric oxide (NO) are nonclassic neurotransmitters that often mediate co-transmission with other classical transmitters (e.g., norepinephrine or ACh). Neurotransmitters typically mediate synaptic transmission in an anterograde fashion; i.e., the transmitter molecules are released from presynaptic terminals and then diffuse through the synaptic cleft to bind to receptors on postsynaptic neurons or cells.

In the last 20 years or so, another group of signals termed *endocannabinoids (eCBs)* have gained more attention as neuromodulators with the potential to modulate numerous physiological and pathological processes

(see review of Pacher and Kunos, 2013). The eCBs are endogenous signal molecules derived from membrane lipids that modulate synaptic function in a retrograde fashion, i.e., they are released by a postsynaptic cell to influence the presynaptic neuron activity. Anandamide (AEA) and 2-arachidonylglycerol (2-AG) are the two most studied eCBs. In the CNS, eCB signaling is mediated by cannabinoid (CB1) receptors, a putative membrane transporter of eCBs, and enzymes involved in both synthesis (diacylglycerol lipase and *N*-acylphosphatidylethanolamine-specific phospholipase D) and inactivation (fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL)) of eCBs. Unlike other neurotransmitters, which are synthesized and stored in synaptic vesicles, the eCBs are synthesized on demand from arachidonic acid in membrane lipids of postsynaptic neurons. Upon depolarization, the eCBs are released from the postsynaptic neuron, and they then diffuse through the synapse to bind and activate CB1 receptors on the presynaptic terminal. eCB synthesis and release can also be stimulated in a receptor-mediated fashion by activation of the G_q protein-coupled metabotropic glutamate receptors and the muscarinic M1 and M3 receptors. Termination of eCB signaling appears to require cellular reuptake via a carrier-mediated transport process. Following cellular reuptake, eCBs are enzymatically degraded. FAAH is distributed throughout the brain and appears to be primarily responsible for AEA hydrolysis, while MAGL appears to be the primary enzyme involved in 2-AG degradation. Both FAAH and MAGL are intracellular enzymes and inhibition of either or both can lead to increases in eCB levels.

Depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) are two forms of synaptic plasticity mediated by eCBs. In DSI, postsynaptic neurons release eCBs, which in turn decrease GABA release in presynaptic neurons and disinhibit postsynaptic cell activity. In DSE, eCBs reduce presynaptic glutamate release, leading to a net suppression of postsynaptic cell activity.

The nervous system is complex both anatomically and functionally. The extensive networks mediating intracellular and intercellular communications make it highly vulnerable to disruption by many toxicants. Moreover, the nervous system possesses additional unique features that can contribute to its higher sensitivity to toxicants, including CWAs.

Special Features of Neurons and High Energy Demand

As noted previously, neurons are composed of a cell body (or soma), extended, branching dendrites, and an elongated axon. Because of this structural complexity, most neurons have relatively large cell volumes compared to other cell types. The unique spatial features of neurons often require the synthesis of a large amount of proteins in the soma and their transport over long distance through processes of axonal transport. The Nissl substance, found exclusively in soma of neurons and formed by clusters of ribosomal complexes, synthesizes the large amount of proteins needed for these cells. Unlike other cells, neurons are excitable cells that continuously generate and transmit electrical signals along their axons and chemical signals across the intercellular synapses. The neurons, therefore, have an absolute requirement for a continuous and abundant supply of energy to support the need for synthesis of large amounts of proteins and neurotransmitters, for transport of proteins, cellular organelles, and cytoskeletal components to distant parts of the cell, and to maintain and restore ionic gradients affected by cycles of depolarization and repolarization.

The nervous system depends heavily on aerobic glycolysis to generate energy. The demand for oxygen and glucose is so high and sustained that even a brief interruption of oxygen, glucose, or both may result in severe adverse consequences, as there is essentially no reserve in the brain. The high oxygen requirement is associated with approximately 20% of the total cardiac output going to the brain. Toxic agents that interrupt the supply of oxygen (e.g., carbon monoxide) or the utilization of oxygen (e.g., cyanide), therefore, can produce catastrophic cellular damage in the nervous system.

The nervous system is a lipid-rich environment. As noted earlier, many elongated axons in the CNS and PNS are insulated by concentric layers of myelin that facilitate conduction of electrical impulses. Because of the density of the lipids, myelinated axons can be more sensitive to lipophilic neurotoxicants. Moreover, axons in adult CNS have a very limited ability to regenerate after injury, at least partially because of myelin-associated inhibitory factors from oligodendrocytes and "glial scars" from reactive astrocytes. In contrast, axons in the PNS have a greater potential to regenerate and regrow after injury under a different environment provided by Schwann cells.

Blood–Brain Barrier

Most of the adult brain and spinal cord is anatomically separated from the circulation by a continuous lining of specialized endothelial cells whose apposed surfaces form tight junctions. This, aided in part by associated glial cells, constitutes a blood–brain barrier (BBB), largely limiting passage of substances based on lipophilicity and active transport mechanisms. The BBB plays a pivotal role in protecting the CNS from various chemical challenges, especially larger, hydrophilic toxicants. In humans, this BBB is incompletely developed at birth, and even less so in premature infants. The

barrier also does not exist in certain brain regions (e.g., the area postrema, hypophysis, hypothalamic regions, pineal body, and the supraoptic crest). The deficiency of the BBB in these areas in the adult brain and the incomplete BBB in the developing brain can be important in terms of differential sensitivity to chemicals, including CWAs. With many substances, such as organophosphorus nerve agents, that are hydrophobic in nature, the BBB plays a minimal role in sensitivity. The integrity of the BBB can be compromised under certain conditions (e.g., anesthesia, stress, metabolic, and pathological disorders (such as adrenocorticoid hypertension and meningitis)), leading to higher sensitivity to some neurotoxicants (Kandel et al., 2012, for more details on all the nervous system overview topics described previously). For further details on effects of chemical and biological warfare agents on the BBB, see Chapter 49.

TYPES OF NEUROTOXICITY

Based on locations of the original "lesion" elicited by a neurotoxicant, neurotoxicity can be classified as neuronopathy, axonopathy, myelinopathy, or transmission-associated toxicity. *Neuronopathy* involves injury or death of neurons, with subsequent degeneration and loss of dendrites, axons, and myelin sheath (when present). Such neuronal degeneration is irreversible and can result in an encephalopathy with global dysfunction, dependent upon the neuronal population(s) affected. *Axonopathy* can be thought of as a "chemical transection" at some point along an axon, such that the distal segment degenerates but the soma remains intact. The critical difference between central and peripheral axonopathies is the ability of the axon to regenerate and reinnervate the target cell over time. An early clinical indicator of axonopathy is loss of sensation (e.g., glove and stocking paresthesia) and disruption in fine, distal motor control. *Myelinopathy* is chemical-induced demyelination (i.e., loss of the myelin sheath) or intramyelinic edema (i.e., separation of the concentric layers of the myelin). Remyelination typically occurs with a much thinner myelin sheath than the original condition. *Neurotransmission-associated toxicity* is typically without any overt morphological change, but related to toxicant-induced neurochemical changes within the synaptic region. Toxicants can disrupt neurotransmission by affecting presynaptic processes, including neurotransmitter synthesis, transport, and storage in synaptic vesicles, neurotransmitter release, reuptake, or degradation, by binding to presynaptic and/or postsynaptic receptors, by affecting ion flux in the presynaptic or postsynaptic cell, or by other mechanisms influencing signal transduction.

Neurotoxicity can also be classified based on the nerve-organ affected, e.g., dysautonomia when the autonomic nervous system is affected, neuromuscular disorders when somatic motor neurons or the neuromuscular junction is affected, sensory neuropathy when the sensory receptors or afferent sensory neurons are the primary targets, cognitive deficits and mood/mental disorders when higher brain/cognitive functions are affected.

SELECTED CWAS THAT AFFECT THE NERVOUS SYSTEM

As briefly reviewed earlier, the nervous system possesses some unique anatomical, biochemical, and physiological characteristics that can contribute to its relatively higher sensitivity to xenobiotics, including some CWAs. Many chemicals are potential weapons of mass destruction, including but not limited to organophosphorus (OP) nerve agents, cyanides, mustards, arsenicals, and the natural products botulinum toxin and ricin. Many CWAs adversely affect the nervous system either directly or indirectly, leading to acute and/or chronic adverse health consequences.

Organophosphorus (OP) Nerve Agents

The OP nerve agents are widely recognized as CWAs and are among the most lethal CWAs ever developed. They are classified as either "G" or "V" series agents. The G agents, including tabun (*O*-ethyl *N*,*N*-dimethylphosphoramidocyanidate; GA), soman (*O*-pinacolyl methylphosphonofluoridate; GD), sarin (isopropyl methylphosphonofluoridate; GB), and cyclosarin (cyclohexyl methylphosphonofluoridate; GF), are volatile and nonpersistent, whereas the V agents, such as VX (*O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonofluoridate) are less volatile and relatively more persistent. Both the United States and the Soviet Union stockpiled many of these agents for military use during the Cold War. However, the first documented military use of nerve agents was not until the Iran–Iraq War of 1980–1988. During this conflict, Iraqi troops employed sarin munitions against both military personnel and civilians, confirmed by detection of trace amounts of a breakdown product, isopropyl methylphosphonic acid, in soil samples taken from a Kurdish village in northern Iraq (Macilwain, 1993). Subsequently, sarin was used in two separate terrorist attacks in Japan by a cult named Aum Shinrikyo (meaning "divine truth" in Japanese). The first attack, on June 27, 1994, occurred in a residential neighborhood in Matsumoto, resulting in 56 hospitalizations and 7 deaths. The second attack, on March 20, 1995, occurred in several subway cars in Tokyo, resulting in 796 hospitalizations and 12 deaths (Yanagisawa et al., 2006).

Among the victims of the sarin attack in Tokyo, Japan, 111 individuals were characterized as severely or moderately injured on admission to the hospital on the day of the attack. They exhibited miosis (99%), headache (75%), respiratory distress (63%), nausea and vomiting (60% and 37%, respectively), eye pain (45%), blurred or dim vision (40% and 38%, respectively), and seizures (approximately 3%) (Okumura et al., 1996). Changes in psychomotor functioning, visual perception and sustained attention, learning and memory, and mood (Murata et al., 1997; Yokoyama et al., 1998) were observed in 18 evaluated survivors 6–8 months later. Depressive behaviors were reported in survivors between 6 months and 7 years after the attack. This included those who had not presented with symptoms of acute toxicity at the time of the incident (Araki et al., 2005). Yamasue et al. (2007) observed smaller regional white matter volumes and diffuse bilateral disruption of white matter integrity 5–6 years after the attacks. Miyaki et al. (2005) reported deficits in attention and gross motor speed 7 years after the attacks.

More than 100,000 US military personnel participating in the Gulf War were potentially exposed to low levels of cyclosarin and sarin following the destruction of a munitions storage facility at Khamisiyah, Iraq, in March 1991 (Smith et al., 2003). Reviews of medical records for Gulf War veterans and of published field accounts revealed no clinical indications of acute OP intoxication (Riddle et al., 2003). However, thousands of veterans later reported various neurological symptoms, referred to as "Gulf War illnesses," characterized by impaired cognition, including depression and insomnia; confusion-ataxia, including problems with thinking and disorientation; and joint/muscle pain, fatigue, and extremity paresthesia (Haley et al., 1997). Deficits in motor dexterity and visuospatial abilities (Proctor et al., 2006) and a twofold increase in the incidence of brain cancer–related deaths (Bullman et al., 2005) were reported in these veterans 4 years later. In addition, a reduction in the overall volume of white matter and an enlargement of the lateral ventricles were observed in the brains of some veterans (Heaton et al., 2007). It has been suggested that exposure to anticholinesterases , including possible low-level exposure to sarin and cyclosarin, prophylactic use of the carbamate drug pyridostigmine bromide, and widespread application of pesticides such as chlorpyrifos, may have played a role in the development of the Gulf War illnesses (Brimfield, 2012).

The most recent documented use of OP nerve agents was in August 2013, in the Ghouta area of Damascus, Syria. Sarin was employed via surface-to-surface rockets, with a considerable number of casualties. Although there are conflicting reports on the numbers, at least 3,600 people went to medical facilities with neurotoxic signs and symptoms, and 588 fatalities were recorded by the Violations Documentation Centre, which reports to the UN (Dolgin, 2013; Enserink, 2013; Patrick et al., 2013).

Organophosphorus compounds, including the nerve agents, elicit acute toxicity primarily via inhibition of acetylcholinesterase (AChE), disrupting ACh-mediated cholinergic signaling in the CNS and PNS. ACh is synthesized in the presynaptic terminal by choline acetyltransferase using choline and acetyl CoA as substrates. Once synthesized, ACh molecules are stored in the synaptic vesicles. Upon depolarization, the vesicles containing ACh fuse with the presynaptic membrane and release ACh into the synapse. The diffusible ACh molecules then interact specifically with cholinergic (i.e., muscarinic and nicotinic) receptors on postsynaptic cell membranes to mediate signal transduction. Muscarinic receptors are G protein–coupled receptors that affect the formation of second messengers (e.g., cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), and inositol triphosphate (IP3)). To date, five muscarinic receptors (M1–M5) have been identified and cloned. M1, M3, and M5 subtypes are coupled to phospholipase C (PLC) via G_q and increase the formation of DAG and IP3 upon activation, whereas the M2 and M4 subtypes are coupled to adenylyl cyclase via G_i and decrease the formation of cAMP once activated. Nicotinic receptors are ligand-gated ion channels that increase Na^+ influx upon stimulation. ACh can also bind to presynaptic muscarinic and nicotinic receptors to modulate the further release of ACh in a negative "feedback" manner. ACh in the synapse is degraded very rapidly by AChE into choline and acetate. The choline molecules are transported back into the presynaptic terminal through a process known as *sodium-dependent high-affinity choline uptake*, the rate-limiting step in ACh synthesis. ACh, therefore, has only a transient opportunity to activate muscarinic or nicotinic receptors, either postsynaptically or presynaptically. Inhibition of AChE prevents the normally efficient breakdown of ACh in the synapse, leading to ACh accumulation and persistent/prolonged stimulation of cholinergic receptors, which in turn elicits signs and symptoms of cholinergic toxicity.

Many of the classical signs of cholinergic toxicity associated with OP poisoning involve the PNS. The well-recognized syndrome of excessive secretions, known as salivation, lacrimation, urination, and defecation (SLUD), and other effects (e.g., miosis) are the result of AChE inhibition and muscarinic receptor activation in peripheral tissues (Espinola et al., 1999). Other common signs of cholinergic toxicity (e.g., muscle fasciculations) involve overstimulation of nicotinic receptors in peripheral tissues (Costa, 1988). The most debilitating effects of anticholinesterase exposures involve the CNS, however (Pope, 2006). Lethality from OP intoxication is typically due to depression of brainstem respiratory control centers, compounded by excessive airway secretions

and dysfunction of diaphragm and intercostal muscles. Rickett et al. (1986) reported that one of the first signs of nerve agent–induced respiratory distress in cats was disruption of the normal firing of respiratory-related neurons in the pons medulla, followed by changes in airflow, diaphragm contraction, diaphragm electromyogram (EMG) and phrenic nerve activity. Spectral analysis of diaphragm EMG indicated that the functional integrity of the diaphragm in guinea pigs following soman exposure (15 μg/kg, s.c.) was not sufficiently compromised to produce respiratory distress despite signs of fatigue (Chang et al., 1990). These studies suggested that respiratory distress following nerve agent exposure is mainly due to disruption of central cholinergic signaling. Moreover, Bajgar et al. (2007) reported that AChE activity in the pons medulla (i.e., the site of respiratory control centers) was more extensively inhibited by soman, sarin, and VX than AChE in many other parts of the brain.

Whole body tremors can result from extensive AChE inhibition in the CNS, with activation of muscarinic receptors in the basal ganglia being of prominent importance (Espinola et al., 1999). With severe intoxications, seizures can be elicited that can lead to irreversible neuropathology (Shih et al., 1991; Kadar et al., 1995; Dekundy et al., 2001, 2007). Shih and McDonough (1999) demonstrated that all five of the classic nerve agents (i.e., cyclosarin, sarin, soman, tabun, and VX) were capable of inducing seizures at lethal dosages. In the case of VX, however, the latency of seizure development was 3–5 times longer than with the other nerve agents. Soman-induced seizures occurred in rats only when cortical AChE inhibition was over 65% (Tonduli et al., 2001). Both anticholinergics (e.g., atropine and biperiden) and benzodiazepines (e.g., diazepam and midazolam) were able to terminate nerve agent–induced seizures in guinea pigs when administered within 5 min of seizure onset, but a higher dose of each was typically required to terminate seizures induced by soman compared to other nerve agents (Shih et al., 2003).

Prolonged seizure activity (i.e., *status epilepticus*) following nerve agent exposure has been shown in several animal models to cause neuropathological lesions that are associated with long-term deficits in cognitive function and other behaviors. Moderate to severe neuropathological lesions were reported in 70% of sarin- (Kadar et al., 1995) and 98% of soman-treated (McDonough et al., 1995) rats, when the seizures lasted 20 min or longer. Pathological lesions were primarily noted in the hippocampus, piriform cortex, and thalamus, but later progressed to other brain regions (e.g., the amygdala). Soman-induced status epilepticus in rats resulted in significant neuronal loss and neurodegeneration (in particular with GABAergic interneurons) in the basolateral nucleus of the amygdala. Moreover, this neuropathology was associated with increased anxiety-like behavior measured in the open-field test and the acoustic startle response 14 days later (Prager et al., 2014). Spatial memory performance in the Morris water maze was directly related to the severity of hippocampal lesions in soman-treated rats. Interestingly, no memory impairment was observed below a threshold of 15% neuronal loss (Filliat et al., 1999). Impaired contextual and cued fear conditioning (fear-based learning and memory) was observed in soman-treated rats and mice that experienced seizures and neuronal degeneration in different brain regions (Coubard et al., 2008; Moffett et al., 2011). These mice also showed anxietylike behaviors measured in unconditioned fear tests (light/dark boxes and elevated plus-maze). Deficits in cognitive function measured in a novelty test, the brightness discrimination task (Myhrer et al., 2005), and the stone maze (Raffaele et al., 1987) were reported in rats following soman-induced convulsions, associated with neuropathological lesions.

Nerve agents such as soman and other OP compounds have been shown to induce anxiety- and depressive-like behaviors in animal models at dosages that do not elicit seizures. A sublethal dosage of soman (0.6–0.8 × LD_{50}) triggered anxiety-like behavior (measured in the open field test and elevated plus maze) in guinea pigs (Mamczarz et al., 2010). Acute intoxication with diisopropylfluorophosphate (a prototype OP and structure analog of sarin) in rats resulted in depressive-like behavior (e.g., increased immobility and decreased swimming in the forced swimming test; Wright et al., 2010). Chen et al. (2011) reported serotonergic-related changes in affective behaviors measured in the elevated plus maze, the Vogel's conflict test, the novelty-suppressed feeding test, and the forced swimming test in adolescent rats exposed to chlorpyrifos (10–160 mg/kg/day for 7 days, subcutaneously). Repeated exposure to methamidophos in the drinking water at levels that did not elicit systemic toxicity induced depressive-like behavior in adult mice (Lima et al., 2009). Malathion exposure has also been reported to be associated with depressive-like behavior in rodents (Ramos et al., 2006; Brocardo et al., 2007; Acker et al., 2009).

Anxiety and depression have also been reported in humans following OP intoxication. A number of epidemiological studies have reported long-term neuropsychological or neuropsychiatric sequelae, such as anxiety and depression, with past anticholinesterase intoxication or exposure (Savage et al., 1988; Rosenstock et al., 1991; McConnell et al., 1994; Steenland et al., 1994; Yokoyama et al., 1998, 2002; Wesseling et al., 2002; Colosio et al., 2003; London et al., 2005; Roldan-Tapia et al., 2006). A cross sectional survey conducted among 761 individuals, representing 479 farms in northeastern Colorado between 1992 and 1997, found that exposure

to pesticides, in particular OPs, at levels sufficient to cause self-reported poisoning symptoms, was associated with a more than fivefold higher rate of depression, independent of other known risk factors (Stallones and Beseler, 2002). A mini-review of 11 studies on depression in relation to pesticides revealed increased odds ratios (ranging from 2 to 6) for developing depression or other psychiatric disorders (Freire and Koifman, 2013). More recently, Wesseling et al. (2010) reported an increased incidence of depression and anxiety, along with other psychological or psychiatric deficits, in workers exposed to OPs that had received medical attention 1–3 years prior to the study.

Thus, central actions of OP anticholinesterases can be critical in both acute toxic responses and long-term neurologic sequelae following acute or repeated exposures. While disruption of cholinergic neurotransmission is a hallmark of OP poisoning, substantial evidence indicates that disruption of noncholinergic signaling contributes to the ultimate outcome. Shih et al. (1991) reported that seizures elicited by soman were initially sensitive but later resistant to the antimuscarinic antidote, atropine. Activation of glutamate signaling in limbic regions (e.g., the hippocampus) appeared particularly important in OP-induced seizures (Lallement et al., 1991). These investigators observed a 78% increase in extracellular glutamate in the CA3 region of the hippocampus within 30 min of seizure onset, and a more robust early increase (180%) followed by a declined but more sustained increase in the hippocampal CA1 region after 50 min of seizure onset in soman-treated rats. Sparenborg et al. (1992) reported that MK-801 (30, 100, or 300 μg/kg, i.m.) blocked soman-induced seizures in a dose-dependent manner, suggesting that NMDA receptor activation may play a critical role in the spread and maintenance of nerve agent-induced seizures. Moreover, NMDA receptor antagonists blocked seizures elicited by OP insecticides (dichlorvos and chlorfenvinphos; Dekundy et al., 2001, 2007). In contrast, it is reported that higher striatal dopamine and GABA levels were correlated with severity of signs of soman toxicity, seizure intensity, and epileptiform bursting (Cassel and Fosbraey, 1996; Jacobsson et al., 1997). The dopamine D1-like antagonist SCH23390 completely blocked soman-induced seizures (Bourne et al., 2001). The activation of noncholinergic signaling pathways, therefore, appears to participate in the expression of OP toxicity, and brain-regional differences in activation of noncholinergic signaling may be important in the ultimate toxic outcome.

Another noncholinergic signaling pathway that may influence the ultimate expression of OP toxicity is mediated by eCBs. The eCB signaling participates in a variety of processes including thermoregulation, food intake, immune function, perception (hearing, color, vision, and taste), cognition (long-term potentiation and short-term memory), and motor function (locomotor activity, proprioception, and muscle tone). Of particular importance, eCBs inhibit the release of a number of neurotransmitters (Miller and Walker, 1995; Gifford and Ashby, 1996; Cadogan et al., 1997; Gessa et al., 1997; Levenes et al., 1998; Cheer et al., 2004). Several studies suggested that eCBs tonically inhibit ACh release in the hippocampus via CB1 receptors; i.e., agonists reduce the release of ACh, while antagonists increase it (Gifford and Ashby, 1996; Gessa et al., 1997; Gifford et al., 2000; Kathmann et al., 2001; Tzavara et. al., 2003; Degroot et al., 2006). Interestingly, ACh release in the striatum is unaffected by eCB agonists and antagonists or by deletion of the CB1 gene in mice (Gifford et al., 2000; Kathmann et al., 2001). Thus differential effects of eCBs on ACh release in different brain regions may be important.

eCB-mediated inhibition of GABA and glutamate release from presynaptic neurons via DSI and DSE may also play an important role in the expression of OP toxicity. As mentioned above, higher levels of striatal GABA and limbic glutamate levels have been correlated with more severe signs of OP toxicity (Lallement et al., 1991, 1992; Cassel and Fosbraey, 1996; Jacobsson et al., 1997). Furthermore, the $GABA_A$ receptor antagonist bicuculline is proconvulsant when directly administered into limbic regions but is anticonvulsant when microinjected into the striatum (Turski et al., 1989, 1991). Thus, changes in GABAergic and glutamatergic signaling and their modulation by eCBs could have regional-specific effects on neurological function after OP intoxication.

While eCBs can regulate the release of ACh and other neurotransmitters, OP toxicants may also affect eCB signaling either indirectly, through AChE inhibition (and neuronal depolarization/activation of M1/M3 receptors), or directly, through binding to cannabinoid receptors, inhibiting cannabinoid metabolizing enzymes, or both (Quistad et al., 2001, 2002, 2006; Segall et al., 2003; Nallapaneni et al., 2006, 2008). Chlorpyrifos oxon, paraoxon, and diisopropylfluorophosphate (DFP) inhibit CB1 receptor binding and the activities of FAAH and MAGL *in vitro*, with chlorpyrifos oxon being more potent. *In vivo*, DFP inhibited FAAH but had little effect on MAGL or CB1 binding (Nallapaneni et al., 2008). More recently, Liu et al. (2013) reported that both chlorpyrifos and parathion (the parent compounds of chlorpyrifos oxon and paraoxon, respectively) inhibited FAAH and MAGL *in vivo*, with more extensive inhibition seen with FAAH by both compounds. Extracellular levels of eCBs in the rat hippocampus were increased by both chlorpyrifos and parathion at dosages that inhibited more than 80% of hippocampal AChE activity.

Pharmacological manipulation of eCB signaling has been shown to affect both acute and long-term toxicity of OPs. The CB1 receptor agonist WIN 55,212-2 (1.5 mg/kg, i.p.) reduced acute paraoxon toxicity with a

single dose, but increased toxicity if paraoxon was given after repeated WIN 55,212-2 dosing (Nallapaneni et al., 2006). Acute DFP toxicity was reduced by WIN 55,212-2, as well as by inhibitors of FAAH and MAGL (URB597 and URB602, respectively) and the eCB reuptake inhibitor (AM404; Nallapaneni et al., 2008). As noted before, acute DFP exposure induced depressive-like behavior in rats. The combination of URB597 and URB602 was shown to have antidepressant-like effects, reversing DFP-induced changes in the forced swimming test (Wright et al., 2010). Interestingly, the CB1 receptor antagonist/inverse agonist AM251 markedly decreased parathion-induced toxicity in rats, while having no influence on functional signs of chlorpyrifos (Liu et al., 2013). Thus, eCB signaling may play differential roles in toxic outcome of OPs based on the modulation of different neurotransmitter systems, possibly related to the time of onset and the duration of the neurotoxic response.

OP anticholinesterases have the potential to markedly disrupt nervous system function, eliciting a broad range of acute and long-term effects. While AChE has been considered the primary target, and downstream changes in cholinergic signaling have been the main focus in understanding mechanisms and exploring countermeasures, noncholinergic pathways following AChE inhibition may play a prominent role in the ultimate outcome following OP exposure.

Cyanides

Cyanides have been used for their toxic potential since ancient Roman times. Cyanogen chloride, cyanogen bromide, and hydrogen cyanide are important cyanide-containing compounds of potential used as CWAs. The use of cyanides in warfare was not implemented until World War I. Due to their high volatility, however, these compounds rarely achieve lethal atmospheric concentrations except in enclosed spaces (Lee, 1997). Hydrogen cyanide was used for mass extermination of prisoners in concentration camps by the Nazis during World War II. Other forms of cyanide-containing compounds have also been used for malicious purposes. For example, potassium cyanide was illicitly placed in capsules of Extra-Strength Tylenol, leading to 7 deaths in the Chicago area in 1982 (Dunea, 1983).

Signs of cyanide exposure include agitation, dizziness, headache, and mental confusion, followed by cardiac disturbances, loss of consciousness, respiratory distress, seizures, and death. While cyanide exposure is often fatal, there are reports of long-term effects with sublethal intoxications. Finelli (1981) described a 30-year-old male who developed choreiform movements in his extremities and impairment in the movement of his left hand one year after he attempted suicide with cyanide. Similarly, Carella et al. (1988) reported a 46-year-old woman who drank a beverage poisoned with cyanide and later developed a dystonic posture of the mouth and tongue that was deviated to the right and twisted. Computed tomography scans showed that both individuals had lesions in the basal ganglia.

Although there are a number of reports on the neuropathological consequences of cyanide exposure, including necrotic lesions in the cerebellar gray matter of dogs (Haymaker et al., 1952) and demyelinating lesions in the corpus callosum and optic nerves of rats (Lessell, 1971), its neurobehavioral sequelae have received relatively little attention. D'Mello (1986) reported that a single exposure to sodium cyanide (4 mg/kg, subcutaneously) impaired swimming ability in guinea pigs. Mathangi and Namasivayam (2000) demonstrated that repeated exposure to sodium cyanide (2 mg/kg/day × 30 days, intraperitoneally) impaired T-maze performance in rats, along with a reduction in dopamine and 5-hydroxytryptamine levels in the hippocampus.

The primary mode of action for cyanide-induced toxicity is inhibition of cytochrome oxidase, the terminal enzyme of the electron transport chain. Inhibition of cytochrome oxidase leads to cytotoxic hypoxia, with a shift from aerobic to anaerobic metabolism, a decrease in ATP synthesis, and an increase in lactic acid production (Way, 1984). The CNS is particularly sensitive to the toxic effects of cyanide due to both extremely limited anaerobic metabolic capacity and high energy dependence. Ikegaya et al. (2001) showed that the inhibition of cytochrome oxidase activity following oral administration of potassium cyanide (10 mg/kg) in rats was higher in the brain than in other organs. Thus, not only is the CNS particularly sensitive to cyanide-induced hypoxia and energy deficit, but the target enzyme itself in the CNS may be more sensitive to inhibition by cyanide compared to enzymes in other tissues.

Oxidative stress may also play a critical role in cyanide-induced toxicity. Potassium cyanide (7 mg/kg, administered subcutaneously) decreased the activities of catalase, glutathione peroxidase, and superoxide dismutase in mouse brain (Ardelt et al., 1989). Potassium cyanide also stimulated the formation of reactive oxygen species (ROS) and increased levels of malondialdehyde in a number of neuronal cell types, including cerebellar granule cells (Gunasekar et al., 1996), primary cortical cells (Li et al., 2002) and rat pheochromocytoma (PC-12) cells (Kanthasamy et al., 1997). Johnson et al. (1986) proposed that a rise in intracellular Ca^{2+} levels following potassium cyanide exposure was responsible for the formation of ROS. In support of this, Gunasekar et al. (1996) reported that removal of Ca^{2+} from the culture medium blocked the formation of ROS in cerebellar granule cells exposed to potassium cyanide (100 µM). Moreover, antioxidants (Muller and Krieglstein, 1995), Ca^{2+} channel blockers (Johnson et al., 1987), cyclooxygenase-2 (COX-2)

inhibitors (Li et al., 2002), as well as the NMDA receptor antagonists (Gunasekar et al., 1996) and phospholipase A2 inhibitors (Kanthasamy et al., 1997), all blocked the formation of ROS in neuronal cell lines exposed to potassium cyanide.

Cyanide-induced cell death may involve selective activation of apoptosis or necrosis in different brain regions or neuronal populations. Mills et al. (1999) reported that cyanide induced cell death in mouse brain via apoptosis in the cortex, but through necrosis in the substantia nigra. Similarly, Prabhakaran et al. (2002) reported that cyanide-induced cell death occurs via apoptosis in primary cortical cells, but by necrosis in primary mesencephalic cells. While potassium cyanide (400 μM) increased the formation of ROS in both cell types, the rates of formation and the nature of the ROS varied. Furthermore, catalase and superoxide dismutase decreased the formation of ROS in cortical cells, but not in mesencephalic cells. These findings suggest that the selective vulnerability of different neuronal populations to cyanide may be related to differences in their susceptibility to oxidative stress.

Dopaminergic neurons are highly sensitive to oxidative stress, possibly due to dopamine autoxidation to quinones and other ROS (Basma et al., 1995; Ben-Shachar et al., 1995). Kanthasamy et al. (1994) reported that mice repeatedly exposed to potassium cyanide (6 mg/kg, twice a day for 7 days, subcutaneously) had a reduced number of TH-positive cells, indicating a loss of dopaminergic neurons in the substantia nigra. Approximately 30% of the cyanide-treated mice exhibited decreased locomotor activity and akinesia, which were alleviated by the administration of L-DOPA (100 mg/kg, intraperitoneally). Cassel and Persson (1992) reported that levels of striatal dopamine and its metabolite homovanillic acid (HVA) were rapidly decreased in rats exposed to sodium cyanide (20 mg/kg, intraperitoneally). However, the *in vivo* synthesis of dopamine, measured as the rate of L-DOPA accumulation after neuronal decarboxylase inhibition, increased in these rats. Kiuchi et al. (1992) noted that perfusion of sodium cyanide (2 mM) into rat striatum produced a transient but marked increase in DA release. In addition, dopamine D1 and D2 receptor binding in the striatum was decreased in rats after the administration of sodium cyanide (2 mg/kg, intraperitoneally) (Cassel et al., 1993). Given that Parkinson's disease is a condition characterized by the selective loss of dopaminergic neurons in substantia nigra and the depletion of striatal dopamine levels (German et al., 1989), these findings suggest that cyanide exposure may lead to the development of signs or symptoms that resemble that disease.

Both glutamatergic and GABAergic neurons may be involved in the development of seizures after cyanide exposure. There is a strong correlation between whole-brain Ca^{2+} levels and cyanide-induced seizures in mice (Johnson et al., 1986). Patel et al. (1992) demonstrated that NMDA receptor activation was responsible for the rise in intracellular Ca^{2+} levels, contributing to cyanide-induced toxicity in cultured hippocampal neurons. Yamamoto and Tang (1996, 1998) reported that cyanide-induced seizures in mice were blocked by MK-801 (2 mg/kg, subcutaneously) and the morphological changes observed in cerebrocortical neurons exposed to potassium cyanide (1 mM) were blocked by 2-amino-7-phosphonoheptanoic acid (AP7; 1 mM): both are selective NMDA receptor antagonists. Persson et al. (1985) reported that striatal GABA levels were decreased in rats exposed to sodium cyanide (20 mg/kg, intraperitoneally), and these decrements were associated with an increased susceptibility to seizures (Cassel et al., 1991). Similarly, Yamamoto (1990) reported that whole-brain GABA levels were markedly decreased and Ca^{2+} levels were increased in cyanide-treated mice exhibiting seizures. All these findings suggested that increased glutamatergic activity, decreased GABAergic activity, or both may contribute to the development of seizures following cyanide exposure.

Sulfur Mustard

Sulfur mustard, also known as HD, is a classic blister agent and an effective incapacitating chemical. It was first used as a CWA in World War I during a German attack on British troops at Ypres, Belgium, in 1915. Subsequently, it has been used in a number of military conflicts, including the Iran–Iraq War of 1980–1988 (Marshall, 1984). During this conflict, Iraqi troops employed sulfur mustard against both military personnel and civilians. Approximately 40,000 victims of sulfur mustard have been documented among the Iranian and Kurdish populations (Hay, 2000; Khateri et al., 2003).

Sulfur mustard exerts local actions on the eyes, respiratory tract, and skin, followed by systemic actions on the nervous, cardiac, gastrointestinal, and hematopoietic systems (Dacre and Goldman, 1996). A moderate exposure to sulfur mustard can cause blisters, conjunctivitis, erythema, lacrimation, nausea, and respiratory inflammation, whereas a severe exposure can cause blindness, bronchitis, bronchopneumonia, corneal damage, leucopenia, seizures, and death. Sulfur mustard is a strong alkylating agent that reacts with thiol, amino, carboxyl, hydroxyl, and primary phosphate groups in DNA and other macromolecules. Alkylation of DNA activates chromosomal poly(ADP-ribose) polymerase, reducing the intracellular supply of NAD^+ and thus inhibiting glycolysis and causing cell death (Papirmeister et al., 1985). More recently, it was suggested that oxidative stress may play a critical role in sulfur mustard-induced toxicity as well (Naghii, 2002). Jafari (2007) demonstrated that

high dosages of sulfur mustard (>10 mg/kg, intraperitoneally) decreased the activities of catalase, glutathione peroxidase, glutathione *S*-transferase, and superoxide dismutase in rat liver and brain, leading to the impairment of antioxidant defense systems.

There is limited information available on the effects of sulfur mustard on the CNS. The majority of the victims exposed to sulfur mustard during the Iran–Iraq War seeking medical treatment at European hospitals suffered from anxiety, confusion, headache, and lethargy (Balali-Mood and Hefazi, 2006). A cohort study conducted on 495 Iranian civilians revealed that individuals exposed to sulfur mustard exhibited a significantly higher incidence of depression, anxiety, hostility, and obsessive-compulsive behavior compared to age-matched counterparts 20 years after exposure (Roshan et al., 2013). Lethal dosages of sulfur mustard (>10 mg/kg, subcutaneously) caused hyperexcitability followed by unsteadiness of gait, muscular weakness, and seizures in dogs (Lynch et al., 1918). Philips and Thiersch (1950) reported that lethal dosages of 2,4,6-tris(ethylenimino)-*S*-triazine (>125 mg/kg, intraperitoneally), which has an ethylenimine moiety analogous to sulfur mustard, caused similar effects in mice. Although the mechanism responsible for these effects is unknown, it is interesting to notice sulfur mustard-induced AChE activity in neuroblastoma cell cultures, suggesting that cholinergic signaling may play a role in its effects on the CNS (Lanks et al., 1975). Thus, while sulfur mustard is typically thought of as a blister agent, clinical and experimental data suggest that it has the potential for eliciting both acute and long-term neurotoxic effects.

3-Quinuclidinyl Benzilate

3-quinuclidinyl benzilate (QNB, also known as BZ,1-azabicyclo[2.2.2]oct-3-yl hydroxy(diphenyl)acetate) is a potent, atropine-like glycolic acid ester that blocks muscarinic receptors in the CNS and PNS (Spencer, 2000). During the 1950s, QNB was explored by both the United States and the Soviet Union as a potential antidote for OP anticholinesterases. However, the marked hallucinogenic actions of QNB led to its potential use as a CWA (Marshall, 1979). In July 1995, the Yugoslav People's Army allegedly used an incapacitating agent that caused hallucinations and irrational behavior against Bosnian refugees fleeing the town of Srebrenica (Hay, 1998). While many believe that this incapacitating agent was QNB, there is no evidence to support this allegation.

QNB acts as a potent, competitive inhibitor of ACh at muscarinic receptors located in the brain, heart, exocrine glands, and smooth muscles in various organs, resulting in a confusional mental state with delusions, hallucinations, mental slowing, erratic behavior, a decrease in intestinal motility and tone, inhibition of bronchial and salivary secretions, sweating, mydriasis, and tachycardia (Ketchum, 1963). Early studies demonstrated QNB was a potent and selective ligand for muscarinic receptors. Yamamura and Snyder (1974) showed that the inhibition of [^{3}H]-QNB binding to homogenates of rat brain by muscarinic drugs correlated with their pharmacological potencies, whereas nicotinic and noncholinergic drugs had negligible affinity. Kuhar and Yamamura (1976) reported that the injection of [^{3}H-]QNB into rat brain led to its localization to muscarinic receptors in the cerebral cortex, hippocampus, nucleus accumbens, and striatum. In addition, Jovic and Zupanc (1973) reported that the subcutaneous administration of QNB (5–20 mg/kg) to rats decreased oxygen consumption in the cerebral cortex and medulla oblongata. Interestingly, while QNB as a radioligand appears to bind to all known subtypes of muscarinic receptors, some evidence suggests that it has higher affinity for the M2 subtype of muscarinic receptors when administered *in vivo* (McRee et al., 1995). QNB thus can produce a variety of neurological effects, presumably through the antagonism of cholinergic muscarinic receptor signaling in the CNS and PNS.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The nervous system has a number of biochemical, physiological, and anatomical characteristics that can make it particularly sensitive to CWAs. Indeed, many CWAs (particularly the nerve agents) can elicit debilitating and devastating neurotoxic responses. In some cases, these neurotoxic effects are elicited by direct interaction with specific target molecules within the nervous system, while in other cases, actions on other organ systems can lead to indirect impairments of neurologic functions. The global importance of the continuous neuronal regulation of vital physiological processes throughout the body makes the nervous system an effective target for disruption by CWAs. Thus, knowledge of mechanisms by which CWAs elicit neurotoxicity can aid in seeking and designing effective countermeasures against CWA exposures.

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CHAPTER

35

Behavioral Toxicity of Nerve Agents

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INTRODUCTION

Behavioral changes in humans exposed to highly toxic organophosphorus (OP) compounds, called nerve agents, have been discussed in numerous reports. The incidence of behavioral effects is higher in individuals who have been severely exposed to nerve agents, but they may occur in individuals who have received low-level exposure below those producing convulsions and other severe clinical signs of toxicity. The behavioral effects usually start within a few hours and last from several days to several weeks or months. The most frequent symptoms include feelings of uneasiness, tenseness, and fatigue. Exposed individuals may be forgetful and generally display impaired memory and learning, poor comprehension, decreased ability to communicate, or occasional mild confusion.

There are a few reports describing behavioral changes in subjects accidentally exposed to nerve agents. They reported sleep disturbance, mood changes, fatigue, jitteriness or tenseness, an inability to read with comprehension, difficulties with thinking and expression, forgetfulness, a feeling of being mentally slow, depression, irritability, giddiness, poor performance in arithmetic tests, minor difficulties in orientation, and frightening dreams. It was observed that the complex of central nervous system (CNS) symptoms may not fully develop until 24 h after exposure. In addition, no correlations between the presence or severity of symptoms and the degree of acetylcholinesterase (AChE) inhibition were seen. Most of the effects of exposure disappear within 3 days. It was concluded that not only severe but also mild intoxication of nerve agents may cause behavioral and psychological disturbances. In general, the behavioral effects have not been permanent but have lasted from weeks to several months, or possibly several years. Long-term behavioral effects after poisoning with nerve agents or OP insecticides have been reported (Levin and Rodnitzki, 1976; Karczmar, 1984; Loh et al., 2010). These reports are based on clinical observations, which are occasionally supported by psychological studies.

METHODS USED TO EVALUATE BEHAVIORAL EFFECTS OF NERVE AGENTS

Functional Observatory Battery

The functional observatory battery (FOB) is a noninvasive and relatively sensitive type of neurobehavioral examination of 40 sensory, motor, and autonomic nervous functions. Some of them are scored (Table 35.1), and the others are measured in absolute units (Frantik and Hornychova, 1995). The first evaluation is performed when nerve agent–exposed or control rats are in the home cage. The observer evaluates each animal's posture, palpebral closure, and gait, and the presence or absence of convulsions is noted. Each rat is then removed from the home cage and briefly held in the hand. The presence or absence of spontaneous vocalization, piloerection, and other fur and skin abnormalities as well as irritability is noted. Lacrimation and salivation are also observed. Other signs such as exophthalmus, crustiness around the eyes, or emaciation are also recorded. The rats are then placed on a flat surface that serves as an open field. A timer is started for 3 min, during which the frequency of rearing responses is recorded. At the same time, gait characteristics are noted and ranked, and activity, tremor, convulsions, and abnormal posture are evaluated. At the end of the third minute, the number of fecal boluses and urine pools on the absorbent pad is registered. Then, a reflex test that consists of recording each rat's response to the frontal approach of the blunt end of a pen, a touch of the pen to the posterior flank, and an auditory click stimulus is used.

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TABLE 35.1 Functional Observational Battery

	Scored Values Only									
Marker	**−2**	**−1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Posture				Sitting or standing	*Rearing*	*Asleep*	Flattened	Lying on side	Crouched over	Head bobbing
Catch difficulty				Passive	*Normal*	Defense	Flight	Escape	Aggression	
Ease of handling				Very easy	Easy	Moderately difficult	Difficult			
Muscular tonus	Atonia	Hypotonia	*Normal*	Hypertonia	Rigidity	Fasciculations				
Lacrimation			*None*	Slight	Severe	Crusta	Colored crusta			
Palpebral closure				*Open*	Slightly	Half-way	Completely shut	Ptosis		
Endoexophthalmus		Endo	*Normal*	Exo						
Piloerection			*No*	Yes						
Skin abnormalities			*Normal*	Pale	Erythema	Cyanosis	Pigmented	Cold	Injury	
Salivation			*None*	Slight	Severe					
Nose secretion			*None*	Slight	Severe	Colored				
Clonic movements			*Normal*	Repetitive	Nonrhythmic	Mild tremors	Severe tremors	Myoclonic	Clonic	
Tonic movements			*Normal*	Contraction of extensors	Opisthotonus	Emprosthotonus	Explosive jumps	Tonic convulsions		
Gait			*Normal*	Ataxia	Overcompensation of hindlimb movement	Feet point outwards from body	Forelimbs are extended	Walks on tiptoes	Hunched body	Body is flattened against surface
Gait score				*Normal*	Slightly impaired	Somewhat impaired	Totally impaired			
Mobility score				*Normal*	Slightly impaired	Somewhat impaired	Totally impaired			
Arousal (level of unprovoked activity)				Very low	Sporadic	Reduced	*Normal*	Enhanced	Permanent	
Tension			*None*	Partial (ears)	Stupor					
Stereotypy			*None*	Head weaving	Body weaving	Grooming	Circling	Others		
Bizarre behavior			*None*	Head	Body	Self-mutilation	Abnormal movements	Others		
Approach response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Touch response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Click response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Tail-pinch response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Pupil size		Miosis	*Normal*	Mydriasis						
Pupil response			No reaction	*Normal reaction*						
Righting reflex				*Normal*	Slightly uncoordinated	Lands on side	Lands on back			

The responsiveness to a pinch on the tail and the ability of pupils to constrict in response to light are then assessed. These measurements are followed by a test for the aerial righting reflex, then by measurements of forelimb and hindlimb grip strength, body weight, rectal temperature, and, finally, hindlimb landing foot splay. The whole battery of tests requires approximately 6–8 min per rat. Motor activity data are collected using an apparatus for testing a spontaneous motor activity of laboratory animals. The animals are placed for a short period (10 min) in the measuring cage and their movement (total horizontal activity, stereotypical activity, rearing, jumping, scratching, and total vertical activity) is recorded.

Performance on the RAM Task

Radial arm maze (RAM) sessions are conducted using an eight-arm commercially available radial maze measuring 137.2 cm in diameter. The center of the maze is a plastic octagon hub measuring 26.67 cm across, with a Plexiglass lid and wire grid-floor. A Plexiglass arm with a wire mesh floor is attached to each of the eight sides of the hub. The entrance to each arm contains a motorized guillotine door allowing access to and from the hub. Each arm's runway contains two floor-mounted switches that are depressed by the weight of the rat when present in the proximal and distal portion of the runway, respectively. The terminal portion of each arm contains a food dispenser for delivering food pellets that is connected to a trough that is outfitted with the photoemitter/detector unit that can detect access by the rats. Experiments are controlled and monitored using a commercial hardware interface and a microcomputer using the L2T2S software control system (Coulbourn Instruments). For the RAM task, four of eight arms are "baited." That is, a single food pellet is available with a nose-poke into the food trough at the terminal portion of four arms. Each rat is randomly assigned a maze configuration of four baited arms from 37 possible configurations that exclude more than two consecutive baited arms. Thus, the same configuration of baited arms is used for a particular rat for each of the sessions, but different configurations can be used for different rats. Sessions begin with the rat placed in the center hub compartment and the doors to the eight arms are raised. The rat is then free to explore the maze to obtain the food rewards available from the four baited arms. The session is terminated when a rat obtains all four food rewards or when 15 min have elapsed. If a rat does not complete the maze within 15 min, then a completion time of 15 min is assigned and errors are analyzed. Failure to complete the maze, however, is infrequent and only occurs during the initial few sessions in the maze. No familiarity training with the maze is conducted before the first session. The major dependent variables characterizing performance on the RAM task are the time to complete the maze and the number of errors made. Errors are designated as occurring when a rat chooses an unbaited arm (reference memory error) or when a rat returns to a baited arm after obtaining the food reward (working memory error) (Genovese et al., 2006).

Acoustic Startle Response and Prepulse Inhibition

The animals are tested for acoustic startle response (ASR) and prepulse inhibition (PPI) in the SM100 Startle Monitor system. The system is usually programmed for six types of white-noise burst stimulus trials: no stimulus (background, 60 dB), prepulse (70 dB), pulse (100 and 120 dB), and prepulse plus pulse (70 dB + 100 dB and 70 dB + 120 dB). Each trial type is presented 10 times in 10 blocks. Stimuli are presented in random order to avoid order effects and habituation. The inter-trial interval can vary from 9 to 16 s. All animals are regularly handled before individual tests to minimize handling-related stress. Animals are pair-matched according to baseline values into the experimental groups using the average of the response to 100 and 120 dB. The tested animals are restrained loosely in holders that are placed on a sensing plate transforming movements of the body (jerks) into an analog signal through an interface. Finally, the percentage PPI measures are calculated as the difference between the pulses alone and multiplied by 100. Percentage scores are typically used to minimize the effect of individual variation of startle amplitude on PPI (Mach et al., 2008).

Performance on Y-Maze

Cognitive functioning can be tested using a Y-maze with averse motivation by a strong electric footshock evaluating learning and spatial memory (Koupilova et al., 1995). The Y-maze is a fully automated apparatus used for the study of behavior of laboratory rats. It is a plastic box consisting of a square start area (285 × 480 mm^2) separated by a Plexiglass sliding door from two trapezoid, black and white arms—choice area (140 × 324 mm^2). The grid-floor at the start and in the choice area is electrifiable. The animal (usually rat) is placed on the start area and after 48 s, electric footshocks (60 V, 50 Hz, duration 0.5 s) are applied at 5-s intervals. The rats try to avoid the shock by escaping to one of two arms. In the case of a rat moving to the wrong (dark) arm, the rat fails to avoid further footshock. The animals are taught spatial discrimination with the preference of the black or white arm in the Y-maze. The latency to enter the correct arm is measured and the number of wrong entries is counted. Before exposure to nerve agent, the rats are trained to avoid footshock by moving to the correct (white) arm

in the Y-maze. It usually takes 4 weeks of training to reach the criterion, which was 80% or more correct averse behavior (moving to the correct arm) within less than 1.5 s. During the training, 10 sessions (two trials/session) per week lasting 4 min are completed. The exposure starts the day after the animals reached this criterion. The latency time to enter the correct arm by nerve agent–exposed rats and the number of entry errors are compared with the values obtained from the control rats exposed to the pure air instead of nerve agent.

Performance on T-Maze

Cognitive functioning can be also tested using a T-maze consisting of five segments, a starting, and a goal compartment to evaluate learning, spatial memory, and spatial orientation (Koupilova and Herink, 1995). The rats are trained, with the food reward, to run through the maze in less than 10 s without entering the side arm. The time necessary to reach the goal box is recorded. Before exposure to nerve agent, the rats are trained to reach the goal box as soon as possible by moving to the correct segment in the T-maze. It usually takes 4–6 weeks of training to reach the criterion, which was 80% or more correct behavior. The exposure starts the day after the animals reaches this criterion. The time of reaching the goal box by rats exposed to nerve agent is compared with the values obtained from the same rats immediately before nerve agent exposure and from control rats exposed to pure air instead of nerve agent.

Performance on Morris Water Maze

The water maze (WM) is often used for the evaluation of effects of various compounds on memory functions, e.g., memory formation, consolidation, and retrieval effects due to its advantages and broad utilization. The Morris WM is a widely used measurement of visuospatial learning that has been demonstrated to have high validity in identifying cognitive effects of various brain lesions and the effects of drugs used to treat cognitive deficits (Morris, 1984; Myhrer, 2003). Special motivation such as food and water deprivation is not required for the WM performance. The effect of odor cue is eliminated in the WM. In addition, rats are forced to swim in the WM. They cannot choose whether to move, so failure to respond is not a confounding issue (Shukitt-Hale et al., 2004). The place-learning version with submerged platform can be used for working memory tests (Myhrer, 2003). The WM can be used to measure spatial learning and memory in the case of the evaluation of cognitive impairment in rats because of the aforementioned advantages.

The rats perform cognitive tasks that require spatial learning and memory—the ability to acquire a cognitive representation of location in space and the ability to effectively navigate the environment in the WM (Shukitt-Hale et al., 2004). Memory alterations appear to occur mostly in secondary memory systems and are reflected in the storage of newly acquired information (Bartus et al., 1989; Joseph, 1992). It is thought that hippocampus mediates allocentric spatial navigation (i.e., place learning) and prefrontal cortex is critical in acquiring the rules that govern performance in particular tasks (i.e., procedural knowledge), whereas the dorsomedial striatum mediates egocentric spatial orientation (i.e., response and cue learning) (McDonald and White, 1994; Oliviera et al., 1997).

The WM consists of a black circular pool (180 cm diameter × 80 cm high) filled to a depth of 25 cm with water at room temperature (Raveh et al., 2002). The pool is imaginarily divided into four equal compartments numbered 1–4 (clockwise). The black antireflective circular escape platform (15-cm diameter) is placed into compartment number 1 or 4, 20 cm off the pool wall. The platform is sunk 2 cm below the water surface so it is not visible to the rats because of the water mirror effect. The yellow rectangle ($30 \times 40\,cm^2$) is fixed on the pool wall that is closest to the platform as the spatial conditional cue (Robinson et al., 2004). Its place is variable according to the platform. Another dark rectangle is randomly fixed on the pool wall in different compartments (without platform) as the negative conditional cue. Around the pool there are several stable extramaze cues in the room that the rat could use to navigate the maze (Morris, 1984). However, the impact of extramaze cues is not significant because of high maze walls.

Performance on Passive Avoidance Test

Passive avoidance is a fear-motivated test classically used to assess short-term or long-term memory on small laboratory animals (rats, mice). Basically, passive avoidance working protocols involve timing of transitions, e.g., time that the animal takes to move from the white compartment to the black one after a conditioning session—during which the entry into the black compartment is punished with a mild inescapable electrical shock—is performed. The animal's position is detected by using high-sensitivity weight transducers providing more effective and reliable detection of animal responses (zones entries) than systems based on photocells beams or on grid-floor displacements. The measured parameter is a latency to enter into the black compartment. A two-compartment step-through passive avoidance apparatus is usually used. The apparatus ($430 \times 220 \times 190\,mm^3$) is divided into bright and dark compartments by a removable divider. The divider is a tunnel with an automatic door. Using a software-controlled magnetic switch, this door can be closed automatically. The gate has to be

opened manually at the start of the experiment. Each compartment is equipped with a light (2.2 W each). The ceiling also contains a common loudspeaker to deliver a sound signal (10 kHz fixed, maximum 100 dB). The animal's position inside the cage is monitored with the help of infrared light barriers. After a specified time interval after drug administration, passive avoidance training is performed. The animals are placed in the bright compartment and allowed to explore for 30 s, at which point the guillotine door was raised to allow the rat to enter the dark compartment. When the animal enters the dark compartment, the guillotine door is closed and an electrical foot shock (0.1–3.0 mA) is delivered. Training sessions are conducted twice (double trainings) during the light phase of the 12-h day/night cycle. The second double-training session is performed immediately after the first session. The animals are placed in the bright compartment and allowed to explore for 30 s, and then the guillotine door will raise. The latency to enter the dark compartment is recorded for up to 300 s (Gacar et al., 2011).

LONG-TERM BEHAVIORAL EFFECTS OF ACUTE HIGH-LEVEL EXPOSURE TO NERVE AGENTS

Many of the data regarding long-term neurological sequelae to exposures to cholinesterase inhibitors in humans have been gathered after accidental exposures to OP compounds (pesticides as well as nerve agents). Nevertheless, the extrapolation from these exposures to prediction of effects from nerve agent is difficult because:

- The cholinergic crisis caused by acute, severe intoxication with the OP pesticides is generally much longer than that caused by OP nerve agents.
- OP pesticides-induced delayed peripheral neuropathy can be caused by nerve agents only at doses many times greater than that of the LD_{50} (Davis et al., 1960).
- A delayed manifestation of OP poisoning has not been described after administration of nerve agents to animals or in instances of nerve agent poisoning in humans (Sidell, 1997).

There have been descriptions of the acute effects in humans that follow high-dose exposure ($\geq LD_{50}$) to nerve agents soman, sarin, and VX (Inoue, 1995; Nozaki et al., 1995; Nakajima et al., 1997). The similar cluster of behavioral symptoms (anxiety, psychomotor depression, intellectual impairment, and sleep disturbance) was observed in the immediate period after resolution of the acute signs of intoxication and then slowly faded with time, sometimes taking months to be fully resolved. The CNS symptoms noted after short-term exposure of humans to diisopropyl fluorophosphate (DFP) were excessive dreaming, insomnia, jitteriness and restlessness, increased tension, emotional lability, subjective tremulousness, nightmares, giddiness, drowsiness, and mental confusion. CNS symptoms were correlated with the depression of red blood cell AChE (EC 3.1.1.7) to 60–70% of original activity and they disappeared within 1–4 days (Grob et al., 1947). It was also noted that more severely exposed individuals and those with multiple exposures tended to display persistent symptoms that included forgetfulness, irritability, and confused thinking, although the duration of these persistent symptoms was never clearly defined (Holmes and Gaon, 1956). These CNS symptoms are virtually identical to those that have been reported to occur after high-level exposure to nerve agents. It was shown in the study of human sarin poisoning that sarin-induced behavioral effects were virtually identical to those reported for DFP. These effects coincided with the depression of plasma ChE and red blood cell AChE activity to approximately 50–60% of original activity (Grob and Harvey, 1958). The behavioral symptoms such as anxiety, psychomotor depression, general intellectual impairment consisting of difficulties in concentration and retention, and sleep impairment generally involving insomnia because of excessive dreaming were also described during human poisoning with nerve agent VX (Bowers et al., 1976).

The exposure to high doses of OP compounds including nerve agents has been demonstrated to result in severe brain neuropathology that involves not only neuronal degeneration and necrosis of various brain regions (Petras, 1981; McLeod et al., 1982; Lemercier et al., 1983) but also persistent severe alteration in behavior and cognitive incapacitation, especially impairments of learning and memory (McDonald et al., 1988; Bushnell et al., 1991). The most significant injury caused by OP poisoning is neuronal degeneration of the hippocampus that is associated with spatial learning and memory. Therefore, impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute OP poisoning (McDonald et al., 1988; Marrs, 1993). In addition, the adverse effects of OP compounds on cognition functions, such as learning and memory, may persist for quite some time after termination of toxicant exposure. The results from several studies have demonstrated the presence of OP compound-induced learning impairments several days after the classic signs of OP toxicity have subsided (McDonald et al., 1988; Buccafusco et al., 1990; Bushnell et al., 1991). Behavioral effects are typically evident before the occurrence of physical symptoms. These effects were associated with whole-blood ChE inhibitions of more than 60%. It was also found that severe poisoning with OP compounds causing general tonic–clonic convulsions altered Ca^{2+} dynamics that could underlie some of the long-term plasticity changes

associated with OP compound toxicity. These changes demonstrated in hippocampal neurons can be responsible for long-term behavioral toxicity of OP compounds (Deshpande et al., 2010).

Several studies of the long-term effects in victims of sarin exposure in Japan have been published. Exposure to nerve agents in humans was found to produce effects that include cognitive deficits and memory loss (Hatta et al., 1996; Hood, 2001; Okudera, 2002). Eighteen victims of the Tokyo subway incident were evaluated at 6–8 months after exposure (Yokoyama et al., 1998). Individuals exposed to sarin scored significantly lower than controls on a digit symbol substitution test and scored significantly higher than controls on a general health questionnaire (GHQ; psychiatric symptoms) and a profile of mood states (POMS; fatigue). The elevated scores on the GHQ and POMS were positively related to the increased posttraumatic stress disorder (PTSD) scores and were considered to be attributable to PTSD (Yokoyama et al., 1998). There have been two brief reports of individuals severely poisoned with nerve agents (one sarin and one VX) in Japan who experienced retrograde amnesia, possibly because of prolonged periods of seizures and/or hypoxia (Nozaki et al., 1995; Hatta et al., 1996). Symptoms related to sarin exposure in Japan still exist 1–3 years after the incident and include fatigue, asthenia, shoulder stiffness, and blurred vision (Abu-Qare and Abou-Donia, 2002).

The existence of long-term behavioral effects after acute exposure to high doses of nerve agents was verified many times with the help of laboratory experiments on animals. There are numerous studies using animals that show that survivors of high-level OP exposure can experience subtle but significant long-term neurological and neuropsychological outcomes that are detectable months or even years after recovery from acute poisoning (Brown and Kelley, 1998). Exposure of animals to nerve agents was shown to produce neurotoxicity in the CNS areas associated with cognition and memory functions (Koplovitz et al., 1992; Petras, 1994). There are a few studies that revealed changes in the brain after sublethal nerve agent exposure that involve not only the cholinergic system but also the glutamatergic system (Lallement et al., 1992; McDonough and Shih, 1997). Excitotoxic injury caused by increased levels of glutamate has repeatedly been shown to cause cognitive dysfunction (O'Dell et al., 2000). Therefore, the disruption of cognitive functions, especially spatial and working memory, seems to be the most frequent and the most observable behavioral effect of nerve agent poisoning. In addition, acute and repeated administration of OP compounds induced anxiogenic and depression-like responses. This fact was assessed on elevated plus-maze and forced-swim test, which are validated animal models for observing anxiety and depression-like behavior (Assissi et al., 2005). The studies show changes in the brain after sublethal nerve agent exposure that leads to memory and attention deficits that normally involve the hippocampus (Hatta et al., 1996; Nishikawi et al., 2001; Miyaki et al., 2005). The role of hippocampus in complex visuospatial learning and memory has been well established. The high concentration of N-methyl-D-aspartate (NMDA) and alpha-amino-3 hydroxy-5 methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, which play a key role in hippocampal-mediated learning and memory, also makes the hippocampus highly vulnerable to glutamate-induced excitotoxic injury from nerve agent poisoning (Shih et al., 1990; Lallement et al., 1992; Filliat et al., 2007).

After a high-dose exposure (more than 0.5 × LD_{50}), seizures are a prominent sign of nerve agent intoxication and these prolonged seizures can produce neural lesions (McDonough and Shih, 1997). Thus, neurological and behavioral deficits are predictable long-term effects after exposure to such doses of nerve agents. Animals exposed to high (convulsive) doses of nerve agent can develop spontaneous seizures and display hyperactive and aggressive behavior and profound deficits in learning and/or performance of a variety of behavioral tasks. Animal studies have demonstrated deficits in acquisition of several types of operant tasks, performance of serial probe recognition task, maze learning, and passive avoidance learning after acute poisoning with nerve agents (McDonough et al., 1986; Raffaele et al., 1987; Modrow and Jaax, 1989).

The inhalation exposure to high-level sarin induced in rats impaired memory processes seen at 1 month after exposure with no recovery of cognitive function during the 6-month follow-up period. In the open field, sarin-exposed rats showed a significant increase in overall activity with no habituation over days. In a working memory paradigm in the WM, the same rats showed impaired working and reference memory processes with no recovery. These data suggest long-lasting impairment of brain functions in surviving rats after a single exposure to sarin. Animals that seem to fully recover from the exposure, and even animals that initially show no toxicity signs, develop some adverse neurobehavioral changes with time (Grauer et al., 2008). These findings are in accord with reports of long-term behavioral impairment after exposure to OP pesticides used in agriculture (Wesseling et al., 2002). Similarly, long-term follow-up of victims of the sarin attacks in Japan demonstrated neurological as well as emotional and cognitive changes up to 7 years after exposure (Ohbu et al., 1997; Yokoyama et al., 1998; Miyaki et al., 2005).

Generally, according to high-dose exposure studies, animals exposed to nerve agents that exhibit seizures that are not promptly controlled develop brain damage and subsequent neurobehavioral problems. Animals

that do not develop seizures or those that are rapidly and effectively treated with drugs that stop the seizures suffer no brain lesion and display no long-term neurobehavioral deficits. Besides classic antidotes (atropine, oximes, benzodiazepines), other drugs such as centrally acting anticholinergic drug scopolamine and a new anticonvulsant drug imidazenil are effective to eliminate or at least reduce behavioral toxicity of nerve agents (Che et al., 2011; Wang et al., 2012).

CHRONIC BEHAVIORAL EFFECTS OF SINGLE OR REPEATED LOW-LEVEL EXPOSURE TO NERVE AGENTS

Anticholinesterase compounds such as nerve agents can alter behavioral functions even after small subtoxic doses. There are very few data regarding human exposures. Based on the data describing the signs and symptoms in accidentally exposed humans, some long-term health effects, including behavioral effects of repeated subclinical exposures to OP compounds, were observed (Wesseling et al., 2002). When the workers were exposed to small amounts of nerve agents they showed mild toxic signs of exposure, including CNS effects such as insomnia, excessive dreaming, restlessness, drowsiness, and weakness (Craig and Freeman, 1953). It was shown that psychological symptoms are probably more common than usually recognized and may persist in more subtle forms for much longer (days, weeks) than physical symptoms (Sidell and Hurst, 1997). Recently, a dose–response association was found between low-dose exposure to sarin and cyclosarin inhalation during the 1991 Gulf War and impaired neurobehavioral functioning as well as subtle CNS pathology, as revealed by MRI study (Proctor et al., 2006; Heaton et al., 2007). It is interesting that functional impairments were detected even in people who initially developed only mild or no signs of sarin or cyclosarin toxicity. These data correspond to the published epidemiological studies showing alterations in cognitive functions, impaired memory, and concentrations in humans after chronic low-dosage occupational exposure to OP insecticides (Stephens et al., 1995; Parrón et al., 1996). Increased reports of forgetfulness and difficulties in thinking, exposure-related increases in work-related tension, sleep disturbance, restlessness, and nervousness have been documented among sheep farmers exposed to OP pesticides (Stephens et al., 1995; Beach et al., 1996).

Based on the experimental animal data, the progression of signs, their neuropharmacological basis, and toxic consequence elicited from acute high-dose exposures have been well characterized (McDonough and Shih, 1993; Shih et al., 2003). However, much less is known about the long-term effects of repeated low-dose nerve agent exposure. Several comprehensive reviews of the long-term health effects of exposure to low-level nerve agent exposure have been published (Moore, 1998; Romano et al., 2001).

It is known that a significant, clinically manifested AChE inhibition in CNS leading to the neuronal degeneration of some brain regions, including the hippocampus, which is associated with spatial learning and memory, is not necessary for clinically manifested cognitive impairments. This fact corresponds with previously published data about neurological and neurophysiological outcomes detectable months or even years after recovery from acute OP poisoning (Savage et al., 1988; Yokoyama et al., 1998). It is very difficult to find the real reason for the memory impairments in the case of low-level nerve agent exposure. Recently, a temporal relationship has been demonstrated between OP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors in specific brain regions (including the hippocampus) after the asymptomatic exposure to OP compounds (Stone et al., 2000). Nerve agent–induced impairment of cognitive functions is probably caused by subsequent desensitization and internalization of cholinergic receptors as a reaction of nerve agent–exposed organisms on hyperstimulation of cholinergic receptors, especially in parts of the brain with a high density of cholinergic synapses such as the hippocampus (McDonald et al., 1988; Stone et al., 2000). This means that a decrease in the number of cholinergic receptors in the hippocampus after low-level exposure to OPs without significant AChE inhibition could cause memory impairments. It was also found that OP compound–induced cognitive deficits may be related to persistent functional changes in brain neurotrophin and cholinergic pathways (Terry et al., 2011, 2014). In addition, the influence of diet on the behavioral toxicity of nerve agents has been demonstrated (Langston and Myer, 2011). It was found that diet composition exacerbates or attenuates behavioral toxicity of nerve agents in exposed rodents. The exacerbated behavioral toxicity of nerve agents in the animals fed with glucose-enriched diet could be due to inactivation of esterases including AChE, BuChE, CaE, and PON by glycation or glycooxidation or by increased acetylcholine (ACh) synthesis and/or utilization. However, ketogenic diet attenuates behavioral toxicity of nerve agents. The mechanism responsible for this effect is unknown, but there is considerable evidence that the ketogenic diet has neuroprotectant and anticonvulsant properties by altered energy or altered neurotransmitter (gamma aminobutyric acid – GABA) synthesis (Bough and Rho, 2007). In addition, moderate benefit of choline supplementation against behavioral toxicity of nerve agents was observed (Langston and Myer, 2011).

In the available literature regarding repeated low-dose exposure to nerve agents, soman is the nerve agent

studied most often. Mice, rats, guinea pigs, and primates were used to investigate repeated low-dose soman exposure. The effects of repeated soman exposures ranged from performance decrements on a well-learned compensatory tracking task (Blick et al., 1994b) to development of attention deficits (Gause et al., 1985) and hyperreactive responses to handling (Shih et al., 1990). In addition, a single subcutaneous exposure of guinea pigs to sublethal doses of soman triggers long-lasting anxiogenesis and decreased locomotor activity (Mamczarz et al., 2010).

Unlike soman, the amount of literature regarding the effects of repeated low-level exposure to sarin is rather sparse and sometimes conflicting. Rhesus monkeys exposed to low levels of intramuscular sarin showed no signs of adverse health or long-term behavioral effects (Burchfiel et al., 1976). In contrast, it has been observed in rats and mice that intraperitoneal injections of subtoxic doses of sarin or soman decreased locomotor activity and altered behavior on the plus-maze and elevated horizontal bridge tests (Nieminen et al., 1990; Sirkka et al., 1990; Baille et al., 2001). Single as well as repeated low-level inhalation exposure of rats to sarin produced a deficit on RAM spatial memory task. The deficit was resolved during the first 3 weeks of acquisition (Genovese et al., 2009). It was also shown that repeated low-level sarin inhalation in rats at clinically asymptomatic doses was disruptive to neurophysiological function and caused long-term memory impairments (Kassa et al., 2001a,b). The results of the study related to the measurement of sarin-induced alteration of behavioral and neurophysiological functions at 3 months after low-level sarin inhalation exposure of rats showed a significant alteration of mobile activity and gait characterized by ataxia and an increase in stereotypical behavior. These signs were observed in rats repeatedly exposed to sarin at clinically asymptomatic doses or singly exposed to sarin at doses causing mild muscarinic signs of exposure. These animals had awkward hindlimbs and their mobility was markedly diminished (Kassa et al., 2001d). Spatial discrimination in the Y-maze was also altered in rats exposed to low levels of sarin. Although spatial orientation of rats singly exposed to clinically asymptomatic doses of sarin was significantly influenced for only a short time (1 or 2 h after exposure), the rats repeatedly exposed to clinically asymptomatic doses of sarin showed a decrease in Y-maze performance for a relatively long time (until the third week after exposure) (Kassa et al., 2004). The significant impairment of spatial memory of rats exposed to clinically asymptomatic concentrations of sarin was also observed when cognitive functions were evaluated with the help of T-maze performance. Rats exposed to low-level sarin showed a significant decrease in T-maze performance for a short time (until the first day after exposure). In addition, the effects of low-level sarin inhalation exposure were dose-dependent. When the rats were exposed to low-level sarin causing moderate signs of poisoning, their time of passage through the maze was longer at 1 and 2 h after the inhalation exposure compared with the rats exposed to clinically asymptomatic levels of sarin (Kassa et al., 2001c).

Single exposure to another nerve agent, cyclosarin, at concentrations that do not produce convulsions or severe clinical signs of toxicity can also produce performance deficits on learned behavioral tasks. However, with repeated exposure, the deficits are not persistent and recovery is complete. In addition, exposure concentrations not producing any evaluated clinical signs of toxicity, other than temporary miosis (in the case of inhalation exposure), do not produce performance deficits on the behavioral tasks (Genovese et al., 2006). Asymptomatic exposure of rats to VX vapors can produce only minor performance effects on previously learned behavioral tasks and on the acquisition of new behavioral tasks (Genovese et al., 2007).

Reports in the literature of animal studies show that nerve agents can be administered repeatedly with minimal overt neurobehavioral effects if care is taken in choosing the dose and the time between doses (Sterri et al., 1980, 1981). The repeated low-level nerve agent exposure made the cognitive impairments longer and higher compared with the single nerve agent exposure. The repeated exposure to low doses of soman can produce small, transient performance decrements only, probably because of the development of a physiological and behavioral tolerance to low levels of ChE activity (Blick et al., 1994a,b). Nevertheless, progressive and long-lasting inhibition of ChE in CNS after repeated administration of low doses of nerve agent soman was demonstrated (Hartgraves and Murphy, 1992). This study was corroborated by using nerve agent sarin (Olson et al., 2000). Generally, repeated or long-term exposure to low levels of nerve agents can cause neurophysiological and behavioral alterations (Abu-Quare and Abou-Donia, 2002).

The rats repeatedly exposed to sarin at doses corresponding to $0.5 \times LD_{50}$ (three times per week, subcutaneous) showed an increase in acoustic startle and a decrease in distance explored in the open field 2 weeks after sarin exposure. However, no effect of sarin exposure on passive avoidance was noted at the same time after sarin poisoning. Brain regional AChE was not affected at any time after sarin exposure, but muscarinic receptors were downregulated in the hippocampus, caudate putamen, and mesencephalon in the sarin group at 2 weeks after sarin exposure. Thus, downregulation of muscarinic receptors in the hippocampus as a reaction to ACh accumulation at muscarinic receptor sites based on AChE inhibition can be considered a cause of behavior performance deficits, especially disruption of cognitive functions (Scremin et al., 2003). In addition,

protracted impairment of cognitive functions in rats exposed repeatedly to low-level OP compounds may be associated with a decreased rate of AChE recovery in the hippocampus (Prendergast et al., 1997).

The results from several studies have demonstrated the presence of OP-induced learning impairments several days after the behavioral signs of OP toxicity have subsided (McDonald et al., 1988; Bushnell et al., 1991). Chronic exposure to OP compounds can also result in specific long-term cognitive deficits even when signs and symptoms of excessive cholinergic activity are not present (Prendergast et al., 1998). Thus, the significant, clinically manifested AChE inhibition in the CNS leading to the neuronal degeneration of some brain regions, including the hippocampus, is not necessary for the clinically manifested cognitive impairments. This conclusion corresponds with previously published data about neurological and neurophysiological outcomes detectable months or even years after recovery from acute OP poisoning (Savage et al., 1988; Yokoyama et al., 1998). A current study attempts to show a temporal relationship between OP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors in specific brain regions caused by asymptomatic exposure to an OP compound (Stone et al., 2000). In addition, low-level OP-induced memory impairment may be associated with a decreased AChE recovery in the hippocampus relative to the cortex. This decreased rate of enzyme recovery may contribute to hippocampal toxicity underlying protracted impairment of working memory and other cognitive functions (Prendergast et al., 1997).

Repeated or chronic low-level nerve agent exposure can cause a prolonged inhibition of extracellular AChE, leading to a prolonged increase in extracellular ACh. The prolonged availability of ACh in the synaptic clefts results in feedback inhibition on muscarinic, presynaptic receptors to decrease further ACh release (Russell et al., 1985). The greater ACh release in the nerve agent–exposed group may be due to the known downregulation of muscarinic receptors in response to chronic nerve agent exposure (Churchill et al., 1984). Neurochemical analyses showed that the normal brain neurotransmitter and receptor homeostasis is disrupted even at 10–12 days after 2 weeks of chronic nerve agent exposure, at least in the striatum, but probably throughout the whole cholinergic system in the brain (Shih et al., 2006).

CONCLUDING REMARKS AND FUTURE DIRECTION

Exposure to high doses of nerve agents has been demonstrated to result in severe brain neuropathology that involves not only neuronal degeneration and necrosis of various brain regions but also persistent severe alterations in behavior and cognitive functions, especially impairment of learning and memory. The most significant injury caused by nerve agent poisoning is neuronal degeneration of the hippocampus, which is associated with spatial learning and memory. Therefore, impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute nerve agent poisoning. In addition, the adverse effects of nerve agents on cognitive functions, such as learning and memory, may persist for a relatively long time after the termination of nerve agent exposure.

Behavioral alterations and impairments of cognitive functions were found after acute exposure to nerve agents with the absence of any classic signs of cholinergic toxicity. It was shown based on the experimental results that not only convulsive doses but also clinically asymptomatic doses of nerve agents can cause subtle long-term neurophysiological and neurobehavioral dysfunctions. The neurological and neurophysiological outcomes are detectable months or even years after the recovery from acute poisoning. It probably means that systems other than the cholinergic nervous system can be involved in nerve agent–induced long-term signs of alteration of neurological and neurophysiological functions. Thus, it is necessary to find new markers describing noncholinergic outcomes of low-level nerve agent exposure.

The long-term behavioral toxicity of nerve agents, especially the alteration of cognitive functions (T-maze, Y-maze, Morris maze test) due to nerve agent-induced delayed toxicity, seems to be connected with the neuropathological damage observed in the hippocampus. Thus, neuropathology of the hippocampus connected with the alteration of cognitive functions can occur after high-level as well as repeated or long-term low-level nerve agent exposure.

Neurochemical analysis of repeated or low-level nerve agent exposure provokes the suggestion that the prolonged nerve agent-induced alteration in brain chemistry may be a pharmacological basis for neurobehavioral changes. Thus, it is necessary to follow brain homeostasis during acute as well as chronic nerve agent exposure.

Repeated or long-term exposure to low levels of nerve agents can cause neurophysiological and behavioral alterations attributable to downregulation of muscarinic receptors in the hippocampus as a reaction to ACh accumulation at muscarinic receptor sites based on AChE inhibition. This phenomenon is considered the cause of behavior performance deficits, especially disruption of cognitive functions.

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CHAPTER

36

The Respiratory Toxicity of Chemical Warfare Agents

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INTRODUCTION

Chemical warfare agents (CWAs) play a significant role in the defense preparation plans of major military organizations worldwide. Human CWA exposure produces unique pulmonary and systemic health effects. The exposure/response relationships have been characterized to some extent, but many of the precise mechanisms are extremely complex and unknown. The physiology, biochemistry, and toxicology of CWA exposure has been studied for decades, but more recently, the interest in this topic has been rejuvenated due to increased threat for potential use of CWAs and development of countermeasures. The health studies involve acute and chronic exposure effects to high or low doses of various agents. The landscape for the administration of medical therapies has evolved from these studies. However, recent history has shown that some of the focus of chemical defense has shifted to the biological agents that have attracted much attention with the deadly anthrax attacks in the early part of this century. While many of these agents pose a different kind of threat with regard to their potential use, this chapter deals specifically with the more common CWAs, including traditional nerve agents such as sarin, soman, and tabun; vesicants such as sulfur mustard; riot control agents (RCAs); metabolic poisons such as cyanide; and choking agents such as phosgene and chlorine. Many of the agents discussed here, while not necessarily regarded as valid CWAs in current plans and scenarios, remain legitimate terrorist weapons.

In an attempt to elucidate the site-specific targets of toxicity and to define the physiological, biochemical, and toxicological mechanisms of an inhalation exposure, rodent models have been extensively utilized. This has been the case with practically all studies that investigate the adverse effects of an inhalation challenge. While these models are extremely useful as a first attempt to identify exposure/response effects, rodents do not accurately represent humans in terms of respiratory structure and function. Therefore, the extrapolation of rodent data to humans should be made carefully with regard to the toxicity of CWAs. The agents discussed herein manifest their toxicities based on the capability of the exposed animal to distribute, detoxify, metabolize, excrete, and override the deleterious effects of an agent challenge, regardless of route of exposure. However, when the toxic load overwhelms the system, potentially adverse outcomes are imminent.

HISTORY OF CWA USE

The use of CWAs on the battlefield gained significant recognition during and after World War I. An excellent and comprehensive history of the use of CWAs is presented by Hilmas et al. (2008) and Salem (2008). The historical use of CWAs stems from work with plant poisons by the ancient Egyptians and Indian civilizations nearly 5000 years ago. One of the earliest recorded uses of chemical warfare was that of Greek fire, as noted in the Ebers Papyrus 3500 years ago. Greek fire may have consisted of pitch, quicklime, and sulfur naptha. Arsenic-based and phosgene-like toxic smokes and "flaming concoctions" were used throughout history by the Greeks, Chinese, and Romans before the common era (BCE) to achieve victories in such settings as the Peloponnesian Wars (431–404 BCE) against tribal mountain rebels, and the Turks over 2000 years ago, respectively. During the fifteenth century, Leonardo da Vinci proposed the use of shells filled with copper acetate, sulfur, and arsenic as a weapon to be

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fired against enemy ships to disable the crew by directly affecting their lung. Similar toxic smokes were used during the Thirty Years War (1618–1648). The use of chlorine gas was suggested during the U.S. Civil War (1861–1865), but it was not actually manifested under battlefield conditions until World War I. By far, the most extensive use of CWAs on the battlefield occurred during World War I. Both Germany and the Allies used projectiles filled with chlorine, phosgene, diphosgene, and sulfur mustard. These were used singly or in combinations, such as chlorine used with phosgene. These agents were well suited for use, as they generally tended to be heavier than air, and as such, caused extensive mortality and morbidity. In the mid-1930s, during the Italian-Ethiopian War, sulfur mustard (and possibly chloropicrin (PS) and phosgene) were used. In the intervening years between World War I (1914–1918) and World War II (1939–1945), significant effort was put into developing therapies and physical barrier protection, such as masks and suits, for the possibility of the chemical warfare attack by warring nations in future conflicts. Although CWAs were stockpiled during World War II, none were used. The development of more deadly chemical agents—namely those of the organophosphorus nerve agents—occurred during this period.

In the 1960s, during the Vietnam War, the United States used defoliating agents such as Agent Orange to uncloak the enemy. While the planned use of sulfur mustard was not applied on the battlefield, the United States implemented the use of nonlethal RCAs. In the Yemen Civil War, the Egyptians used mustard gas, phosgene, nerve agents, and RCAs on a regular basis from 1963 to 1967. In the mid-1980s, sulfur mustard and nerve agents were used by the Iraqis on Iranian troops, causing massive casualties and deaths. Over 35 years later, the survivors of these gas attacks continue to have exposure-related health issues. More recently, in 2002, the Russians used the incapacitating agent fentanyl to subdue Chechnyan terrorists holding Russian civilians hostage in a movie theater. This attempt to defuse the situation resulted in over 118 deaths due to asphyxiation. In 2007, terrorists exploded homemade chlorine canisters, causing numerous casualties in Iraq. Even more recently, in 2013, there was the alleged use of the nerve gas sarin by the Syrian government in the Syrian Civil War that presumably killed hundreds of civilians. Virtually all these agents, regardless of their route of exposure, can injure the respiratory system.

THE RESPIRATORY SYSTEM

The structure of the respiratory system is complex, so the effects produced by inhaled CWAs can vary depending on the chemistry and deposition efficiencies of these substances. The respiratory system is comprised of the airway compartment, to transport air, and the alveolar compartment, to perform the gas-exchange function. The airway begins with the nose, which through the pharynx carries air to the trachea, divides into two main bronchi and then several small bronchioles within each lung. Epithelial cells of different types line the airways and perform cell-specific functions. The pseudostratified, columnar ciliated cells predominate in the airways, and while guiding the airflow, function to remove particles encountered through inhaled air and protect the lung from injury. Dome-shaped, secretory cells within small airways (known as *Clara cells*) secrete uteroglobin, possess drug-metabolizing enzymes, and function as stem cells to replenish lost ciliated epithelial cells. In addition, goblet cells produce and secrete mucus at the apical surface of the airway epithelium. The mucous layer helps remove particulates and pathogens via mucociliary clearance and modulates the innate immune response. The thickness of the mucous layer is proportional to the diameter of the airway and the density of goblet cells in a given area. At the basal sector of the airway epithelial cells is the basement membrane, which supports the epithelial cells and interstitial space and allows communication to the smooth muscle layer. Dendritic cells that project between airway epithelial cells function as antigen-presenting cells which, upon recognition of antigens (particulate, microbial, or soluble substances), produce innate and humoral responses (Cook and Bottomly, 2007). Sensory, vagal C-fibers innervate the airways at the airway epithelium and evoke a classical reflex reaction when stimulated, leading to bronchoconstriction and coughing, which in turn enhances parasympathetic tone, causing bradycardia and hypotension (Pisi et al., 2009). The airway surface layer is the first to encounter inhaled soluble and insoluble respirable toxicants that pass through the nose after inhalation.

The terminal airways, called *respiratory bronchioles* in humans, lead into small alveolar sacs, which are lined with type 1 and type 2 alveolar epithelial cells. Thin type 1 epithelial cells cover the alveolar surface, while cuboidal type 2 cells function to secrete proteins and surfactant material that is stored in lamellar bodies. The pulmonary arteries, which bring blood from the heart to the lung for oxygenation, form a capillary network surrounding the alveolar sacs. Type 1 cells are close to the capillary walls, allowing the diffusion of carbon dioxide from the blood to the air and oxygen from the air to the blood to occur (Galambos and Demello, 2008; Herzog et al., 2008). Myofibroblasts are found within the interstitial tissue supporting the alveolar compartments and capillary network and are involved in various functions, including synthesis of collagen, elastin, and other extracellular matrix proteins. The alveolar sacs, interstitial matrix, and capillary network are encapsulated by the

pleural mesothelial layer, which provides anatomical structure to each lung.

The surfactant material produced within type II cells is composed of about 80% phospholipids and about 20% neutral lipids and proteins. Once secreted, it layers thinly over the entire alveolar epithelial surface and provides stability to the alveoli, prevents collapse, and preserves patency. In addition to maintaining surface tension, surfactants play an important role in host defense. Of the four surfactant proteins (SPs), SP-A, SP-B, SP-C, and SP-D, SP-A and SP-D play a role in host defense, whereas hydrophobic SP-B and SP-C are involved in the adsorption and spreading of the surfactant material along the alveolar lining (Griese, 1999; Enhorning, 2008). Alveolar macrophages within the alveoli protect the lung from inhaled pathogens by engulfing bacteria, particles, damaged surfactants, and dead neutrophils or other cells after an acute lung injury. In addition, macrophages perform important innate immune functions by expressing cytokines that are involved in mounting an inflammatory response, as well as in the resolution of inflammation. A variety of warfare agents and pathogens can produce acute lung injury and alveolar edema and lead to respiratory collapse when exposures are encountered at high levels.

PULMONARY AGENTS

Practically all CWAs can be classified as airway and lung toxicants. When inhaled, CWAs can penetrate to various levels of the respiratory system, from the nasal passages to the lung periphery, and cause toxicities specific to lung cells. More importantly, the development of many CWAs were specifically designed to cause significant mortality and morbidity due to compromised respiratory function. These include phosgene, chlorine, diphosgene, and chloropicrin, all of which were used either singly or in combination during World War I and other conflicts worldwide. While, these compounds may be found in old agent stockpiles, both phosgene and chlorine pose industrial and occupational hazards because they are also employed in many industrial chemical manufacturing processes. They are also toxic combustion by-products. Their heavy industrial usage classifies them as toxic industrial compounds (TICs), with the significant potential to affect large residential or rural areas if exploited. In this chapter, we describe the respiratory toxicity of the known warfare agents for which inhalation is the primary route of exposure.

Arsine

Arsine (also known as *arsenic trihydride*, *arsenous hydride*, and *hydrogen arsenide*) has been described as the most toxic form of arsenic. Its chemical formula is AsH_3, and it has a molecular weight of 77.95. It is 2.5 times as dense as air, which makes it highly suitable for a "state-of-the-art" trench-type warfare agent. Arsine most likely would now be classified as a low toxicity and high persistence CWA, at a level equivalent to chlorine (Table 36.1). However, due to its low toxicity (10 times less toxic than phosgene), its battlefield usage was halted during World War I. It is colorless and can have the mild odor of garlic. AsH_3 is soluble in chloroform and benzene, but only slightly soluble in water. Currently, arsine gas is used in the microchip industry to plate semiconductors with arsenic. Other workplace activities where arsine is used are for galvanizing, soldering, etching, and lead plating. Arsine is a strong reducing agent and can be formed through the following arsenide, Zn_3As_2, and acid hydrolysis reaction (Eq. (36.1)):

TABLE 36.1 Classification of CWAs and Their Military Symbols Based on Environmental Persistence and Lethality

Potency	High Persistence	Low Persistence
Low lethality	Sulfur mustard (HD), lewisite (L), mustard-lewisite (HL), nitrogen mustard (HN1–3), cyanogen chloride (CK), diphenylchloroarsine (DA), arsine (AsH_3), chlorine (Cl_2)	*o*-Chlorobenzylmalonitrile (CS), dibenz(*b*,*f*)-1,4-oxazepine (CR), chloracetophenone (CN), chloropicrin (PS)
High lethality	Soman (GB), cyclosarin (GF), VX	Phosgene (CG), sarin (GB), tabun (GA), hydrogen cyanide (AC)

$$Zn_3As_2 + 6H^+ \rightarrow 2AsH_3 + 3Zn^{2+} \qquad (36.1)$$

Exposure Physiology

The single most important route of exposure to arsine is through inhalation. Generally, no discomfort occurs from exposure to arsine, as it is a nonirritating compound. The extent and nature of the symptoms depend largely upon the concentration and duration of exposure. Romeo et al. (1997) report that exposure levels of 10–32 mg/m^3 for up to several hours may produce symptoms consistent with arsine toxicity (described next). An earlier study (Morse and Setterlind, 1950) indicated that exposures ranging from 23 to 970 mg/m^3 may be linked to lethal results in people. Many symptoms are latent in nature. Within 1–24 h after inhalation at a high concentration, persons become ill and may experience hematuria (the voiding of dark, bloody urine). Massive hematuria may lead to anuria, which can become fatal. Within 24 h, symptoms may also include headache, vomiting, muscle weakness, dyspnea, nausea, confusion, wheezing, and jaundice. Fowler and Weissberg (1974) have shown that arsine

causes tachycardia, tachypnea, hepatic enlargement, and abdominal rigidity and tenderness. In severe cases, acute respiratory distress syndrome (ARDS) could become evident along with hyperthermia, hypotension, and paresthesias in the extremities. According to the Centers for Disease Control and Prevention (CDC), high levels of inhaled arsine can also produce convulsions, loss of consciousness, paralysis, and respiratory failure, ultimately leading to death. Pulmonary edema and circulatory collapse may also result in a fatal outcome. However, at low concentrations of 10 ppm for 6 h/day for 4 days, arsine exposure to pregnant mice and rats resulted in no developmental effects (Morrissey et al., 1990). The chronic aftereffects of arsine poisoning include renal injury, polyneuritis, memory loss, and agitation. The most significant finding of arsine toxicity is the fulminant lysis of erythrocytes. It has been reported that arsine at estimated concentrations of 750–1,500 ppm × min will cause extensive hemolysis (Caravati, 2004). The LCt_{50} for arsine gas is estimated to be 5,000 mg × min/m^3 (Seto, 2011).

Exposure Biochemistry

The effect of arsine on the blood has been studied in several animal inhalation exposure models. Blair et al. (1990a,b) exposed male and female mice to arsine at concentrations totaling 9–900 ppm administered for 6 h/day for 5 days over 13 weeks. Pathophysiological results at the highest total dose showed significant decreases in hematocrit (HCT) and hemoglobin (HGB) with increases in mean corpuscular hemoglobin at 5 days post-exposure. Between 15 and 90 days, a regenerative process appeared to have been activated by elevated reticulocytes and mean corpuscular volume. At 90 days, denatured proteins such as Heinz bodies were present. Also at 90 days, the formation of methemoglobin suggests that the oxidation of ferrous heme iron (Fe^{2+}) to ferric heme iron (Fe^{3+}) had occurred. When red blood cells (RBCs) were exposed to arsine gas *in vitro*, reduced glutathione (GSH) levels were diminished by 60% after a 4-h exposure (Blair et al., 1990a,b). The authors concluded that an enhanced oxidative environment may be the source of the oxidation of membrane-bound sulfhydryl groups on the HGB molecule. This conclusion was corroborated to some extent by the addition of the sulfhydryl inhibitor N-ethylmaleimide, 2 h after arsine exposure, which resulted in less hemolysis (Rael et al., 2000). Ionic gradient hemostasis is also affected by arsine exposure in RBCs. Rael et al. (2000) have shown that intracellular K^+ and Mg^{2+} decreased, whereas Na^+, Cl^-, and Ca^{2+} increased. Based on these data, the increase in Ca^{2+} influx may be responsible for arsine-induced hemolysis of RBCs. However, the role of oxidized GSH and an amplified oxidizing environment has been questioned by some investigators as the primary reason for arsine-induced hemolysis (Hatlelid et al., 1995; Winski et al., 1997). Pulmonary toxicity of arsine has not been reported, although inhalation exposures have been used in many studies, except for one where intraperitoneal (i.p.) injections of dimethylarsine and trimethylarsine have resulted in lung tumor formation (Yamanaka et al., 2009).

Exposure Histopathology

A search of literature did not reveal any lung pathological reports for arsine gas toxicity. As stated earlier, the most prominent event following an inhalation challenge is erythrocyte hemolysis.

Chlorine

Similar to phosgene, chlorine is considered a "choking" agent. Under ambient conditions, chlorine, also known as *dichlorine* (molecular weight/MW = 71), is a pungent, noncombustible, yellow-green gas. Chlorine is heavier than air, which enables it to settle in low-lying areas, making it a possible persistent agent, and yet it does not have the toxicity of phosgene (Table 36.1). It is slightly soluble in water in which it can form hydrochloric and hypochlorous acids (HOCl). Chlorine can also bind with alkenes, alcohols, and ammonia. Due to high electronegativity, chlorine can react with numerous biomolecules present in cells and tissues. Reactions with Cl_2 can occur by either radical or ionic processes that form one or two chlorine atom bioproducts. As a nonmetal halogen, Cl_2 is not formed in nature due to its high reactivity. After fluorine and oxygen, it is the third most electronegative element. It was one of the first widely used CWAs, having been employed early in World War I. Presently, the majority of exposures to chlorine occur through accidental occurrences such as train derailments, industrial process failures, and recently, the intentional use of these substances by terrorists in Iraq in 2007 (Weill, et al., 1969; Hilmas, et al., 2008; Van Sickel et al., 2009).

Exposure Physiology

The principal route of exposure to chlorine is through respiration. Chlorine is classified as a pulmonary irritant that can affect both the central and peripheral airway compartments. Unlike phosgene, chlorine does not obey Haber's rule; i.e., that a constant concentration × time ([*C*] × *t*) product leads to a consistent physiological/toxicological outcome (Hoyle, et al., 2010). Acute lung injury is not necessarily caused by chlorine itself, but by the aqueous reaction products formed in the mucus membrane of the airways. These include chlorine dioxide, chloramine, and HOCl. General sequelae include, but are not limited to, lacrimation, rhinorrhea, conjunctival irritation, cough, sore throat, laryngeal edema, dyspnea, stridor, pulmonary edema, and ARDS, all of which can lead to respiratory collapse. Furthermore, inhalation

leads to abrupt airway bronchoconstriction, increased airway resistance along with decreased compliance, epithelial cell necrosis, and microvascular permeability. Chlorine exposure has also been linked to irritant-induced asthma, or what is commonly known as *reactive airways dysfunction syndrome (RADS)*. RADS is characterized by airway hyperreactivity, fibrosis, and airway obstruction. Thus, the responses to chlorine are generally nonspecific, as they are with most irritant compounds. The LCt_{50}, the amount of chlorine inhalation needed to cause 50% mortality in exposed animals or humans, is estimated to be 19,000 mg × min/m^3 (Seto, 2011).

To understand the physiological and mechanistic responses to chlorine inhalation, exposure-response studies have been conducted in rodents. Acute respiratory responses following exposure of female mice to chlorine were examined by Morris et al. (2005). In this study, mice were exposed for 15 min at total chlorine concentrations ranging from 12 to 57 ppm × min. It is not surprising that in obligate nose-breathers, the results indicate that chlorine was scrubbed with excellent efficiency in the upper respiratory tract (URT) at 97%. Resistance in the URT beginning at 15–17 days after exposure was also significantly higher with chlorine than with deep lung irritants. The RD_{50} (the 50% reduction in respiration rate as measured by frequency), at 57 ppm/min of chlorine was significantly increased from baseline to twofold during the last 6 min of 15 min total exposure. Mo et al. (2013) reported an impairment of lung antimicrobial activity in male mice exposed to a total concentration of 14,400 ppm × min chlorine and then challenged with *Aspergillus fumigatus* 24 h post-exposure. In these mice, significant increases in the recruitment of lymphocytes, monocytes, and neutrophils were measured compared to chlorine exposure alone. The authors show that increased neutrophils and concentrations of reactive oxygen species (ROS) may be responsible for the inability of exposed mice to mount an immune challenge to *A. fumigatus*.

Exposure Biochemistry

Chlorine inhalational damage is not restricted to particular cell types, such as epithelial cells. Injury caused by inhaled chlorine can be complex and involves multiple pathways (Figure 36.1). The loss of vascular tone following chlorine exposure has been linked to dysfunctional nitric oxide (NO)–dependent mechanisms and resulting vasodilation (Honavar et al., 2011). To address the role of NO, Honavar et al. (2014) found that when rats were exposed to a total chlorine concentration of 12,000 ppm × min, isolated pulmonary artery studies showed disruption of vascular tone due to disrupted NO signaling. The balance between endothelial nitric oxide synthase (eNOS)- and inducible nitric oxide synthase (iNOS)-derived NO was disrupted by chlorine. The expression and activation of eNOS and iNOS through the interaction between p38 MAPK- and PI3/AKT-dependent pathways may also have an effect on endothelial permeability in lung injury. However, the precise mechanism by which eNOS and iNOS might be linked to functional impairment of pulmonary vascular tone, and bronchoconstriction is not well understood.

Exposure to chlorine in mice induces inflammatory pathways, resulting in the recruitment of neutrophils and the production of cytokines such as GROα/CINC/KC, IL-6, and TNFα (Tian et al., 2008; Song et al., 2011). Li, et al. (2013) have determined that exposure of mice to chlorine gas, 400 ppm × 30 min upregulates unfolded protein response (UPR) in the lung for up to 6 h post-exposure. UPR elements are regulated by a defensin-like peptide through the disruption of iron homeostasis. The authors speculate that inflammatory mediators such as TNFα, IL-6, and hepcidin might also be involved. The link between UPR elements and inflammation has been demonstrated in other studies (Xue et al., 2005). Chlorine exposure also affects regulatory Na^+ channels in epithelial lung cells (Lazrak, et al., 2012). Epithelial Na^+ channels (ENaC), which are present on the apical surface, were inhibited in mouse lung slices and Type II epithelial cells after exposure to 400 ppm × 30 min of Cl_2. The data suggests that Cl_2-activated ERK1/2 expression (extracellular signal-related kinase) in Type II cells *in vitro* and *in vivo* might be involved. The data suggests that Cl_2 exposure results in compromised Na^+ regulatory activity, partly responsible in fluid clearance mechanisms. Post-exposure treatment with reactive species scavengers ameliorated the problem (Xue et al., 2005). The common thread among these studies is that chlorine-induced inflammation produces reactive intermediates and induces cytokine release, which can ramp up destructive pathophysiological responses. Chloramine, a reactive by-product of chlorine metabolism, is also capable of initiating reactive processes. The degree of chlorine-induced injury and lung inflammation has been shown to differ between mouse strains (Tian et al., 2008).

Exposure Histopathology

In humans who have died from severe chlorine exposure, postmortem results have shown the presence of massive pulmonary edema, ulcerative bronchiolitis, and cardiotoxicity (White and Martin, 2010). In mice exposed to chlorine at 400 ppm × 15 min, profound changes in lung pathology were observed (Hoyle et al., 2010). This study looked at lung damage through a range of concentrations over various points in time that produced a constant concentration × time ([C] × *t*) product. There was significant damage to the airway epithelium. At 6 h post-exposure, the injury was more widespread, encompassing large airways and causing denudation. At 24 h post-exposure, sloughed epithelial tissue fragments were present in the airway lumen. There was

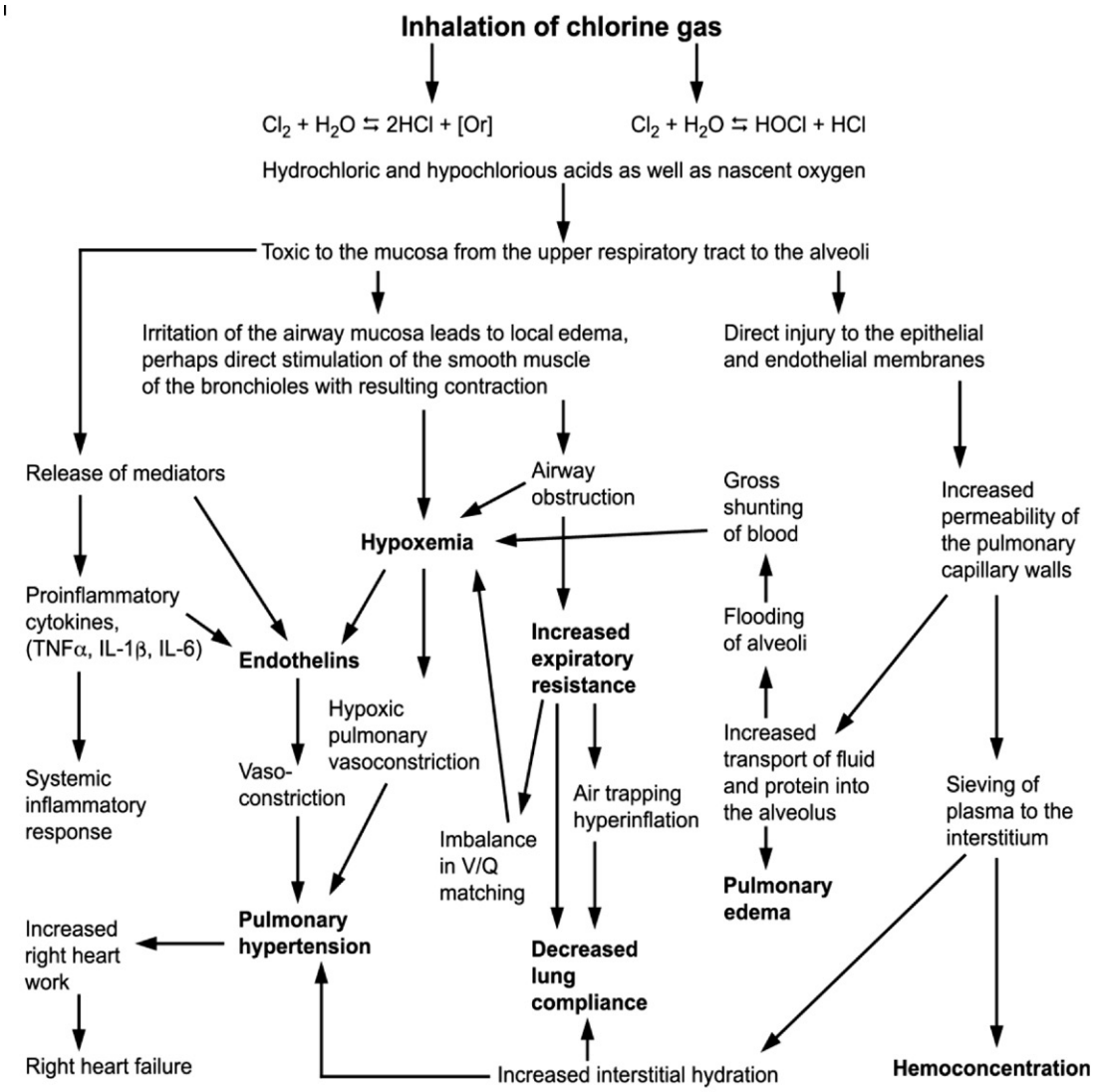


FIGURE 36.1 General mechanistic pathways involved in the toxicity of inhaled chlorine following exposure. IL: interleukin; TNF: tumor necrosis factor; V/Q: ventilation profusion ratio. *Source: Reprinted from* Textbooks of Military Medicine: Medical Aspects of Chemical Warfare, *Published in 2008 by the Office of the Surgeon General at TMM Publications.*

also evidence of neutrophilic inflammation that is consistent with inhaled chlorine toxicity. The magnitude of the histopathological response was not necessarily [*C*] × *t*-dependent. These data corroborate a comparable study by Tian et al. (2008) where similar histological responses were observed. However, at a later time point after exposure (48 h) repair processes are likely activated. Thus, exposure to high concentration of chlorine can produce profound lung injury and pathology.

Phosgene

Phosgene (also known as *carbonyl dichloride, carbon dichloride oxide, carbon oxychloride, chloroformyl chloride, dichloroformaldehyde*, and *dichloromethanone*), whose chemical formula of $COCl_2$ (MW = 98.9) was used as a CWA during World War I. In addition to its weapons applications, it has a variety of industrial uses, such as in the synthesis of pharmaceuticals and organic materials (Wyatt and Allister, 1995). Because of its potential for toxicity, phosgene is often produced and used captively in the same chemical facility to avoid potential hazard. Although it is less toxic than nerve gases such as sarin and tabun, it is still regarded as a viable CWA because it is so easy to produce. Phosgene is generally produced in industry by the interaction of carbon monoxide and chlorine gas, using activated carbon as a catalyst. It can also be produced from chloroform in the presence of oxygen and ultraviolet (UV) light. When phosgene reacts with water, it decomposes into hydrogen chloride

(HCl) and carbon dioxide. Accidental exposures to phosgene in humans at high concentrations cause pulmonary edema, sensory irritation, and associated rapid breathing (Diller, 1985a). Patients complain of dyspnea upon exertion and reduced ability to exercise for several months to years after an accidental exposure. Normalization of lung function can take several years (Diller, 1985b).

Exposure Physiology

Phosgene can be widely and easily dispersed by the wind. Being heavier than air, it tends to sink into trenches and ditches making it fairly nonpersistent on the ground (Table 36.1). Phosgene is deposited in deeper regions of the lung, as opposed to nitrogen oxide, sulfur oxide, and chlorine, which are more water-soluble and are deposited primarily in the upper airways. Inhaled phosgene causes chest pain, burning throat, and persistent cough. It affects numerous metabolic pathways critical to cell and tissue survival. The average LCt_{50} of phosgene in acutely exposed rats has been reported to be 1,741 $mg/m^3 \times$ min (Pauluhn, 2006a). The respiratory toxicity of phosgene is well studied in laboratory rodents, and large animal species such as dogs and sheep. There is a steep acute [*C*] × time mortality relationship for phosgene gas in rats (Pauluhn et al., 2007).

Exposure to phosgene was associated with early bronchoconstriction, an obstructive injury pattern, and disruption of mechanical rhythm of breathing, which were largely attributed to the progressive production of pulmonary edema in mice (Sciuto et al., 2003). Increased expiratory resistance and decreased dynamic compliance were noted in rats exposed to phosgene (Ghio et al., 2005). In pigs exposed to a high concentration of phosgene, a transient decrease in oxygen saturation and cardiac stroke volume index was observed during the exposure period, while significant decreases in arterial pH, P_aO_2, and lung compliance were noted 6 h after exposure (Brown et al., 2002). Thus, the acute physiological effects are likely mediated by sensory irritation and lung edema.

Exposure Biochemistry

Once inhaled, phosgene penetrates deep into the alveolar region, where it transits through the airway surface and is hydrolyzed to CO_2 and HCl; however, it has been shown that the concentration of HCl is not sufficient enough to produce the damage that is typically seen after an acute phosgene exposure (Pauluhn et al., 2007). In contrast to soluble gases, the less-soluble phosgene gas penetrates the lower respiratory tract without marked retention in the conducting airways. It has been hypothesized that phosgene-induced acylation of nucleophilic amino, hydroxyl, and sulfhydryl moieties of the fluid components of the alveolar lining, rather than HCl itself, results in rapid alveolar injury and inflammation (Pauluhn et al., 2007). Through this acylation reaction, phosgene instantaneously interacts with antioxidants such as GSH, resulting in its depletion (Sciuto et al., 2003; Sciuto et al., 2005). It has been proposed that surfactant destabilization and generation of reactive oxidation by-products results in rapid intra-alveolar pulmonary edema. The surfactant abnormalities have been presumed to initiate events leading to acute respiratory failure (Mautone et al., 1985).

Many experimental studies have investigated transient surfactant abnormalities soon after phosgene exposure (Jugg et al., 1999). The surfactant, a mixture of lipids and proteins, performs an important function of maintaining surface tension between air liquid interphase within the alveoli and prevents alveolar collapse. It also regulates innate immune response and host defense (Wright, 2003). Proteins leaked from the vasculature into the alveoli due to phosgene-induced damage to the surfactant and alveolar cells can further inactivate surfactant material. Upon exposure to phosgene, increases in the bronchoalveolar lavage fluid (BALF) levels of proteins are attained on day 1, while lipids increase on day 3, suggesting that these proteins are the likely cause of surfactant destabilization, leading to further vascular leakage, inducing apoptosis, and inflammatory signaling cascade via an innate immune response (Pauluhn et al., 2007). Evidence from studies using large animal models such as dogs and pigs suggests that phosgene inhalation causes a high-permeability type of lung edema brought on by high surface tension and the compensatory interstitial perimicrovascular hydrostatic pressure, resulting in alveolar flooding (Pauluhn, 2006a,b). It has been postulated that the latency between phosgene entry into the lung and edema are due to the time required for increased fluid distribution between interstitial, lymphatic, and perimicrovascular compartments within the alveoli. The alveolar epithelial and interstitial cells (but not endothelial cells) are the primary targets of phosgene-induced injury, further supporting the hypothesis that pulmonary edema results from a pressure gradient across the air liquid interphase (reviewed in Pauluhn et al., 2007).

Increased pulmonary edema induces a sequence of events that results in the release of inflammatory cytokines, apoptosis, extravasation of inflammatory cells, and inflammation that is linked to extracellular matrix remodeling and fibrosis observed in a number of studies, specifically those involving low-level, longer duration exposures. The degree of inflammation and subsequent fibrosis could be directly related to the concentration of phosgene, the longevity of exposure, and the type of laboratory animal model being exposed (Kodavanti et al., 1997; Pauluhn et al., 2007). The removal of pulmonary liquid and inflammatory cells may facilitate quick reestablishment of the homeostasis. A number of

processes, such as mucociliary clearance, fluid clearance through pulmonary circulation, and inflammatory cell signaling, are initiated within alveolar and lower airway structures. Although acute pulmonary toxicity of phosgene is fairly well established, there are no effective antidotes other than supportive management of symptoms. A number of different therapeutic approaches have been tested experimentally (Sciuto and Hurt, 2004).

Exposure Histology

Histopathological lesions have been reported after an acute exposure to phosgene, which are characterized by alveolar and interstitial edema, hemorrhage, fibrin deposition, alveolar and interstitial flooding, and inflammatory cell infiltration. Focal bronchiolar and terminal airway degeneration and necrosis have also been reported. Resolution of inflammation and edema have been noted in a number of animal studies (Gross et al., 1965; Hatch et al., 2001; Duniho et al., 2002). At relatively low concentrations and longer exposures, phosgene can induce alveolar inflammation and fibrosis in a concentration-dependent manner (Kodavanti et al., 1997). The histologic changes in the bronchioalveolar regions in rats exposed to phosgene at 0.1 ppm for 4 weeks were characterized by a small but apparent thickening and mild inflammation, which were progressive with increased concentrations. Masson's trichrome staining indicated increased collagen deposition at the terminal bronchiolar sites and increased pulmonary hydroxyproline, a measure of collagen deposition, at high concentration of 1 ppm. These lesions were also seen in animals exposed for 12 weeks and persisted after a 4-week nonexposure recovery period. Histological changes, including collagen deposition after long-term phosgene exposure, have also been reported by Pauluhn (2006a). Histopathology in pigs exposed to high concentrations of phosgene revealed areas of widespread pulmonary edema, petechial hemorrhage, and bronchial epithelial necrosis (Brown et al., 2002). Thus, phosgene-induced injury is likely to cause pulmonary fibrosis and scarring of the peripheral lung tissue, which might affect breathing.

Nerve Agents

The nerve agents consist of a family of compounds whose role in chemical warfare was to cause significant morbidity and mortality among soldiers. As such, the most likely application of nerve agents was initially for military purposes. However, as recently as 2013, nerve agents were allegedly used against civilians in conflicts in the Middle East (Sellstrom et al., 2013). The syntheses and development of nerve agents took place in Germany between World War I and World War II. Nerve agents are highly toxic and are classified as organophosphates (OPs). OPs are esters of phosphoric acid and are the most toxic of the known chemical CWAs. Members of this family also include parathion, malathion, and mipafox, which are commonly used worldwide in agriculture as well.

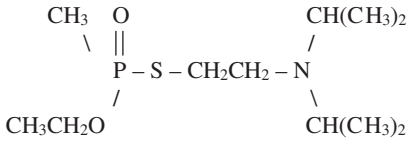
The principal chemical varieties of chemical warfare nerve agents are shown in Figure 36.2. Nerve agents are generally odorless and colorless. See Table 36.2 for physical chemical characteristics. Sarin (GB), tabun (GA), cyclosarin (GF), and soman (GD) are known as the *G agents*, where *G* denotes "Germany." These agents volatilize fairly rapidly. Whereas the *V agents* (where *V* denotes "venomous"), such as *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX) and VR, are classified as nonvapor hazards. VR (also known as *Russian VX*) is a close stereoisomer of VX. Classifying V agents as nonvapor threat agents may not be entirely appropriate, as these agents can penetrate the airways if they adhere to dusts, mists, and fog particles, thereby causing significant effects. As a class of threat agents, they are meant to be used as offensive weapons and can be delivered via rockets, bombshells, mortar rounds, and other devices. Usually dispersion is in the forms of sprays, aerosols, and vapor, and possibly the combination of aerosol and vapor. The toxicity response produced by a mixture of OPs can be multifaceted with respect to how these agents are absorbed and deposited in the airway. Punte et al. (1958) makes the statement that OPs in the aerosol form may be more toxic than an equivalent amount delivered by a gas exposure. Owing to their rapid uptake, nerve agents can be absorbed by inhalation, dermal, ocular, and oral routes of exposure. The neuronal effects of nerve agent intoxication include physiological, toxicological, and biochemical responses, which are well studied, but the pulmonary effects are less well examined. These effects are common to all the agents with structural similarities, as shown in Figure 36.2.

Regardless of the route of exposure, nerve agents cause a variety of physiological and toxicological effects, as discussed next. Many of these occur within minutes of exposure, especially with the more volatile G agents. Studies involving inhaled nerve agents have been conducted for many years with a range of animal models. Many factors affect the toxicity response—for example, whole-body exposure versus head-out or nose-only, animal species, agent type, time of day of exposure, duration of exposure, aerosol versus vapor (as these enter the airspaces at different rates and in different locations), dilution vehicle used, agent concentrations and particle size (in the case of an aerosol), and most important, the target tissue dose. Pulmonary toxicities of different classes of nerve agents are described next.

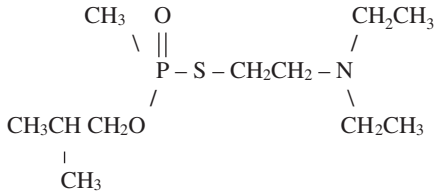
Volatile Agents

G agents include GA, also known as *ethyl dimethylamidocyanophosphate, EA1205* (tabun); GB, known as *isopropyl methylphosphonofluoridate, trilone, MFI, TL1 618, T144*, and

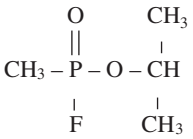
A. VX (*O*-ethyl *S*-(2-(diisopropylamino) ethyl) methlyphosphonothioate)



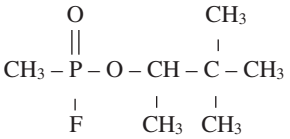
B. VR (O-isobutyl S-[2-(diethylamino)ethyl] methylphosphonothioate)



C. Sarin ([GB], isopropyl methylphosphonofluoridate)



D. Soman ([GD], pinacolyl methyl phosphonofluoridate)



E. Tabun ([GA], ethyl *N*-dimethyl phosphoroamidocyanidate)

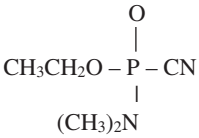


FIGURE 36.2 Chemical structures of nerve agents.

T2106 (sarin); and GD, known as *pinacolyl methylphosphonofluoridate* (soman). They are among the most toxic CWAs. Listed in descending order of ease of volatilization, it is GB, GD, and GA. GA is colorless and may have a fruity odor, which can change to bitter almonds (similar to cyanide) upon decomposition. GA is soluble in organic solvents such as ethanol, diethyl ether, and chloroform. Hydrolysis of GA can produce cyanide and hydrocyanic acid. GA has a $t_{½}$ of 8.5 h at a pH of 7 in an aqueous solution. GB is colorless and odorless, and it is the most volatile of the G agents. It is miscible in water and, like GA, is hydrolyzed in both acidic and basic conditions. This rapidly acting compound has a $t_{½}$ of about 1.5 min. at a pH of 11 and 25°C. However, one report shows an estimated $t_{½}$ to be 40 h (Garigan, 1996). GB comes in two enantiomeric forms (+) and (−), with the (+) form twice as toxic as the (−) form (Christen and Van den Muysenberg, 1965). GD is also a colorless liquid with a fruity odor. It is about 20% soluble in water at 25°C. In water at 20°C, and a pH of 7, it hydrolyzes with $t_{½}$ of about 80 h. GD has two isomers, C (±)P(−) is more toxic than C(±)P(+), each possessing different rates of hydrolysis, with C(±)P(+) being faster. GF and GA are the least studied of the G agents and will not be discussed herein. Chemical properties of the G agents are shown in Table 36.2.

Exposure Physiology

Considerable valuable and relevant data have come from many experimental models using the inhalational approach to assess G agent toxicity. The organ distribution of G agents can take on different patterns following exposure with regard to the volume of distribution. GB is distributed to the brain, kidney, liver, and blood plasma of mice (Little et al., 1986). Within the brain, both GA and GD are detected in the hypothalamus (Wolthuis

TABLE 36.2 Nerve Agent Physicochemical Properties

Property	Sarin (GB)	Soman (GD)	Tabun (GA)	VX/VR
Molecular weight	140.1	182.2	162.1	267.4
Specific gravity at 25°C	1.0887	1.022	1.08	1.0083
Melting point (°C)	−56	−80	−50	−39-calculated
Boiling point (°C)	147	167	245	300
VAPOR PRESSURE (MMHG)				
0°C	0.52	0.044	0.004	
10°C	1.07	0.11	0.013	
20°C	2.1	0.27	0.036	0.00044
25°C	2.9	0.4		0.0007
30°C	3.93	0.61	0.094	
40°C	7.1		0.23	
50°C	12.3	2.6	0.56	
Guinea pig LD_{50}, mg/kg body weight, s.c.[a], at 24 h	42	30	117	8/11.3

Source: Adapted from Maynard and Chilcott (2009).
Missing values indicate lack of information available.
[a]*s.c., subcutaneous administration.*

et al., 1986). GD can partition into fat, which delays the morbidity response in animals. In addition to distribution, elimination pathways of nerve agents consists of covalent binding and enzymatic hydrolysis. A number of studies have examined the toxicity of these agents. Van Helden et al. (2004) exposed marmosets and guinea pigs to low vapor concentrations of GB, 7–150 µg/m^3 over 10–300 min, to determine the earliest physical response to exposure. Results showed that GB, at levels that were undetected by the fielded alarm systems used, produced significant neuronal effects in the exposed animals. This suggested that acute effects of GB can occur at very low levels. Lung microinstillation exposures of guinea pigs for 4 min to GB caused increased weight loss, lung edema, decreased O_2 saturation, decreased peak inspiratory and expiratory flows, and increased minute volume within 4 h (Conti et al., 2009). Decreased flows are indicative of increased airway resistance. High-dose inhalation exposure to GB (a total dose of 130–150 mg/m^3 × min over 10 min) caused severe bronchoconstriction in rats (Gundavarapu et al., 2014). Che et al. (2008) showed that microinstillation exposure of GB in guinea pigs caused immediate inhibition in BALF in acetylcholinesterase (AChE) activity, followed by a reversal of this effect within an hour despite continued inhibition of blood AChE. This was likely due to the high lipophilicity and rapid absorption of GB. In a 3-month study using rats exposed to GB vapor at 33–35 µg/L for 10 min daily, Allon et al. (2005) determined that there was an increased vulnerability to cardiac arrhythmias after each challenge to GB. This study pointed to the potential of delayed effects of GB exposure; however, long-term consequences of single high-dose GB exposures are not well studied. In a limited study, Husain et al. (1993) showed that mice exposed to nebulized GB at 100 mg/m^3×min per day for 10 days developed muscular weakness of the limbs and slight ataxia on day 14. GB and GD exposure-response effects on hemodynamics and lung function were assessed in baboons following an inhalation challenge. GD at 13.14 µg/kg and GB at 30 µg/kg were vaporized into the upper airway of baboons. Both agents caused increased development of apnea due to decreased phrenic nerve signals, cardiac arrhythmias, and a decrease in mean systemic blood pressure (Anzueto et al., 1990).

In an acute *in vivo* guinea pig lung microinstillation model, Katos et al. (2009) demonstrated that exposure to GD produces prominent respiratory dynamic changes. At 24 h post-exposure, tidal volume increased along with respiratory frequency. In contrast to the study by Conti et al. (2009), peak inspiratory flow was increased, possibly suggesting agent-specific effects on lung airflow patterns. Using a head-out vapor exposure system, Perkins et al. (2013) investigated the toxicity of inhaled GD at concentrations ranging between 520 and 1,410 mg × min/m^3 in the conscious rat. All animals exposed to the higher two doses died. Significant increases in BALF protein occurred with dose-dependent inhibition of AChE activity in the lung and brain within 24 h after challenge. Cholinergic crises were evident based on dose-dependent changes in cholinergic symptoms compared to controls. It can be presumed that the systemic effects of acute nerve agent exposure may be more likely when the RBC–AChE levels drop by 75–80% (Sidell, 1992). Systemic OP-induced depletion of AChE could be directly responsible for increases in mortality resulting from heightened convulsive and seizurogenic activity. However, a number of studies have shown the effects independent of AChE inhibition. Willems (1981) demonstrated that in OP pesticide-exposed individuals, there was no correlation between AChE inhibition and the extent of neuronal symptoms. Moreover, an acute non-inhalation exposure study with sarin, VX, and soman in male and female guinea pigs demonstrated that respiratory toxicity of these compounds did not correlate with AChE activity (Fawcett et al., 2009). Mutagenicity studies with GB and GD have shown that they do not produce adverse effects in the Ames *Salmonella*, Chinese hamster ovary, and mouse lymphoma assays. However, GA was deemed slightly mutagenic in the Chinese hamster ovary and Ames bacterial system assays (Nasr et al., 1988; Goldman and Dacre, 1989).

Thus far, human data have largely come from low-dose exposure of volunteers and from nonfatal accidental exposures. Exposed humans have experienced coughing, wheezing, rhinorrhea, and nonexertional and exertional dyspnea, along with a feeling of increasing pressure in the thoracic region. Craig and Freeman (1953) provided evidence that accidental exposure to GA or GB causes behavioral changes, sleep disturbances, fatigue, and mood changes. Indeed, the concentrations of these agents required to produce mortality in humans are not precisely known. However, the estimated LCt_{50} of GA, GB, and GD are believed to be 150, 70–100, and 40–60 mg × min/m^3, respectively.

Exposure Biochemistry

The nerve agents are known to bind and functionally disable AChE regardless of the agent form (i.e., vapor or spray) or the route of exposure. The extent of AChE inhibition is a function of dose and duration of exposure, basically the product of $[C] \times t$. AChE is found at the synaptic junctions of nerve endings and is responsible for hydrolyzing excess acetylcholine (ACh), which is critical to synaptic nerve transmission. The failure to metabolize or break down ACh causes an accumulation of ACh at the nerve terminals, leading to increased cholinergic stimulation and many of the toxidromic symptoms typical of nerve agent poisoning, known medically as a *cholinergic crisis.* Increased cholinergic stimulation leads to persistent stimulation of muscarinic receptors (M_1–M_3 in the lung) within the parasympathetic neurons. In mammalian species, parasympathetic nerves innervate the airways. Muscarinic receptors belong to the G-protein coupled receptor family presenting throughout the airways. Overstimulation of muscarinic receptors initiates increased salivation, lacrimation, urination, and defecation (commonly known as *SLUD*), along with bronchoconstriction, bradycardia, increased nasal secretions, emesis, and dyspnea. Nicotinic receptors, a member of the ligand-gated ion channel family, occur in the somatic or sympathetic nervous system and are also affected by OPs. Symptoms include tachycardia, mydriasis, fasciculations, meiosis, skeletal muscle paralysis, hypertension, slurred speech, irritability, fatigue, impaired judgment, insomnia, and diaphragmatic weakness. More severe poisoning is associated with more profound CNS responses such as ataxia, convulsions, seizures, and death by asphyxiation.

Neurotransmitter pathways may be directly involved in the sequelae of G agent-induced toxicity. In the CNS, G agents can also act directly on glutamate receptors. Chebabo et al. (1999) demonstrated in an *in vitro* model that nM amounts of GB reduced the amplitude of gamma-aminobutyric acid (GABA), which is a neurotransmitter. It has been suggested that overstimulation of glutamatergic receptors by GD in the brain may be responsible for the modulation of seizure activity (Lallement et al., 1991a,b). Affected neurotransmission pathway may have far-reaching consequences on behavior and induction of convulsions. For an in-depth discussion of nerve agent exposure-response effects and case histories, refer to the excellent overview by Sidell et al. (2008). Some investigations have centered on the effects of repeated exposure to nerve agents. Kalra et al. (2002) studied the effects of subclinical repeated inhalation nose-only exposures to GB of 0.2 and 0.4 mg/m^3 on immune cell responses in rats. GB exposure suppressed T-cell mitogenesis, conconavalin A, and anti-αβ-T receptor-dependent antibody-forming cell responses. In addition, there was a reduction in glucocorticoid production. Their data indicated that repeated exposures to GB over 5–10 days caused changes in T-cell responsiveness mediated by GB's effect on the autonomic nervous system.

Inhaled G agents cause significant respiratory problems. The respiratory effects occur within minutes and clinically can resemble a severe asthmatic attack. A nerve agent-induced death is normally linked to pulmonary dysfunction. Enhanced secretions are the result of vagal efferent activity resulting from increased ACh concentration, substance P, and vasoactive intestinal peptide release. The inhalation of a high concentration of vapor will result in loss of consciousness, apnea, flaccid paralysis, and seizures (status epilepticus) within a period ranging from seconds to several minutes. Peak effects can occur within 30 min, followed by an asphyxiating death (Berkenstadt et al., 1991).

One of the major differences between the principal nerve agents GA, GB, and GD is associated with aging of the AChE enzyme. This AChE aging is basically a chemical reaction resulting in the complexation of the nerve agent with the AChE enzyme that prevents the reactivation of the enzyme. GB-complexed AChE ages over about 5 h, whereas GD takes only minutes (Garigan, 1996). Aging half-time estimates can vary depending on the experimental model used such as *in vitro* versus *in vivo* (Sidell et al., 2008).

In addition to binding with AChE, the detoxification process can lead to interaction of OPs with other cholinesterases such as butyrylcholinesterase (BuChE) and carboxylcholinesterase (CaChE) in the plasma and tissues. Levels of these enzymes can vary from organ to organ. In humans, CaChE is present in cells rather than in plasma, as is measured in other animal species. OPs can also interact with other enzymes such as paraoxonase and arylesterase. For a more comprehensive review of these secondary effects, see Casida and Quinstad (2004).

Exposure to G agents in particular GD may change the antioxidant-to-oxidant ratio by affecting free-radical scavenging pathways that are abundant in mammals. Klaidman et al. (2003) showed that an intramuscular injection of GD in rats decreased protein sulfhydryls in

the piriform cortex and the hippocampus during seizures at 1 h post-exposure, whereas at 24 h, post-exposure GSH levels decreased nearly 50% in the piriform cortex. These results suggest that free-radical formation may be contributing to seizurogenic pathophysiology. Furthermore, subcutaneous challenge to GD increased lipid peroxidation and formation of nitrogen oxides within 30 min of administration in rat brain, especially the hippocampus, thalamus, and medulla-pons (Jacobsson et al., 1999). Since the medulla-pons region controls respiration, it is likely that neuronal mechanisms might also contribute to respiratory effects of OPs. Despite the fact that the two models cited here do not involve the inhalation route of exposure, it is clear that once the agent crosses into the systemic circulation, similar physiological responses occur regardless of the route of exposure.

Exposure to G agents can also cause changes in transcriptional pathways. For instance, RamaRao et al. (2011) showed that GD caused perturbations in the phosphorylation levels of cAMP response element binding protein (CREB), c-Jun, and NF-κB, all of which control a variety of pathological processes. Dillman et al. (2009) demonstrated using microarrays that GD markedly modulated p38 mitogen-activated protein kinase (MAPK) and extracellular receptor kinase (ERK) signaling pathways. While these were not inhalation studies, it should be noted that once the agent enters the systemic circulation, multiple comparable side effects are initiated and propagated. Exposure to G agents causes perturbations in calcium regulatory signaling pathways, which can affect a variety of physiological responses. Destabilization of protein kinase C, which is responsible for brain ion fluxes and eventual neurotransmitter release at different rates in various brain regions, suggests that brain regions respond differently to nerve agent challenges. Exposure to GB also induces inflammatory gene expression within the lung. GB increases lung tissue expression of IL-2, TNFα, IL-1β, hypoxia-induced factor HIF-α, and eotaxin 24 h following exposure, suggesting a cytokine surge resulting from highly active inflammatory processes (Gundavarapu et al., 2014). For these and other nerve agent-induced expression changes in inflammatory processes, see RamaRao and Bhattacharya (2012).

Exposure Histopathology

Inhalational effects of G agents on lung tissue following exposure can range from basically none to severe based on the $[C] \times t$ of exposure. In rats exposed to 768 mg × min/m^3 aerosolized GB, major changes in the pathology at 4 days after exposure were observed. Primary changes were seen in the epithelial lining of the lobar bronchi. Lung histology showed increases in interstitial mononuclear cells and thickening of the alveolar septa. Masses of exudate, inflammatory cell infiltration, and bronchial epithelial damage were also seen (Pant et al., 1993). In a study featuring 10-day repeated exposure to GB, on day 14 post-exposure spinal cord pathology showed clear evidence of degenerative axons (Husain et al., 1993). Brain lesions and cardiomyopathy were observed in rats exposed to GD or GB in a subcutaneous injection model (Singer et al., 1987; Tryphonas and Clement, 1995). In Perkins et al. (2013), rats exposed to 4–6 min GD vapor at 600 mg × min/m^3 had compromised lung tissue pathology compared with controls. At 24 h after exposure, alveolar hemorrhage, inflammation, and histiocytosis scored twofold higher than controls. Neutrophilic exudate and alveolar destruction were also observed. An intramuscular challenge with GD showed astrocytic degeneration, neuronal necrosis, and liquefaction necrosis of the CNS. The cerebral cortex, limbic system, thalamus, and substantia nigra appeared to be targets of toxicity in the rat (Tryphonas and Clement, 1995). Pulmonary pathology changes after systemic administration of nerve agents are not well characterized.

Nonvolatile Agents

Nonvolatile agents, such as VX and its structural isomer VR, have the same molecular weight (as shown in Table 36.2) and are not considered to be vapor hazards. As a result, they are judged to be low inhalational threats to humans (the agents, listed in order of volatility are GB, GD, GA, VX, and VR). Most research on V agents have centered on VX, which is considered the "gold standard" against which the toxicities of all other agents (GA, GB, and GD) are compared.

VX is an odorless and colorless compound when in pure form. It is an oily liquid that is slightly soluble in water. These characteristics are largely responsible for its capacity to be classified as a persistent agent (Table 36.1). Although VX and VR are similar in structure, Collins et al. (2013) demonstrated in a rat aerosol exposure model that after a single 10-min exposure to VX or VR, the VR lethal concentration was nearly half that of VX (367 versus 632 mg × min/m^3) making it more toxic. These data support results from an earlier study by Chang et al. (2002), where they conclude that it may take higher doses of atropine to treat those exposed to VR. While there may be differences between VR and VX in terms of lethality, the physiological, biochemical, histopathological, and toxicological outcomes are very similar. Therefore, unless specifically pointed out, mostly VX data are reported. The reported estimates of lethality for VX is 40 mg × min/m^3 (Seto, 2011).

Exposure Physiology

While VX and VR pose more of a percutaneous threat, exposure by inhalation should be given more serious consideration, as the agent can directly come into contact

with airway surfaces when bound to mists, dust particles, fog, and other substances. As a result, entry into the airspaces can be accelerated. Exposure of airway surface can lead to almost immediate reactions culminating in respiratory failure if the [C] × t product is sufficiently high. Bronchoconstriction, a hallmark of nerve agent exposure, in animals caused by the inhaled V agents most likely is a result of mechanisms common to those of G agents. Bronchospasms may be caused by local effects on the respiratory center of the CNS, as opposed to direct effects. It is likely that more persistent agents have a tendency to deposit in the lung compartment and are slowly released over time as previously described for GD. Exposure to VX or VR produces similar hypercholinergic effects such as SLUD, incapacitation, seizures, and cardiorespiratory depression (bradycardia, dyspnea, and convulsions).

Lung exposure studies in animals using microinstilled VX have provided evidence of the nature of pulmonary effects. In guinea pigs exposed for 5 min to VX doses ranging between 50 and 90 μg/m^3, acute effects varied from an increase in lung wet/dry weight ratio with increases in the numbers of macrophages/monocytes in BALF at 24 h post-exposure (Wright et al., 2006). In a similar exposure model of local pulmonary instillation of VX, Graham et al. (2006) demonstrated that BuChE activity, although inhibited at 5 min post exposure, was quantifiably present in BALF at 24 h post-exposure even at the highest dose of VX. The authors speculate that the presence of BuChE, which is synthesized in the liver and circulates in the plasma, may be a marker of damaged air–blood barrier integrity at later time points. Katos et al. (2007) found a significant inhibition of AChE in the esophagus and intestine following microinstilled VX in the lung. From these studies, it is apparent that VX can localize to the gastrointestinal tract, causing an "irritable bowel-like" condition as a result of its likely clearance through the gut. In guinea pig studies investigating the long-term effects of a 10 min. VX exposure on changes in pulmonary respiratory dynamics, Rezk et al. (2007) demonstrated that end-expiratory pause (EEP); i.e., the length of time between the end of expiration and the beginning of the inspiratory cycle was increased at 48 h post-exposure. This study also indicated that most of the altered respiratory function returned to near control levels by day 7, except for EEP. EEP did return to normal levels by day 18. These data suggest that VX can persist in the body due to tissue-specific compartmentalization and produce long-term effects.

A direct 10-min intratracheal VX exposure of anesthetized rats, bypassing the nose (preventing neuronal translocation to brain through nose), resulted in acute lung injury and tissue damage, suggesting direct tissue-specific effect. In this work, VX aerosol was administered at 514 mg/min/m^3 and produced significant increases in BALF protein concentrations at 6 and 24 h after exposure, suggesting the prevalence of pulmonary edema. Airway resistance was significantly increased at 20 min and 6 h post-exposure at all VX concentrations tested and at the two highest doses, 343 and 514 mg × min/m^3, 24 h post-exposure (Peng et al., 2014). The authors concluded that VX inhalation impairs pulmonary function through an obstruction associated with increased pause, expiration time, and nearly a 50% decrease in expired flow rate, decreased respiratory rate and tidal volume, leading to a drop in minute ventilation. These are all indicators of airway flow obstruction. VX inhalation could transiently disturb lung function through airway smooth muscle spasm, paralysis, or both, and when combined with mucus oversecretion, could result in respiratory failure; however, the precise mechanisms are not well understood.

Additionally, a preliminary assessment of the VX exposure effects on cellular profiles in BALF was undertaken. Study time points were 3, 6, 24 h, and 1 and 2 weeks post-exposure. Blood analyses indicated that VX exposure at 343 mg × min/m^3 increased circulating white blood cells (WBCs), erythrocytes (RBCs), HCT, HGB, neutrophils, platelets (PLTs), and eosinophils at 6 h post-VX inhalation compared with naïve controls. Lymphocytes, basophils, monocytes (MONO), HCTs, and RBCs remained elevated at 1 week. Monocytes and PLTs continued to be elevated at 2 weeks after exposure compared to naïve controls (Sciuto, 2014). While further work is needed in this area to determine how these changes are associated with pulmonary pathology and the chronicity of the damage, these data indicate that variable effects of V agent inhalation on cell populations are not limited to acute effects. This substantiates the fact that V agents may deposit in tissues and get released over a long period of time after a single exposure. It is also possible that there may be a lag-time in the immune response induced by V agent exposure as part of a compensatory mechanism.

Exposure Biochemistry

Limited published studies are available investigating the effects of V agent exposure on biochemical pathways. Similar to G agents, V agents likewise inhibit blood and lung tissue AChE activity. In the aerosolized VX rat exposure model of Peng et al. (2014), blood AChE activity was decreased 41% at 343 and 52% at 514 mg × min/m^3 24 h post-exposure. Lung tissue AChE activity was also inhibited by VX. However, using a similar exposure model testing the effects of VR, AChE mRNA transcription in the lungs was up-regulated by 19% for 143 and 30% for the 286 mg × min/m^3 VR groups compared to vehicle at 24 h (Sciuto, 2014). Up-regulation of lung tissue AChE mRNA may indicate the cellular response to accumulation of nonfunctional enzyme. In

addition, we observed by immunohistochemistry a positive staining for iNOS in alveolar epithelial cells together with decreased staining of surfactant D in lung tissue 24 h post-inhalation exposure to 514 mg × min/m^3 VX.

In these VX aerosol inhalation studies, Western blot analyses showed that xanthine oxidoreductase, an enzyme-producing superoxide, is activated in response to VX inhalation exposure at 343 mg × min/m^3. VX inhalation also triggered IL-6 expression in rat lung tissue. In VR-exposed rats, lung lavage assays showed decreased GSH concentration and superoxide dismutase (SOD) activity, which indicates a local oxidative stress environment. Western blot analyses of lung tissue 6 h post-exposure demonstrated an increased expression of xanthine oxidase, increased IL-1β expression, and activation of phosphorylation of p38 and Akt suggesting stimulation of inflammatory mechanisms.

Exposure Histopathology

There is minimal if any lung damage within 24 h after VX exposure in rats. Lung and tracheal lesions were generally less severe in the 3- and 6-h VX post-exposure groups than in the 24-h VX post-exposure groups consistent with its delayed effects. This delay could be due to the time it takes for extravasation of inflammatory cells, such as neutrophils and macrophages (Peng et al., 2014). The tracheal lesions in these animals showed evidence of ulceration accompanied by neutrophilic inflammation and necrotic epithelial lining (necrotic membrane). Similar findings were observed in a VX-exposed guinea pig inhalation model (Nambiar et al., 2007). Peng et al. (2014) also showed that at high levels of inhaled aerosolized VX, exposure produced prominent alterations in airway and lung pathology at 24 h in the rat. A 10-min exposure to 514 mg × min/m^3 VX-induced architectural changes in the trachea and lung. The most significant findings were perivascular inflammation, histiocytosis, alveolar exudate, alveolar epithelial necrosis, septal edema, and bronchiolar inflammatory infiltrates.

VX exposure also causes lung parenchymal pathology. VX-induced histological changes are persistent for 1–2 weeks post-exposure. In general, the pulmonary histologic changes described are likely secondary to nebulized agents. The prevalence of lesions suggest that VX, when administered by inhalation at concentrations of 171 and 343 mg × min/m^3, can induce mild but persistent pulmonary changes over several days post exposure. Wright et al. (2006) showed similar patterns in the lungs of guinea pigs microinstilled with VX for 5 min.

Cyanides

Cyanides were used as CWAs and are potential terrorist agents (Magnum and Skipper, 1942; NRC, 1999). Cyanides are also used industrially for electroplating and the extraction of gold and silver. They can be released into the atmosphere from volcanoes, fungi, and bacteria. Common food items such as pears, peach, sweet potatoes, peas, apples, and lima beans are sources of cyanogenic compounds.

Exposure Physiology

The cyanides, formerly known as "blood agents," consist of hydrogen cyanide (NATO code designation AC, HCN: MW 27.04) and cyanogen chloride (CK, ClCN: MW 61.5). The common metabolic by-product is the toxic CN^- anion, which is largely a systemic toxicant. Vapors from these agents are heavier than air and spread across the ground like phosgene and can pose a problem in low-lying, confined areas (Table 36.1). A mild exposure to these agents through the inhalation route can cause headache, loss of consciousness, ataxia, and confusion. Palpitations and respiratory tract irritation with labored breathing (dyspnea) can lead to hyperpnea in some cases. For exposures classified as severe, serious CNS effects may occur. These include coma, seizures, and mydriasis. Dysrhythmias, low blood pressure, and eventual life-threatening cardiac arrest may follow. The occurrence of pulmonary edema followed by respiratory insufficiency can be a late manifestation following a severe inhalation exposure episode. The toxic load of inhaled HCN can be enhanced through its effect on increased minute volume (Purser et al., 1984).

Animal studies have provided evidence of the tissue distribution of inhaled cyanide. These studies have indicated that the target organs are the lung and heart, followed by the brain; all highly perfused. For example, in the rat following an acute inhalation exposure to HCN, the highest concentrations were measured in the blood, brain, heart, and lung with very little in the liver (Ballantyne, 1994). The estimated human inhaled 50% lethal concentration of HCN is 2,500–5,000 mg × min/m^3 and for cyanogen chloride, it is approximately 11,000 mg × min/m^3. These data indicate that via the inhalation route, the cyanides are less toxic compared to their effects as systemic poisons (McNamara, 1976).

Exposure Biochemistry

It is well known that HCN vapor readily penetrates the epithelium with little difficulty due to its low ionization and low molecular weight. This allows for the rapid absorption through pulmonary alveolar membrane during exposure. The basic metabolic chemical reactions involving CN^- have been studied for decades (Sykes, 1981). CN^- binds to and inactivates enzymes involved in oxidative phosphorylation within mitochondria. The mitochondria are the primary target organelles involved in HCN poisoning. Lethal cytotoxic anoxia results from the interaction of CN^- with cytochrome c-oxidase, which is an important regulator of cellular respiration. This

reaction, which occurs within minutes, inhibits aerobic metabolism by binding to the binuclear heme center. Additionally, there is loss of the formation of ATP, which is critical to supplying cells with the energy required for normal cellular processes (Keilin, 1929; Klein and Olsen, 1947). This adverse reaction prevents the binding of electrons to the molecular oxygen. As such, aerobic cell metabolism is halted even in the presence of well-oxygenated blood. In a futile attempt to regain the loss of ATP, cells increase glycolysis, especially in organs rich in mitochondria, such as the brain and heart. This results in metabolic acidosis characterized by increase in lactic acid. This, along with severely decreased ATP production, is responsible for dampened neurotransmission and altered perceptive abilities (Lindahl et al., 2004). In the brains of mice exposed to cyanides, lactic acid increases, and accumulation of adenosine diphosphate (ADP) and phosphates also lead to overall CNS metabolic impairment (Estler, 1965; Isom et al., 1975). CN^- can be removed through metal-complexing interactions with cobalt, molybdenum, and organic compounds prior to cell entry. Cyanide also forms cyanomethemoglobin as a result of its interaction with methemoglobin within erythrocytes. This process can remove cyanide from the plasma. Detoxification of CN^- occurs through the formation of the less acutely toxic thiocyanate (SCN) via the mitochondrial enzyme rhodanese which catalyzes the transfer of a sulfane sulfur atom from sulfur donors to sulfur acceptors (Eq. (36.2) and Figure 36.3). The end reaction with the formation of SCN^- is generally irreversible.

$$CN^- + S_2O_3^{2-} \rightarrow SCN^- + SO_3^{2-} \qquad (36.2)$$

β-mercaptopyruvate-cyanide transulfurases, which are present in the liver, kidney, and blood are also capable of detoxifying cyanides forming SCN^- (Westley, 1981; Westley et al., 1983). A third enzymatic system present in the kidney may likewise play a role in the detoxification of CN^-. The sulfotransferase, called *cystathionine γ-lyase*, has been shown to be effective in cyanide-clearing processes (Wrobel et al., 2004) (Figure 36.3).

Exposure Histopathology

Inhalation exposure to cyanide-forming agents leave scant identifiable markers of tissue or cellular damage in target organs such as the lung, heart, and brain following challenge. This is mostly because once in the body, it diffuses rapidly into the bloodstream and acts essentially as a systemic toxicant, regardless of route of exposure. Several earlier animal studies showed that acute or repeated exposure to CN^- causes neuropathological changes, such as gray matter necrosis and degenerative changes in ganglion and Purkinje cells, in dogs (Haymaker et al., 1952). Encephalopathy and optic nerve neuropathy are other sites of toxicity following inhalational exposure (Hirano et al., 1967; Lessel, 1971). Thus, inhalational exposure might result in more severe neuronal impact, since the brain is rich in mitochondria. Systemic administration of cyanides, however, has been shown to affect the heart, and lung in addition to brain (Ballantyne and Salem, 2008).

Riot Control Agents

RCAs, also known as *sternutators* (from the Latin term meaning "sneezing") and *lacrimators*, consist of a specific class of irritant compounds. Agents (and NATO codes) in this class are 2 chlorobenzylidene malononitrile (CS: MW 188.6), dibenz (*b,f*)–1:4-oxazepine (CR: MW 195.3), oleoresin capscicum (OC: MW 305.4), 10-chloro-5,10-diphenylaminochlorarsine (DM: MW 277.6), chloropicrin (PS: MW 164.4), and 1-chlorocaetophenone (CN, also known as MACE: MW 154.6). Some of these agents, such as PS, DM, and CN, are CWAs (Figure 36.4).

RCAs have the general characteristics of causing rapid incapacitation, ease of dissemination, and relative low toxicity. They are usually disseminated as mists, aerosols, smoke, or volatilized M18 thermal grenades. They have been used widely as CWAs (Hilmas et al., 2008; Salem et al., 2008). CN was the original tear gas, which eventually was replaced by CS. In general, these agents possess actions that are short-lived, with the exposed becoming adapted to the effects within about 30 min of exposure. However, death has been known to occur with high doses and in confined spaces. Pharmacologically, they fall across classes that include emetics, hypnotics, serotonin antagonists, neuromuscular blockers, and sedatives. The toxicological effects of RCAs are readily apparent in various anatomical areas, such as the eye, gastrointestinal tract, nasal, oral and neuronal tissues, lung, and skin. Complex interactions among the sites described make RCAs potent candidates for crowd dispersion and control. Even though all of these anatomical sites are critical with regard to aftereffects, we will focus primarily on inhalation toxicology. For an outstanding and extensive review of RCA toxicity, see Olajos and Salem (2001).

2-Chlorobenzylidene Malononitrile (CS)

CS is a white crystalline solid with a relatively low solubility in water, but a rapid rate of hydrolysis. It is soluble in most polar organic solvents. Harris (1993) describes RCAs as nonlethal, making them most effective as crowd dispersants on unprotected personnel. RCAs can enter the respiratory tract in the form of vapor or aerosol.

Exposure Physiology

When inhaled, these agents are capable of sensory nerve receptor irritation, causing the Kratschmer reflex,

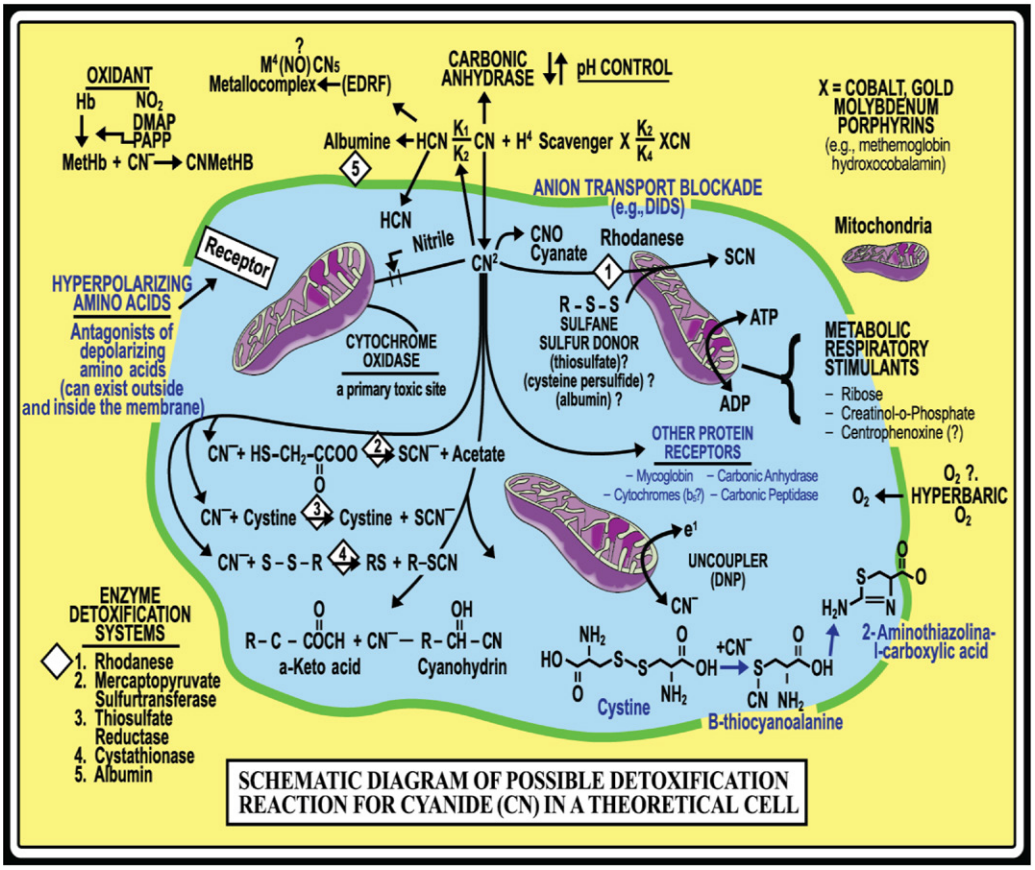


FIGURE 36.3 Likely detoxification reactions for cyanide within a cell. The enzyme detoxification systems are as follows: (1) Rhodanese: an intra-mitochondrial enzyme that catalyzes the transfer of sulfur from a donor molecule to cyanide to form thiocyanate. Rhodanese is a key regulator of cyanide detoxification. (2) Mercaptopyruvate sulfurtransferases: a group of enzymes widely distributed in the body that catalyze the transfer of a sulfane sulfur atom from a donor molecule to a thiophilic acceptor substrate for the limitation of cyanide. (3) Thiosulfate reductase: enzymes found in the liver, kidney, heart, brain, intestine, and testis that use electrons from thiols to reduce the sulfane sulfur atoms of inorganic thiosulfate and organic thiosulfonate anions to sulfide. Sulfide production from these thiol-dependent reductases is thought to be used in the synthesis of Fe-S proteins. (4) Cystathionase: enzymes widely distributed in the body that can transfer sulfur from one cysteine to another, generating thiocysteine and pyruvate. (5) Albumin: molecules that act like an enzyme in the detoxification of cyanide. Albumin contains sulfur moieties that bind to and limit cyanide formation. ADP: adenosine diphosphate; ATP: adenosine triphosphate; ATCA: 2-aminothiazoline-4-carboxylic acid; CN: cyanide ion; CNMetHb: cyanomethemoglobin; CNO: cyanide; DIDS: 4,4′-diisothiocyano-2,2′-disulfonic stilbene; DMAP: dimethylaminophenol; DNP: deoxyribonucleoprotein; EDRF: endothelium-derived relaxing factor; Hb: hemoglobin; MetHb: methemoglobin; PAPP: p-aminopropiophenone; R: reduction factor; S: substrate. *Source: Reprinted from* Textbooks of Military Medicine: Medical Aspects of Chemical Warfare, *Published in 2008 by the Office of the Surgeon General, TMM Publications.*

which may result in cessation of respiratory function. Initial responses include sneezing, coughing, a burning sensation, and excessive rhinorrhea. Additional responses can also include dizziness and disorientation. While these are protective mechanisms to reduce the effects of inhalation, they may be accompanied by bradycardia and biphasic changes in aortic blood pressure. The half-life of CS following inhalation is less than 30 seconds (Olajos and Salem, 2001). Because of its low water solubility CS is environmentally persistent (Table 36.1). To further increase environmental persistence for up to 2 weeks, two hydrophobic forms were synthesized, CS1 and CS2, which are rapidly absorbed from the respiratory tract. In rat inhalation studies, repeated exposure to CS1 caused aggressive behavior and hyperreactivity. In other studies with concentrations reaching 25,000–68,000 mg × min/m^3, there were no changes in blood electrolyte levels; however, pulmonary edema and necrosis of the respiratory and gastrointestinal tracts were observed in the rats that died after CS exposure. Death most likely consisted of the combined result of hypoxia, circulatory failure, and obstructed airways (Salem, et al., 2006). In human volunteers, exposures to a range of CS concentrations showed no evidence of deleterious changes in airway resistance, vital capacity, or tidal volume (Beswick, et al., 1972). The LCt_{50} values for humans have been estimated to range between 25,000 and 100,000 mg × min/m^3 (WHO, 1970).

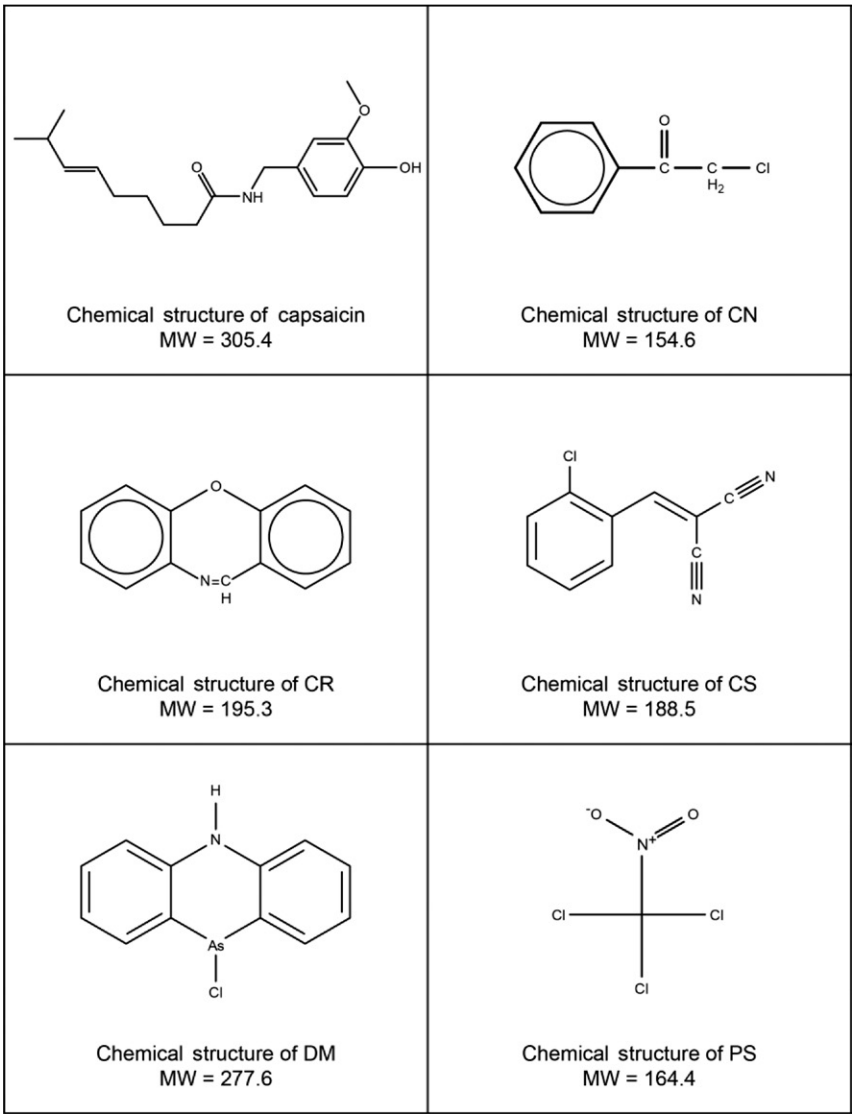


FIGURE 36.4 Chemical structures and formula weights of the most commonly studied RCAs.

However, there have been no reported human mortalities due to CS exposure. The high levels of estimated concentrations of CS required to be lethal in humans clearly places it in the low lethality category (Table 36.1). While lethality is not measured during the course of human exposure, CS exposure has been linked to RADS, which can result from a single intentional exposure (Hu and Christiani, 1992). The respiratory irritation effects of CS exposure may be linked to the release of bradykinin (McNamara et al., 1969: Cucinell et al., 1971).

Exposure Biochemistry

As noted previously, CS is rapidly absorbed during inhalation. It has a very short half-life and is metabolized and detoxified in the blood and other organs. Its disappearance follows first-order kinetics, and it spontaneously hydrolyzes to form malononitrile, which is then metabolized to thiocyanide (Nash et al., 1950). However, it is believed that in the case of inhalation exposure, the amount of formed thiocyanide may have little if any role in human toxicity (Ballantyne, 2006). Metabolites can be found in the blood only after high exposures. CS is an SN_2-alkylating agent with active halogens, which react with nucleophilic sites. A common metabolite 2-chlorobenzaldehyde was found in the blood of rodents and humans after exposure. CS reacts rapidly with plasma proteins and GSH, all of which are possible cofactors responsible for detoxification (Cucinell et al., 1971). Sulfhydryl-containing enzymes, such as lactic dehydrogenase, pyruvic decarboxylase, and glutamic dehydrogenase, are targets of the alkylating effects of CS (Lovre and Cucinell, 1970). The i.p. administration of CS to rats results in its excretion in the urine as mercapturic acid (Rietveld et al., 1983). It is not known if

this occurs following inhalation. On the other hand, it has been reported that aerosol exposure to CS leads to an excretion of thiocyanate. The release of cyanide has also been shown to occur following i.p. injection in the rat (Frankenburg and Sorbo, 1973).

Exposure Histopathology

Overall, CS does not produce significant respiratory tract or systemic lesions. In mice and rats, exposure to CS caused squamous metaplasia of the olfactory epithelium and hyperplasia and metaplasia in the respiratory epithelium. In 1990, the National Toxicology Program (NTP) tested CS for genotoxicity and carcinogenicity and found no evidence of either effect in rodents (NIH, NTP, 1990). In inhalation experiments using rats and hamsters, with CS concentrations ranging between 150 and 750 mg/m^3 and lasting from 30 to 120 min, a small degree of lung pathology was seen for up to 28 days post-exposure. The hallmark of exposure at high levels in the lung was congested alveolar capillaries, pulmonary edema, and hemorrhagic lesions. Secondary effects in extrapulmonary organs were renal tubular and hepatic cellular necrosis (Salem et al., 2006). In 120-day inhalation studies using guinea pigs, acute alveolitis was observed (Ballantyne and Callaway, 1972; Ballantyne and Swanston, 1978).

Dibenz (b,f)-1:4-oxazepine (CR)

CR is a pale yellow solid with a strong, pepperlike odor. Exposure-effect responses of CR are similar to CS. CR is the most potent lacrimator with the least toxicity. It is the parent compound of the antipsychotic drug loxapine (Blaine, 2003).

Exposure Physiology

Upon inhalation, CR is rapidly absorbed through the lung and into the bloodstream. It has a plasma half-life of about 5 min. (Upshall, 1977). CR can be dispersed as an aerosol or as grenade-generated smoke. For the most part, animals exposed to CR exhibit physical dysfunctions such as ataxia, loss of coordination, tachypnea, and convulsions. In human volunteers, low-level CR aerosol exposure produced bronchoconstriction and increased pulmonary blood flow within about 20 min. (Ashton, et al., 1978). These effects are believed to be due to the stimulation of irritant receptors, the effects of which usually subside within an hour after exposure. Ballantyne studied the effects of inhaled CR aerosol and CR smoke in rats. Concentrations ranged between 13,050 and 428,000 mg × min/m^3. Excessive nasal discharge and blepharospasms occurred, which also subsided in about 60 min. (Ballantyne, 1977). No mortalities were recorded. Investigations involving the effects of CR exposure on teratogenicity (specifically, embryo toxicity) revealed no adverse developmental toxicology (Upshall, 1974). Carcinogenicity and genotoxicity assessment using various strains of *Salmonella typhimurium* reverse mutation tests, CHO gene mutation system (V79/HGPRT), mouse lymphoma assay (L5178Y/TK+/TK−), and micronucleus testing also indicated no toxicity (Colgrave et al., 1979). Data from Kumar et al. (1995, 2006) provides evidence that CN is much more toxic than CR at the 5% LC_{50} level and upon repeated exposure in rats and mice.

Exposure Biochemistry

In rat tissue, CR is metabolized to 4-, 7-, and 9-hydroxylactams via oxidative processes, followed by ring hydroxylation and sulfate conjugation, and then excreted via urine (French et al., 1983). Metabolites and excretion profiles of CR are similar among many species regardless of exposure route. Intravascularly dosed mice suggested rapid uptake of CR from the blood and into the kidney, small intestine, bile, and liver, which was in line with data from rats indicating rapid absorption, hepatic metabolism, and renal excretion (French et al., 1983).

Exposure Histopathology

CR administered by aerosol inhalation at 68,400 mg × min/m^3 did not cause lethality in mice, rabbits, or guinea pigs (Ballantyne, 1977). However, congestion in the capillaries and alveolar hemorrhaging were noted. Pattle et al. (1974) exposed rats to CR at a concentration of 115,000 mg × min/m^3 to determine whether a high concentration produced adverse effects on cell organelles such as lamellar bodies. Electron microscopy indicated no effects of inhaled CR on lamellar bodies. Colgrave et al. (1979) exposed animals to a range of CR aerosols at dosages from 78,200 to 161,300 mg × min/m^3 and reported no abnormalities in the lung. However, subsequent assessment after microscopic examination revealed mild lung congestion, hemorrhage, and emphysema. Long-term effects of inhaled CR aerosol were conducted in hamsters and mice using dosages of 204 ppm (5 min), 236 ppm (8.6 min), and 267 ppm (15.8 min) 5 days/week for 18 weeks. Survivors were sacrificed and dissected 1 year after exposure, with the only pathology being chronic laryngeal inflammation (Marrs et al., 1983).

10-Chloro-5,10-diphenylaminochlorarsine (DM-Adamsite)

DM, an organoarsenical compound, is a yellowish-green crystalline solid with low volatility. DM and other compounds, such as PS, diphenylchloroarsine (DA), and diphenylcyanoarsine (DC), are known as *vomiting agents.* DM is considered odorless, but it can contain a faint odor of bitter almonds. It is more soluble in organic solvents, such as benzene, toluene, and alcohols, than in water. It can be released as a dry powder using thermal or explosive techniques.

Exposure Physiology

Respiratory effects of inhaled DM include increased nasal congestion, salivation, coughing, and sneezing. Irritation of the mucous membranes of the eyes and airway lining can occur. Persistent vomiting and headaches are also exhibited after DM exposure. Compared to other RCAs, the effects of inhaled DM can take as long as 3 min and last up to several hours (BMOD, 1972). Punte et al. (1962) studied the effects of a number of RCAs in animals that were exposed for periods ranging from 5 to 90 min. With DM, there were no abnormalities observed below concentrations of 500 mg × min/m^3. Estimated LCt_{50} values were as follows: for mouse, 22,400 mg × min/m^3; rat, 3,700 mg × min/m^3; and guinea pig, 7,900 mg × min/m^3.

Striker et al. (1967) exposed nonhuman primates to various [C] × *t* levels of DM. An inhaled dose of 2,565 mg × min/m^3 was well tolerated except for one animal that exhibited nasal discharge. At a dose level of [C] × *t* of 8,540 mg × min/m^3, nasal discharge and facial erythema were noted, but these symptoms were resolved by 24 h post-exposure. When the exposure dose was increased to 28,765 mg × min/m^3, hyperactivity ensued, with significant nasal discharge and respiratory distress in all animals. Striker et al. (1967) concluded that death in some animals was caused by respiratory failure. In human volunteers, doses thought to cause adverse physiological responses such as nausea and vomiting were tested. Some studies found that humans can tolerate DM in the range of 22–92 mg/m^3 for at least 1 min with a 50% tolerance level of up to 220 mg/m^3 (Punte et al., 1962). In this study, the definition of tolerability was the desire to vacate the exposure space as quickly as possible. From these studies and others, it was shown that exposure to inhaled DM produced a range of effects including pain in the eyes, throat, nose, and upper airway; coughing; and salivation similar to a cholinergic response seen with nerve agent exposure. If the exposure is severe enough and sufficient in duration, death may ensue. There is one known human death caused by inhaled DM (Owens et al., 1967). Exposure times of 5–30 min at concentrations of 1,130–2,260 mg/m^3 ([C] × *t* of 5,650 to 67,800 mg × min/m^3) were estimated. For the most part, this is well above the estimated LCt_{50} of 11,000 mg × min/m^3 for humans reported by Sidell (1997).

Exposure Biochemistry

There is only one study that suggests the possible metabolic and detoxification fate of DM (Hass et al., 2004). As an organoarsenical compound, it would seem likely that its toxicity may be linked to the metabolism of arsenic. Through the oxidation of As (III) by manganese peroxide, DM is broken down into As (V). This reaction releases chloride with the subsequent incorporation of two oxygen molecules into the parent compound (Haas et al., 2004). Arsenic released in the metabolic process with a specific valance state could inhibit succinic dehydrogenase activity and cause the uncoupling of oxidative phosphorylation and reduced ATP levels.

Exposure Histopathology

As stated previously, there remains little doubt that the target organ showing significant and consistent debilitating effects of inhaled DM is the lung. In DM-exposed nonhuman primates, gross postmortem examination showed evidence of edematous and severely congested lungs, whereas microscopic evaluation revealed ulceration of the tracheobronchial tree and alveolar edema (Striker et al., 1967). In the human death mentioned previously, postmortem analysis revealed edema of the subcutaneous tissue of the neck, mediastinum, pleura, and pericardium, along with emphysematous bullae widespread in the lung. Histologically, bronchopneumonia, hemorrhage, pseudomembrane formation in the trachea and bronchi, and congestion were observed throughout the entire respiratory tract (Owens et al., 1967).

Oleoresin of Capsicum (OC—Pepper Spray)

OC is a reddish-brown, oily resin derived from capsicum plants, commonly known as nightshade (Figure 36.5). Capsaicinoids can exist in several derivative forms such as dihydrocapsaicin, homocapsaicin, and nordihydrocapsaicins. Capsaicinoids are isolated through volatile extraction of the dried, ripened fruit of chili peppers. Once dried, the OCs are predominantly capsaicin, followed by nordihydrocapsaicin and homocapsaicin (Salem et al., 2006). However, other potentially irritating components are present, such as acids, esters, and phenolic agents. Capsaicins at high concentrations can cause a variety of potent effects such as dermatitis, nasal, ocular, pulmonary, and gastrointestinal tissue injury. The active ingredient of capsaicin is 8-methyl-*N*-vanillyl 1,6, nonenamide.

Exposure Physiology

Like all other RCAs, aerosol-dispersed OC causes erythema; burning of the eyes, nose, and throat; sneezing; coughing; and blepharospasm. Acute exposure to inhaled OCs results in pulmonary edema, bronchospasm, respiratory arrest, hypertensive crises, and hyperthermia. OC causes excitement, convulsions, dyspnea, and death due to respiratory failure. While pepper spray is considered relatively safe, fatalities have been reported due to airway obstruction (Synman et al., 2001). Inhalation of capsaicin activates the Kratschmer reflex, as mentioned earlier, accompanied by bradycardia, apnea, and a biphasic rise and fall of aortic blood pressure. The pulmonary effects of capsaicin may be species-related. In guinea pigs exposed to capsaicin via aerosol, bronchoconstriction occurred, suggesting both a

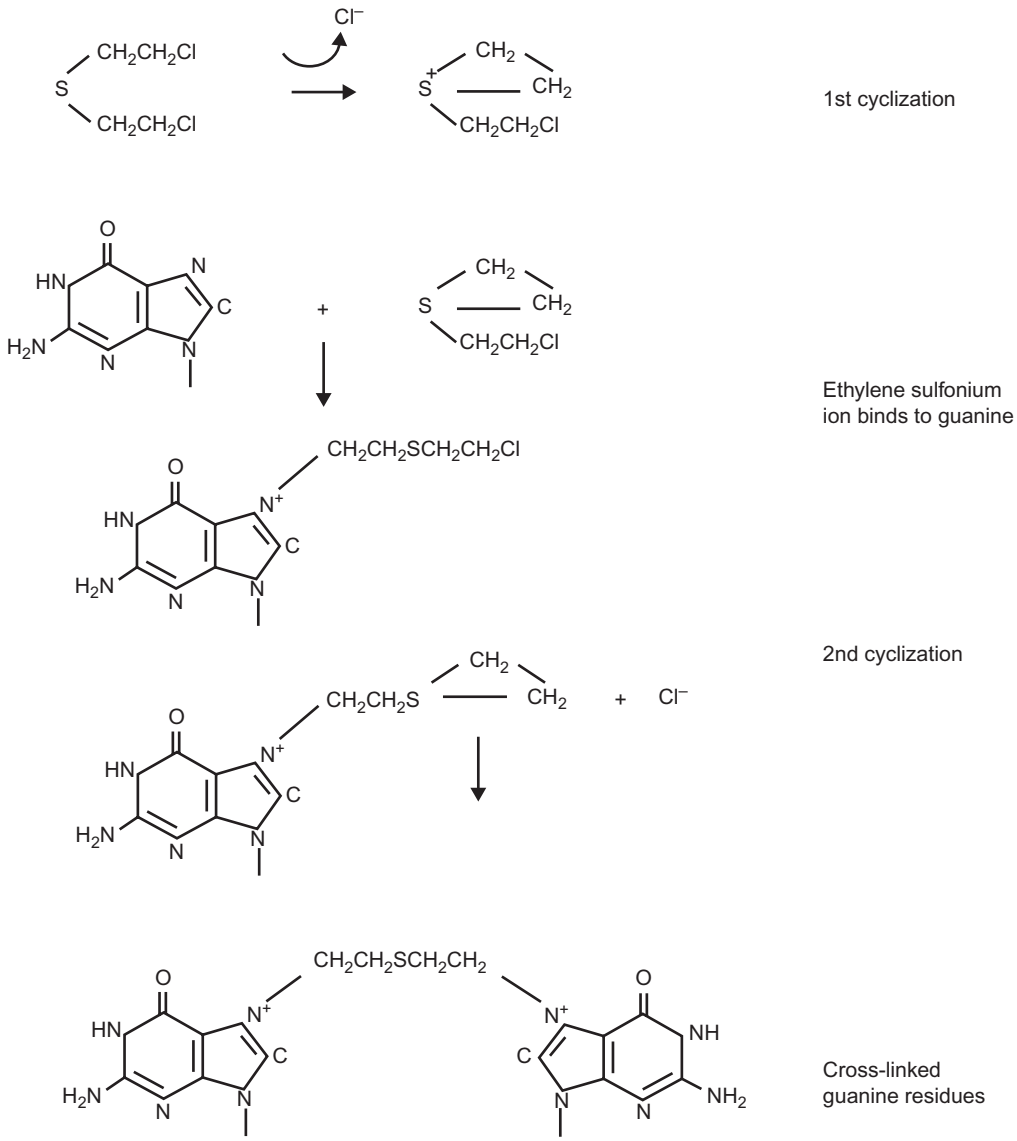


FIGURE 36.5 Reactive chemical mechanisms involved in HD hydrolysis, formation of sulfonium ion, and the alkylation of DNA through guanine cross-linked residues.

vagal-cholinergic as well as a noncholinergic local axonal reflex (Buchan and Adcock, 1992), whereas in cats using a similar exposure method, bronchoconstriction was determined to be due to a direct vagal-cholinergic response (Adcock and Smith, 1989). Glinsukon et al. (1980) determined the LD_{50} in several animal species using various routes of delivery. The concentrations ranged from 0.56 mg/kg intravenously (i.v.) to 1.6 mg/kg by intratracheal instillation. An LCt_{50} value for capsaicin of 13,000 mg/m^3×min has been published by Seto (2011).

Exposure Biochemistry

One of the mechanisms responsible for capsaicin toxicity is the stimulation of neurons consisting of neuropeptide-containing afferent nerves that are capable of activating specific vanilloid receptors (Szallasi and Blumberg, 1999). Stimulated nerves are of the Aδ and C fiber-unmyelinated types. The activation through ligand-gated mechanisms opens cation channels (Marsh et al., 1987) and increases the influx of Ca^{2+} and Na^+ in cells, which leads to depolarization and the release of neuropeptides such as substance P. Protective reflexes are activated via autonomic motor neurons. Other transmitters of biological responses to capsaicin included calcitonin gene-related peptide (CGRP) and neurokinin A (a tachykinin), both of which are released from neurons and are responsible for pain transmission and neurogenic inflammation. For an extensive list of capsaicin-sensitive markers, see Holzer (1991).

Mediator-induced inflammatory processes can lead to increased vascular permeability, neurogenic inflammation of airways and blood vessels, chemotaxis, and bronchospasm (Smith and Topford, 1999). Furthermore, capsaicin activates the vanilloid receptor family TRPV1 (transient receptor potential cation channel subfamily V) within sensory neurons. Activation of these receptors leads to prolonged refractory periods and to a nonconducting desensitization. One study suggests that TRPV pathways may not be entirely responsible for all of the adverse reactions caused by capsaicin (Holzer, 1991).

Exposure Histopathology

There have been no reports or reviews assessing the histopathology results from an inhalation challenge to capsaicins or OCs. The only data from toxicity studies was from Glinsukon et al. (1980), who found visceral organ hyperemia without hemorrhage, gastric desquamation, and increased mucus.

Chloropicrin (PS)

PS produces many of the effects consistent with RCAs. In addition, it has been classed with choking agents such as phosgene and chlorine due to its effect on the upper airways. PS is an oil ranging from colorless to light green, with a stinging odor classified as aniselike. It has low solubility in water, but it is readily soluble in most solvents such as acetone and chloroform. At high temperatures, PS decomposes to phosgene, nitrogen dioxide, and other irritants. During World War I, it was labeled as a choking agent and mixed with sulfur mustard to lower its freezing point. PS is a lung irritant and is used as a soil fumigant for its biocidal and fungicidal properties. As a result of commercial usage, exposure threats are by way of the inhalation and dermal routes in persons living and working within the operational area (O'Malley et al., 2004).

Exposure Physiology

Mechanisms of PS toxicity are not that well understood. Largely, PS is irritating to the respiratory system, mucous membrane, gastrointestinal tract, skin, and eyes. Airway inflammation, lacrimation, nausea, and vomiting are the hallmarks of exposure. In severe respiratory exposures, pulmonary edema can ensue, ultimately causing death. In mice inhalation studies, Buckley et al. (1984) assessed the RD_{50} (the dose of material required to cause a 50% reduction in respiration rate), which was 8 ppm with exposures of 6 h/day over 5 days. Exfoliation, ulceration, and necrosis of the respiratory epithelium were observed. The estimated LCt_{50} is 2,000 mg × min/m^3. A 20-min exposure to PS in pigs and cats resulted in an LD_{50} value of 800 mg/m^3 or 16,000 mg × min/m^3, respectively; and for mice, 15,840 mg × min/m^3 over a 4-h exposure (NIOSH, 2007). For humans, the permissible exposure limit (PEL) established by the National Institute of Occupational Safety and Health (NIOSH)/Occupational Safety and Health Administration (OSHA) for a work-related, time-weighted average is 0.7 mg/m^3 (1 ppm = 6.72 mg/m^3). With regard to the capacity of PS to be genotoxic, carcinogenic, or both, studies have shown mixed results.

Exposure Biochemistry

As with several RCAs, the absorption, distribution, metabolism, and excretion of PS are not well elucidated. However, in two studies by Sparks et al. (1997, 2000), data indicated that PS can form adducts with thiol groups. After oral or i.p. injection in mice, PS is rapidly absorbed and distributed to the blood, liver, and kidneys. Metabolites identified include thiophosgene dichloronitromethane, chloronitromethane, and nitromethane. Castro et al. (1988) and Sparks et al. (1997, 2000) both suggest that enzymatic reactions may be driven by β-lyase, cytochrome P-450s, and GSH-S-transferases. Additional evidence suggests that PS may be converted to raphanusamic acid (Sparks et al. 1997).

Exposure Histopathology

In postmortem assessment of exposure to inhaled PS, Buckley et al. (1984), recorded that PS was only one of several inhalants that produced lower respiratory tract lesions. In a case of homicidal poisoning resulting in an exposure to PS, spotty discoloration and pulmonary edema were found in the postmortem examination (Gonmori et al., 1987). Death in this case occurred within 3–4 h of exposure.

1-Chloroacetophenone (CN)

CN is a gray, crystalline solid compound with the odor of apple blossoms. Known also as Mace, it was specifically developed as an RCA. A potent lacrimator, it was developed just after World War I. As a vapor, it is 5 times heavier than air. Dissemination of CN can be achieved by grenade, steam dispensers, projectile cartridges, and aerosols.

Exposure Physiology

CN is a much more effective lacrimator than either CS or CR, causing serious injury to the ocular targets. Blepharospasm, corneal edema, erosion, and ulceration, as well as focal hemorrhages, can occur. As a potent skin toxicant it produces severe skin edema. Irritation of the airway, including sneezing, coughing, nasal secretions, and congestion, may persist for up to 20 min. after a challenge. The primary cause of death is from damage to the lung.

Exposure Biochemistry

The LCt_{50} for several species using the aerosol delivery method are as follows: dog, 7,033 mg × min/m^3; rat,

8,878 mg × min/m^3; and guinea pig, 7,984 mg × min/m^3. Multiple exposures using nonhuman primates and guinea pigs demonstrated that lethality decreases with repeated exposures over days or weeks, suggesting adaptation to CN toxicity. Using guinea pigs, dogs, and nonhuman primates in a similar experimental design, with a 10-day concentration totaling 88,000 mg × min/m^3, mortality was observed in animals. In one of the few comparative inhalation studies, Ballantyne and Swanston (1978) performed acute toxicity testing on rats, mice, guinea pigs, and rabbits using CN and CS aerosols. The duration of exposure ranged from 15 to 60 min, with lethality occurring within 14 days. The respective LCt_{50} values for these animals were 8,750, 18,200, 13,140, and 11,480 mg × min/m^3. For all species, CN was much more toxic than CS. Equitoxic doses of CN and CS produced similar findings using the i.v. and i.p. challenge routes. These data suggests that for appropriate toxicity screening, multispecies and multiagent comparisons are critical. For further toxicological reviews, see McNamara et al. (1969).

CN has been shown to increase lung sphingomyelin and decrease phosphatidylcholine and ethanolamine in rats (Kumar et al., 1995). Based on the increased bacterial retention in the lung in mice exposed to CN, the authors concluded that CN might increase susceptibility to infection. This may be attributed to the immunotoxic effects of CN through altered, T-cell-mediated macrophage functions (Kumar et al., 1992).

Exposure Histopathology

Animals exposed to high concentrations of CN that resulted in mortality at 48 h showed evidence of tracheal congestion and pulmonary edema (Ballantyne and Swanston (1978). Histopathology revealed congestion of alveolar capillaries and intrapulmonary veins, inter- and intra-alveolar hemorrhages, and excessive secretions in the bronchioles. CN was reported to produce degeneration in the epithelium of the bronchiole and alveolar septal-wall thickening and the presence of an increased number of mononuclear cells in rats (Kumar et al., 1995). Necrosis of the liver, kidney, small intestine, and spleen were also observed.

DA and DC

As previously stated, DA and DC are known as *vomiting agents.* Like other RCAs, they manifest their toxicity by irritating the airway mucous membranes, as well as the eyes. Irritation results in sneezing, coughing, vomiting, and headache. They have a bond between trivalent arsenic and chlorine (in the case of DA) or cyanide (in the case of DC) and are less toxic than CN.

DA, a sternutator, is a white crystalline solid with a MW of 265. As was noted for DM, the toxicity of these compounds is attributable to the presence of arsenic in the form of As (III). The general, physiological outcome of exposure to organoarsenicals can be manifested as severe conjunctival membrane irritation, swelling of the cornea, and sloughing of respiratory mucosa forming mucus plugs in the lower airways. DA can produce these effects, and it leads to death if exposure occurs in unventilated and confined spaces (Ochi et al., 2004). Studies by Ishi et al. (2004) postulated that metabolic byproducts of DA and DC, such as diphenylarsenic acid, might be associated with developmental abnormalities in humans. The estimated LCt_{50} values for DA and DC are 15,000 and 10,000 mg × min/m^3, respectively (Seto, 2011). It should be noted, however, that the toxicity of DA as a CWA is lower than the vesicants discussed next. Limited information is available on the pulmonary toxicity of these agents.

Vesicating Agents

Sulfur mustard was used extensively in World War I and was responsible for thousands of casualties. The most recent significant intentional use of vesicants was that of sulfur mustard (HD) during the Iran-Iraq war (1984–1988). Vesicants are compounds that produce chemical burns. Members of this group of vesicating agents include HD, lewisite (also known as *Agent L*) (an arsenical) and phosgene oxime (CG). CG exposure produces hivelike, urticarial skin reactions on contact. HD is a persistent agent as it can remain on the soil for more than a day (Table 36.1). Persistence is dependent on pH, soil type, and hydrophilicity. At high temperatures, such as those that occur in the desert (38–49°C), it is a considerable inhalation hazard. There is a great deal of literature regarding the toxicology, physiology and biochemistry of HD.

Sulfur Mustard—bis-(2-chloroethyl) Sulfide (HD)

Sulfur mustard, or mustard gas, is a colorless oil with a MW of 159. Impurities can cause HD to give off a garlic-like odor. It is 5.4 times as dense as air, which enables it to settle in trenches (and hence making it well suited for war). Although it is a low volatility agent, the HD vapor was responsible for about 80% of the chemical exposure deaths during World War I. HD has also been known as "Yellow Cross," "Lost," "S-Lost," "H," or "Yperite." Because HD is denser than water and does not readily hydrolyze, it can remain a threat for a while in various aqueous environments and has caused skin blisters when handled many years after submergence (Aasted et al., 1985; Wulf et al., 1985). The formation of fluid-filled blisters by HD can cause immediate tissue damage yet latent pain that may take several hours. Clinical effects may occur hours later.

Exposure Physiology

Although skin is considered to be an important route of exposure for HD, many of the fatalities ascribed to HD exposure during World War I and the Iran–Iraq war were the result of inhalation. Inhaled HD is largely absorbed in the upper airway and rarely penetrates to the lung parenchyma unless the concentration or duration of exposure are sufficiently high. In humans, following inhalation exposure to HD, reports have identified bronchopneumonia, chest tightness, and, in long-term survivors, chronic bronchitis, lung fibrosis, productive cough, and chronic obstructive pulmonary disease (Ghanei and Harandi, 2007). Injury can develop slowly, becoming much more intense over time. One important issue with HD-induced lung damage is the proper maintenance of blood oxygenation. Hypoxia is usually secondary to inflammation and bronchitis. Other consequences following exposure include tracheobronchial stenosis and life-threatening pseudomembrane (casts) formation. Leukopenia can also occur several days after exposure and suggests compromise of the immune pathway that can progress to suppressed bacterial clearance and sepsis. HD exposure has also been shown to affect the CNS. Most of the casualties observed following an inhalation exposure to HD were from pulmonary damage generally due to complications of bronchopneumonia, sepsis, and immunosuppression. Experiments done in animals decades ago employed various routes of administration, including inhalation, which produced convulsions, hyperactivity, and abnormal movements (Vedder, 1925). Anderson et al. (1996) demonstrated the formation of mucus casts within 24 h of exposure in a HD vapor rat inhalation model. Casts can cause ventilation perfusion mismatch due to clogged airspaces and lead to respiratory failure. Heully and Gruninger (1956) reported that three children accidently exposed to HD had abnormal muscular activity with one child alternating between coma and agitation. Two of these children died.

In large animal studies, such as with swine, 6 h after a 10-min exposure to HD vapor from 67 to 157 µg/kg body weight resulted in respiratory acidosis, increased shunt fraction, and hypoxemia at the higher concentrations. Elevated bronchoalveolar lavage (BAL) levels of IL-8 and IL-1β were measured in the high-dose exposed animals suggesting a pro-inflammatory stimulation associated with airway injury (Fairhall et al., 2010). The LCt_{50} of HD has been estimated to be 1,500 mg × min/m^3 (Seto, 2011).

Exposure Biochemistry

The mechanism of biotransformation of HD has been elucidated. Once HD enters the target organ and blood, it forms an intermediate sulfonium ion which is transformed into a carbonium ion considered to be a strong electrophilic species capable of reacting with highly nucleophilic sites (Figure 36.5). These are robust alkylating agents that react with cellular constituents, such as SH^- and NH^- in DNA, RNA, lipids, and proteins. Byrne and Stites (1995) have shown that HD forms crosslinks with cysteine residues in proteins. HD reacts with RNA and DNA molecules at such sites as N7 guanine, N3 adenine, and O^6-guanine. The O^6 position is particularly critical for the action of O^6-alkyl-guanine-DNA-alkyltransferase in DNA repair (Ludlum et al., 1986) (Figure 36.5). HD also forms DNA crosslinks. Kehe and Szinic (2005) have discussed the likely role of HD in alkylating membrane-bound proteins and enzymes. Zhang et al. (1995) have assessed the effects of HD on basement membrane components. Their results indicated that HD can alkylate and form crosslinks with membrane adhesion molecules such as laminin. This may have important consequences in the case of a high-dose inhalation exposure to HD, whereby it can reach and directly affect the alveolar epithelial cell layer. Bone marrow, a site of cell proliferation, is also a target of HD toxicity (Dacre and Goldman, 1996). In 2008, Kan et al. provided some evidence that there may be causal link between the inhalation of HD and cancer in rats exposed to a single high dose of HD (3 mg total dose over 10 min) (Kan et al., 2008). The conclusions of this limited study have yet to be supported by continued research. However, the results obtained may provide the incentive for future studies.

In rodent studies using intravascularly administered radiolabeled HD, distribution occurred in highly perfused organs such as the kidney, heart, liver, intestine, and lung within 1 h after injection (Maisonneuve et al., 1994). These organs are sites of biotransformation. Capacio et al. (2008) showed that HD-plasma protein adducts can be measured in the blood following a single 10-min exposure to graded increases of HD in a rat aerosol inhalation model.

The role of GSH in detoxification of HD has been somewhat controversial. Sciuto et al. (2007) have shown that there may be a biphasic response to HD intoxication. Testing for pulmonary toxicity using an intravascular HD injection model in rats, it was shown that at 1 h post-exposure, BAL revealed a dose-dependent inflammatory stage that decreased over time from 3 to 24 h. Macrophage inflammatory protein (MIP-2) was the predominant chemokine in the BAL followed by IL1β, IL10, and TNFα. A second phase involved changes in the antioxidant response pathway, which was mostly affected at 6 h post-challenge. HD exposure increased BALF protein, GSH, and the activities of GSH peroxidase (GPX), catalase, GSH reductase, and SOD (Sciuto et al., 2007). Laskin et al. (2010) summarized that in some animal models of acute lung injury with HD and HD-like compounds, antioxidant markers such as GPX and GSH

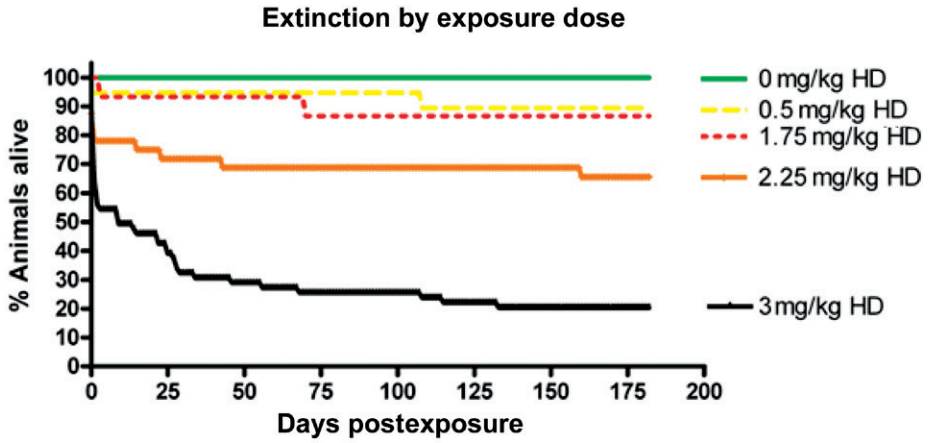


FIGURE 36.6 Shown here are 26-week survival curves of male rats exposed to a single 10 min inhaled HD. Concentrations represent the total dose delivered during exposure. Data indicate a delay in lethality based on dose. Exposures were done via intratracheal administration of aerosol in ventilated and anesthetized animals to avoid scrubbing in the nasal passages and providing a direct challenge to the lung. To control the rate of aerosol delivery, ventilation parameters (such as the tidal volume and the respiratory rate) were held constant for all exposed animals. *Source: (Olivera and Sciuto, 2014, unpublished results).*

increased, suggesting enhanced endogenous compensatory antioxidant activity. Brimfield et al., (2012) investigated mustard-induced, oxygen free-radical formation in an *in vitro* system using the spin-trapping technique. They concluded that the presence of peroxyl and hydroxyl free radicals could arise only when oxygen is reduced by carbon-based mustard radicals. The electron paramagnetic resonance (EPR) ROS signals disappeared by adding catalase and SOD to the reaction system.

Increased autophagy has also been reported in rats exposed to HD. Increases in LC3B-II and LC3B I were noted in rat lungs as early as 6 h and as late as 48 h post-exposure (Malaviya et al., 2010). Since this process might involve mitochondria, it is possible that HD might induce alterations in cell energy and metabolism (Uchiyama et al., 2008, from Malaviya et al., 2010). Dillman et al. (2005) showed that apoptotic pathways are also activated within 3 h of intravascular injection of HD in rats. This damage subsequently resulted in enhanced expression of proteins that arrest cell cycle progression and enhance programmed cell death. Presumably, apoptotic activation in the face of extensive DNA damage may be intended to avert multiple mutations that could lead to cancer formation. It is also apparent that tissue and cell damage is dose-related and does not appear to decrease over time, suggesting a long-term effect in the lung from a single HD challenge.

It is apparent from battle casualties (most recently from the Iran–Iraq war) that both soldiers and civilians continue to suffer debilitating compromised lung function 30+ years after initial exposure (Ghanei and Harandi, 2007). However, the long-term consequences of HD inhalation exposure have not been well examined in animals. Mortality at 3–8 and 15–19 weeks following a single HD intravenous exposure (3 mg over 10 min) occurred in rats (Figure 36.6). This dose approximately translates to LCt_{70} and LCt_{80}, respectively, over these time intervals. The data indicated that rats surviving a first phase with respect to lethality are not completely free of problems later. These data are supported by depressed respiratory function, which also suggests a link with temporal morbidity outcomes. Results from this long-term study in HD-exposed rats also revealed compromised respiratory function and symptoms of chronic obstructive pulmonary disease in rats that are similar to those described in soldiers and civilians exposed to HD during the Iran-Iraq war.

Biomarkers of HD exposure have focused on blood and urine evaluations. Historically, the metabolite thiodiglycol (TDG) was used as an indicator of exposure. However, the significance of linking TDG to HD exposure became problematic since TDG levels in the urine can be naturally elevated as a result of normal metabolism. However, as analytical techniques improved, more refined analyses revealed other metabolites that were indicative of actual HD exposure. In a large swine HD inhalation study, the monosulfoxide β-lyase metabolite of HD [1-methylsulfinyl-2-[2(methylthio) ethylsulfonyl) ethane] (MSMTESE)] was discovered in the urine (Fairhall et al., 2010). MSMTESE was present for up to 6 h post-exposure following a single 10-min inhalation challenge to 158 µg/kg HD. The detection of MSMTESE in the urine served as a valid biomarker for an HD inhalation exposure in this model.

Exposure Histopathology

In 15-year survivors of the Iran–Iraq war, histopathological examination of lung tissues revealed evidence of

increased asthma, chronic bronchitis, emphysema, airway stenosis, bronchiectasis, and lung cancer, and possibly lung fibrosis as well. The contributions of smoking and the presence of preexisting pulmonary disease as potential confounders against these conclusions were not taken into account.

Lewisite—b-Chlorovinyldichloroarsine (Agent L)

Lewisite (also called *Agent L;* MW = 207.3) is a colorless, oily liquid. Impure Agent L may have a faint odor of geraniums and could be blue-black in color. It is classified as a blistering, but not vesicating, agent due to its effects on the skin and eyes. Compared to HD, Agent L forms fluid-filled blisters accompanied by immediate pain. Exposure occurs through inhalation, ocular, or dermal routes. After acute dermal exposure, the liver, gallbladder, urinary bladder, lung, and kidneys are affected as well (Snider et al., 1990). The appearance of blisters can begin at any time, ranging from seconds to minutes based on exposure $[C] \times t$. Lesions resulting from various exposure routes are similar to HD. Hydrolysis products are more persistent than HD, and it is about 10 times as volatile. It is soluble in organic solvents and is readily absorbed by paint, rubber, and porous surfaces. As an organoarsenical, the enhanced reactivity of Agent L is linked to labile chlorine atoms, trivalent arsenic, and its capacity to form multiple chemical bonds with proteins. Agent L contains about 0.8–1.3 mg/mL of arsenic.

Exposure Physiology

Exposure to Agent L can occur through oral, dermal, ocular, ingestion, and inhalation pathways. Lewisite shock is observed following exposures to high levels. This mostly results from protein and plasma leakage from the capillaries, hypotension, and hemoconcentration. Agent L can readily penetrate the skin in both vapor and liquid form, causing systemic exposure; as little as 0.5 mL can cause serious problems, and at 2 mL, lethality can occur in humans. Vapor inhalation causes rhinorrhea, violent sneezing, and irritation to the nasal passages. These may be followed by expectoration of blood and coughing. According to Sidell (1997), Agent L does not cause bone marrow damage or immunosuppression. Clinically, large doses of arsenical compounds may lead to leukopenia. Since trivalent arsenic, As (III), is a major metabolite of Agent L, acute exposures might cause neurological damage. These may include loss of memory, problems in concentrating, anxiety, and confusion (Rodriguez et al., 2003). The inhaled LCt_{50} is estimated to be 1,500 mg × min/m^3 in mammalian species (Seto, 2011). These are close to earlier estimates of inhalation studies in mice, where the LCt_{50} for a 10-min exposure was 1,900–2,000 mg × min/m^3 (Silver and McGrath, 1943).

Currently, there is insufficient data on the carcinogenic effects of Agent L. Combustion by-products of Agent L, such as arsenic trichloride, vinyl chloride, and arsenic trioxide, have the potential to induce cancer. Arsenic trioxide and vinyl chloride are labeled Class A carcinogens by the U.S. Environmental Protection Agency (EPA 1984). However, it is not known if Agent L has any direct effect on cancer development. See Goldman and Dacre (1989) for a more detailed review on the chemistry of lewisite toxicity.

Exposure Biochemistry

Agent L will react with water to form chlorovinylarsenous acid (CVAA), which is slowly converted to arsinoxide. Metabolites can be found in the urine after exposure (Waters and Williams, 1950). It is highly distributed systemically, even after inhalation, due to its high lipophilicity. The reaction by-products include unstable chlorine atoms, As (III), and carbon. It is most likely that the metabolite As (III) is responsible for the observed systemic toxicity. As (III) can react as a nucleophile on thiol groups in proteins and lipids, particularly on lipoic acid, eventually forming alkylarsine sulfides. The binding of Agent L to sulfhydryl-containing proteins and enzymes inhibit pyruvate dehydrogenase activity, which is critical to glucose and fatty acid metabolism (Black, 2008). Agent L metabolites produce antioxidant imbalance, calcium metabolism imbalance, lipid peroxidation associated with oxidative stress, membrane impairment, and ultimately cell death (Sidell et al., 1997). Fidder et al. (2000) incubated labeled Agent L with human erythrocytes and found that 25–50% of the bound isotope formed protein adducts at cysteine residues of 93 and 112 of the β-globin molecule (Noort et al., 2002). The major excretion by-products of Agent L, including CVAA, are quantifiable markers in the blood that can be useful in detecting exposure.

Exposure Histopathology

There have been very limited studies on the toxicity of inhaled Agent L, specifically in the lungs. Decades ago, Vedder (1925) showed that in dogs exposed to Agent L, considerable nasal secretions, lacrimation, vomiting, and labored breathing were present until death. At postmortem, lungs were found to be edematous, with pseudomembranes extending from the nasal area to the bronchi. Airway mucosa was congested and edematous. Bronchopneumonia was also present. No other studies have systematically examined pulmonary pathological changes in animals exposed to Agent L.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The potential for human exposure due to intentional use or accidental release of CWAs and toxic industrial

chemicals will likely persist for many years. History has shown that exposure to these chemicals, regardless of the source, can be problematic for both civilians and military personnel. As such, continued study of the toxicity of these agents is of the utmost importance. All agents have the potential to cause significant morbidity and mortality by way of severely compromised pulmonary physiological homeostatic processes. The key target organ is the lung, and in many cases, regardless of the exposure route, respiratory failure is the cause of death. Subsequent exposure-related effects can involve altered respiratory function, compromised vital cellular metabolic/detoxification pathways, mitochondrial toxicity, hematotoxicity, and CNS toxicity. Other effects include immune response and associated release of cytokines and chemokines, altered antioxidant/oxidant pathways, increased free-radical formation, destruction of lung solute transport channels, and disruption of adaptive repair processes. All of the processes discussed here appear to be affected in a concentration-dependent manner; often with a latency period. For example, vesicant inhalation significantly alters the architecture of the lung to the extent that attempts to counteract the effects of damage through the innate repair process are compromised, leading to an obstructive pulmonary disorder.

The works cited herein show that although progress has been made in elucidating the toxicological, physiological, biochemical, and histopathological processes responsible for respiratory toxicity, much more effort is required to understand chemical-specific mechanisms and develop effective therapeutic measures. Three major issues will need to be addressed by future studies: (i) the development of a reliable, and appropriate animal inhalation exposure systems and the availability of human relevant animal models; (ii) examination of molecular mechanisms to identify potential therapeutic targets; and (iii) assessment of the long-term effects of inhaled CWAs. It is hoped that future efforts in this area will lead to the successful determination of therapeutic windows, treatment modalities, and effective medical countermeasures. A single therapeutic medical intervention likely will not be sufficient, but combinational therapies may be needed to counter the respiratory and systemic toxicities of CWAs.

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The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and the policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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CHAPTER

37

Cardiovascular System as a Target of Chemical Warfare Agents

Csaba K. Zoltani

INTRODUCTION

On contact with toxicants, the heart reacts immediately with changes in its electrical homeostasis. Ancillary indicators, such as changes in the metabolites in body fluids, are time-delayed and initially less useful for diagnosis and for selection of countermeasures. This chapter describes how the heart is affected by and reacts to various chemical warfare agents (Balazs, 1981; Baskin, 1991; Acosta, 2001). The first section gives an overview of the anatomy and functioning of the heart (Opie, 1998), with emphasis on the constituents most vulnerable to toxic presence. The importance of cardiac neuronal changes as possible indicators of exogenous effects is underscored. The normal electrical state and signature of cardiac toxicity are described, followed in detail by the palette of military warfare agents and their modes of action at the cellular level, including description of their expression in the changed morphology of the tissue and modified electrical state (Ellison, 2008; Romano et al., 2008). The effect of the different poisons on the heart is strongly dose-related. The references provide a good summary of the expected effects of each of the anticipated conditions. Electrocardiograms (ECGs) (Wexler et al., 1947; Karki et al., 2004; Delk et al., 2007) illustrating the effect of the toxicants are provided when available. New biomarkers such as miRNAs, which are short noncoding RNA sequences that regulate gene expression and control cellular processes, are revolutionizing the detection and treatment of cardiotoxicity. Approaches to therapy and recommended antidotes for some of the discussed toxicants are given.

BACKGROUND

Cardiac Anatomy

The timed delivery of oxygenated blood to all parts of the body is the function of the four-chambered pump, the heart. It is enclosed in a double-layered sac, the pericardium, with the inner layer, the visceral pericardium, anchoring the heart and the outer layer attached to the sternum. A cardiac skeleton anchors the four heart valves and the atria and ventricles. The thick wall separating the two ventricles, the interventricular septum, houses the Purkinje fibers, which play an important part in the electrical activity of the heart.

Deoxygenated blood from the body enters the heart by the vena cava and empties into the right atrium. Through the tricuspid valve, blood then transits into the right ventricle and via the pulmonic valve enters the lungs, where it is oxygenated. It returns to the heart through the pulmonary veins and the atrium to the left ventricle. This muscular pump redistributes the oxygenated blood to all parts of the body.

The heart is mediated by an electrical system that is easily disturbed by toxicants changing the timing, flow, and magnitude of the electrical pulses, and thus interfering with the required pumping action. The activity of the heart is myogenic (the activity is initiated by the heart itself). Various components of the heart, such as the sinoatrial (SA) node, atrioventricular (AV) node, and the Purkinje fibers, are capable of pacemaker activity. Healthy atrial and ventricular tissue does not engage in pacemaker activity. The primary pacemaker is the SA node, but in diseased or affected states the secondary pacemakers take over.

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The SA node lies in the right atrium in an epicardial location and functions as the primary source of the electrical impulse formation. It consists of specialized muscle cells smaller than ventricular cells containing few contractile elements. Because of its location, the action potential generated traverses the atria first, causing the primary contraction. The action potential is relayed to the AV node, the conduit for the electrification of the ventricles. The signal must pass through the AV node; otherwise, the atria and the ventricles are electrically insulated from each other. A time delay in transmission is needed for the atria to be emptied of blood.

The AV node is a subendocardial right atrial construct that is located within a fibrous stroma in the triangle of Koch. It is connected to both sympathetic and parasympathetic nerves. The bundle of His arises from the distal section of the AV node and goes to the summit of the ventricular septum. It is the only nonpathologic electrical conduction between the ventricles. The bundle of His divides into the left and right bundle branches that then become the Purkinje fibers that interweave the contractile cells of the ventricle and speed the excitation throughout the ventricles.

Action potential of the cardiac myocytes is initiated by inward sodium current in atria and ventricles, with inward calcium currents contributing to the upstroke in the SA and AV nodes. Slow inward currents result in lower resting and activation potentials. In the latter, membrane potentials range from −40 to −70 mV and activation threshold lies in the range of −30 to −40 mV and shows phase 4 depolarization. These cells are modulated by acetylcholine (ACh) and catecholamine. The duration of the action potential depends on the outgoing potassium current durations with the inward calcium currents of lesser importance. It is longest in the Purkinje fibers. Resting potentials of atrial and ventricular cells range from −80 to −90 mV, with activation thresholds in the range of −60 to −70 mV. Impulse conduction ranges up to 300 V/s in atrial and ventricular cells and up to 900 V/s in the Purkinje tissue.

B Innervation of the Heart

The autonomic nervous system guides the electrical and mechanical functions of the heart. The heart is innervated by both the sympathetic and parasympathetic systems, which have opposite effects and are activated reciprocally. They play important roles in arrhythmia susceptibility. Sympathetic stimulation originates from the intermediolateral column of the thoracic spinal cord. Its neurotransmitter, norepinephrine, is released from neurons of postganglionic fibers of stellate ganglia and epinephrine is released from the adrenal medulla. Both of these act on cardiac β-adrenergic receptors. Sympathetic nerves are predominantly on the epicardial surface. Receptors for norepinephrine on cardiac muscle are of the β-adrenergic kind. Postganglionic sympathetic neurons innervate the SA and AV nodes, the conduction system, and the myocardial fibers in the ventricles. Epinephrine, an amine, is the primary endogenous catecholamine produced in the adrenal medulla and regulates organic metabolism. It stimulates β_1 receptors, enhances ventricular contractility, and enhances SA nodal cell phase 4 depolarization, that is, impulse generation.

The cholinergic (i.e., parasympathetic system) acts through the vagal nerves by release of ACh that opposes the sympathetic stimulation. Parasympathetic preganglionic neurons originate in the medulla. Parasympathetic fibers terminate mainly on cells of the atria. Parasympathetic innervation is denser in the SA and AV nodes than the left ventricle. The right vagus nerve innervates the SA node. The neurotransmitter ACh and adenosine promote susceptibility to atrial fibrillation and shorten atrial refractoriness. Excessive stimulation causes bradyarrhythmia. The left vagus nerve innervates the AV node, where excessive stimulation results in AV block. Receptors for ACh are of the M2 muscarinic type and ACh binding to muscarinic receptors inhibits cAMP production. ACh has a negative chronotropic effect (slows heart), slows conduction (negative dromotropic effect), and also has a negative inotropic effect (decreases strength of contraction). The latter is through the activation of the current $I_{K,ACh}$, resulting also in shortening the action potential. ACh has three actions on cardiac muscle: it activates ACh-sensitive K-current, $I_{K,ACh}$; it inhibits the voltage time-dependent inward calcium current I_{Ca}; and it inhibits the hyperpolarization-activated inward current I_f, important for pacemaking. Ventricular muscle is not affected by vagal stimulation.

There are anatomical physiological differences between the sympathetic and parasympathetic systems. The parasympathetic ganglia lie within or close to the organ that postganglionic nerves innervate, whereas sympathetic ganglia lie closer to the spinal cord. There is limited parasympathetic innervation of the ventricles compared with the atria. The vagal nerve fibers are mostly intramural. The heightened adrenergic activation in the ventricles is potentially arrhythmogenic.

All catecholamine receptors are metabotropic. They act by initiating metabolic processes affecting cellular functions. β-adrenergic receptors, receptors for epinephrine, and norepinephrine act by stimulatory G proteins to increase cAMP in the postsynaptic cell. cAMP binds to and activates protein-kinase enzyme.

Disturbed balance between the parasympathetic and the sympathetic systems can result in disturbances in cardiac function. As discussed later, chemical warfare agents play an important role in disturbing this balance.

Neuropeptides

Vasoactive intestinal peptide (VIP), a neurotransmitter, is found in extrinsic and intrinsic nerves of the heart. VIP is released by the vagal nerve, and its effect is to increase I_f and pacemaker rates (Chang et al., 1994; Accili et al., 1996). VIP release takes place under high-frequency stimulation. As an internal brake, it limits the ability of ACh to excessively suppress the sinus node and other pacemakers. It also has an effect on the calcium-activated potassium channel.

VIP acts as a parasympathetic neurotransmitter in its involvement in the postsynaptic control of the heart. VIP is exactly opposite in its action to that of ACh. Vagal activity, causing release of ACh, slows the heart but co-release of VIP leads to tachycardia due to VIP-preferring receptors, leading to an increase in adenylate cyclase and accumulation of cAMP.

Neuropeptide Y (NPY) suppresses the pacemaker current I_f. Colocalized with norepinephrine in sympathetic nerve terminals on the heart, it is released with catecholamines during sympathetic neural activation. NPYs on cardiomyocytes have surface membrane binding sites and suppress contractility in concentrations of 10^{-9} or more. In ventricular myocytes, the L-type Ca^{2+} current as well as the delayed rectifier potassium currents are suppressed.

Energetics of the Heart

The heart requires a continuous supply of energy to be able to sustain its pumping action. Most of the energy is derived from fatty acids. Under ischemic or anaerobic conditions, glycolysis comes into play, consuming large amounts of glucose with the adverse effect of the formation of lactic acid (Jafri et al., 2001).

More than 95% of the metabolic energy is used in the form of adenosine triphosphate (ATP). Its concentration in a myocyte is approximately 10 mM. ATP is synthesized by oxidative phosphorylation in the mitochondria. There, acetyl CoA is broken down to CO_2 and hydrogen atoms. Electrons are pumped out to form a proton gradient across the mitochondrial membrane. The protons re-enter the mitochondria and combine with oxygen, eventually forming water.

The glycolytic pathway under hypoxic conditions produces only a limited amount of ATP. Pyruvate formed in glycolysis is transported into the mitochondria, where CO_2 is formed through cellular respiration. From each glucose molecule, 28 ATP molecules are formed in anaerobic glucose metabolism. The heart also has an energy reserve in the form of phosphocreatine (PCr), which is an immediate precursor of ATP. In the reverse Lohman reaction, creatine kinase (CK) favors the maintenance of the ATP concentration at required levels. The energy released during hydrolysis of the phosphoanhydride bond in ATP powers energetically unfavorable processes, such as the transport of molecules against a concentration gradient.

During conditions of hypoxia, ATP is first degraded to adenosine diphosphate, then to adenosine monophosphate, and then to adenosine. The latter diffuses into the circulating blood, where adenosine concentration rapidly increases under conditions of cellular damage. The action of adenosine is anti-inflammatory as well as inhibitory.

Electrophysiology

The sinoatrial node (SA), consisting of spindle-shaped cells, initiates the electrical activity of the heart. From its location in the right atrium in proximity to the superior vena cava, the electrical activity spreads to the atria, whose cells are larger than those of the SA. The pulse from the atria spreads to the atrioventricular node (AV), the gateway to the ventricles. The atria and the ventricles are electrically isolated. The AV node also slows the electrical activity, giving the atria time to fill. The bundle of His is the upper end of the electrical path, which through the Purkinje fibers allows the electrical signal to activate the ventricles and thus to pump the blood.

Each cell's activity goes through four phases of the action potential (Grant, 2009). The morphology of each type of cell is different. Also, the kinds of electrolytes moved and their quantity and speed are dictated by environmental conditions (Carmeliet and Vereecke, 2002). Xenobiotic interference with the transmission of the ions changes the electrical homeostasis and commences breakdown in the state of the tissue. The membrane currents, pump, and exchanger of importance in this regard are given in Table 37.1. Anionic chloride

TABLE 37.1 Membrane Currents, Pump and Exchanger of Importance

Current	Function/Origin	Effect
I_{Na}	Voltage-gated Na^+ current	Depolarization
I_{K}^+	Voltage-gated K^+ channel	
I_{Ca}^{2+}	L-type Ca^{2+} current	
I_{Cl^-swell}	Activated by cell swelling	Cell swelling
$I_{K,ATP}$	Activated by fall in intracellular ATP	Fall in ATP
I_f	Hyperpolarization-activated current carried by Na^+ and K^+ in sinoatrial and AV node cells and His-Purkinje cells and contributing to phase 4 depolarization	
$I_{K,ACh}$	Parasympathetic control of the heart	ACh
Na^+/K^+-ATPase	Moves Na^+ out, K^+ into cell against concentration gradient using ATP for energy	Stimulated by α, β recap. via PKA, PKC
Na/Ca	Exchanges intracellular Ca^{2+} for extracellular Na^+	Responsible for DAD

currents, especially swelling-activated, play an important role in the case of cyanide intoxication (Baumgarten and Clemo, 2003).

SIGNATURES OF CARDIAC TOXICITY

The ECG as a Diagnostic Tool for Poisoning

Recorded Morphological Changes on the ECG

At present, morphological changes of an ECG are insufficient to make a definite diagnosis regarding the nature of a poisoning; however, they are useful in giving guidance regarding which of the ion currents are affected and thus to the possible nature of the toxicity (Yates and Manini, 2012). Without access to and comparison with an ECG before an incident and the multitude of factors that need to be considered, the diagnosis may be erroneous. However, poisons cause definite changes in timing and morphology of the ECG of affected individuals (Dalvi et al., 1986; Gussak et al., 2004; Delk et al., 2007; Zoltani and Baskin, 2007), and thus an ECG constitutes an important tool in the initial evaluation.

Disequilibrium in the electrolyte balance can provide diagnostic clues. For example, hyperkalemia causes tall T-waves in leads II, III, and V_2 to V_4 when the potassium balance exceeds 5.5 mmol/L. In conjunction, the amplitude of the P-wave is reduced and QRS is widened. Hyperkalemia is usually present when the amplitude of the T-wave is higher than that of the R-wave. With increasing potassium concentration, P-waves widen and eventually disappear. Accentuated hyperkalemia results in asystole.

Hypokalemia results in decreased T-wave amplitude and ST-segment depression; however, accurate QT interval measurement is difficult. Malignant ventricular arrhythmias result when potassium concentrations become very low. Hypercalcemia shortens the QT interval, whereas hypocalcemia produces ST-segment prolongation.

Left bundle branch block is characteristic of poisoning and is defined by Zimetbaum et al. (2004) as QRS more than 0.12 s with delayed intrinsicoid deflection in the V_1, V_5, and V_6 leads more than 0.05 s. The risk of arrhythmia is greatest when QRS is 0.11 s or more. Right bundle branch block more than 0.12 s is a fairly good predictor of arrhythmic death.

Blockade of potassium, sodium, calcium channels, β-adrenergic receptor sites, and the Na^+/K^+-ATPase pump change the ECG and may give an indication of the type of poisoning present in the tissue (Delk et al., 2007).

Reductions in the outward potassium ion flow prolong QT, a harbinger of Torsade de Pointes (TdP), and ventricular fibrillation (VF). Sodium channel blockers delay the entry of sodium ions, widening the QRS complex. In extreme cases, asystole ensues. A subsidiary event may be ventricular tachycardia degenerating into VF.

For control of tachyarrhythmia, calcium channel blockers are used. Blockade of these channels decreases contractility and SA and AV node depolarization. Blocking β_1 receptor sites within the myocardium reduces the intracellular calcium concentration and the contractility. The potassium concentration is increased, which is an undesirable effect when the β_2 receptors are blocked.

Inhibition of the Na^+/K^+-ATPase pump results in $[K^+]_o$ and intercellular $[Na^+]_i$ increases, thus increasing the intracellular calcium concentrations.

Long QT

Important predictors of arrhythmia are changes in the duration of the QT trace, the time for ventricular repolarization, displacement of the ST-segment, and changes in the pattern of T-waves that may sometimes be seen as a T-wave and U-wave (Roden, 2008). It can be linked to ventricular tachycardia, including TdP. Lengthened QT increases the time available for intracellular calcium accumulation, enabling early after-depolarization (EAD) in the Purkinje fibers, and activates calmodulin (CaM) and calmodulin kinase (CaMK). CaMK is believed to enhance after-depolarization, triggering TdP. CaMK increases L-type calcium channel activity. Anderson (2006) reported that inhibition of CaMKII can prevent cellular arrhythmia when QT-prolongation is present (Zoltani and Baskin, 2007).

During the plateau phase of the action potential, there is a delicate balance between the L-type Ca^{2+} channels and the slowly and rapidly activating cardiac delayed rectifier currents, I_{Ks} and I_{Kr}. Due to the slow onset of I_{Ks} activation during the rectification of I_{Kr}, a limited amount of repolarizing current flows. The L-type Ca^{2+} channels are time-dependently inactivated while an outflowing potassium current occurs. With a net outflow of ions, repolarization takes place. It is critical that the I_{Kr} deactivates slowly. Thus, small changes in the inward or outward conductance, for example, the amount of current leaving or entering, have a deciding influence on the length of the repolarization. The effect of poisons on these processes plays an important role in determining the QT-segment length.

Biochemical Markers of Tissue Injury

Conventional Biomarkers

Protein markers released into the bloodstream have been used as indicators of cardiac injury. The gold-standard marker currently is troponin. Three types of troponin, as well as elevated creatine kinase–MB (CK–MB) levels and peripherally myoglobin, are used as indicators of cardiac tissue damage (Apak et al., 2005;

O'Brien, 2008). Biomarkers of heart failure (Mittmann et al., 1998), including natriuretic peptides that increase in response to wall stress and ST2, reflective of ventricular remodeling and cardiac fibrosis may also be a consequence of toxicity and are detailed by Maisel (2012).

Cardiac troponin complex consists of three parts. Troponin T facilitates contraction, troponin I (cTnI) inhibits actin–myosin interactions, and troponin C binds to calcium ions. Troponin I and T are specific to the heart, but cTnT is also expressed by injured skeletal muscle. In the course of cell damage, cardiac troponin is released from myocytes, facilitated by increased membrane permeability that allows smaller troponin fragments to traverse the membrane. Complicating the use of troponin levels is the fact that in cases where there is cardiac injury without cardiac cell membrane disruption, serum troponin level can increase. Also, altered ion homeostasis may not be reflected in troponin levels.

In cancer patients undergoing high-dose chemotherapy, elevated troponin I and CK–MB are predictors of ventricular systolic dysfunction, and thus are indicators of cardiac damage. Immunological ultraviolet-array is used for the determination of the level of CK–MB, where an elevation greater than twice normal is taken as an indicator of myocardial infarction (MI). Cardiac troponin is measured by electrochemiluminescent immunoassay. Cardiac impairment is indicated by the presence of troponin. The release of the enzymes has been linked to a mismatch between oxygen demand and expenditure in the tissue, lessening the time available for diastolic coronary perfusion, catecholamine release, and intense sympathetic stimulation. Warfare agent–associated cardiac toxicity is characterized by these conditions.

Is the increased level of troponin due to damage to the heart muscle? In-depth studies note that stroke victims without acute MI have elevated troponin levels. Severe renal dysfunction may also be a cause. Thus, until convincing proof is presented for noncardiac causes, increase in the presence of marker enzymes can be taken as an indicator of damage due to toxins in the cardiac tissue.

CK is an enzyme necessary for ATP generation. One of its forms, CK–MB, is found mainly in the myocardium and, on tissue damage, such as MI, becomes elevated. It takes up to 24 h for the elevated level to reach its peak. A difficulty resides in the fact that unlike troponin, an assay does not distinguish between cardiac and skeletal muscle damage. Also, in approximately one-third of MI cases, although CK–MB levels stay neutral, troponin elevation is noted. The normal level of troponin in the blood for troponin I is less than 10 μg/L and that for troponin T is 0–0.1 μg/L, whereas for CK–MB it is less than 3.0 ng/mL. For humans, the CK is in the range of 55–170 IU/L (international units per liter) and is less specific than CK–MB for cardiac tissue damage.

miRNA

Traditional biomarkers lack tissue specificity and their appearance in body fluids is time-delayed. For example, troponin levels are elevated between 3 and 12 h after myocardial injury. Recently, microRNAs have gained acceptance as alternate biomarkers (Yamakuchi, 2012; Gidlof et al., 2013; Sahu, 2013; Vickers, et al., 2014). Their tissue specificity, sensitivity, stability, and timeliness underlie their usefulness and have opened new therapeutic approaches. These noncoding RNAs play an important role as gene expression regulators, and miRNAs regulate fundamental cellular functions and toxicological outcomes. The number of identified miRNAs is large, and they regulate the majority of mRNAs.

miRNAs are short, 22-nucleotides-long, and modulate protein expression by binding to mRNA, thereby inhibiting translation or targeting them for degradation. As regulators of physiological processes, they play an important part in the developing pathology (Kim, 2013; Vacchi-Suzzi et al., 2013).

Biogenesis of miRNAs starts in the nucleus of the cell where miRNA genes are transcribed by RNA polymerase into primary miRNAs. The Drosha enzyme then creates the precursor miRNA. After export to the cytoplasm, cleavage by the Dicer enzyme produces the miRNA. The mature miRNA is incorporated into RISC (the RNA-induced silencing complex). The miRNA of the RISC complex binds to the target messenger RNAs preventing translation. After myocardial infarction, for example, miRNA levels increase signaling-reduced systolic function when the left ventricle ejection fraction is less than 50%.

Microvesicles shed from the cell, such as exomes, export the miRNAs and transport them to other locations, in effect constituting the intercellular communication and thereby regulating genetic function and protein generation.

In drug toxicity (Yokoi and Nakajima, 2013) as well as oncology, miRNAs are becoming useful tools in making diagnoses. As shown in Section VII, miRNAs offer new therapeutic approaches for warfare agent–caused cardiac toxicity.

INDICES OF THE TOXICITY OF WARFARE AGENTS

Classes of Warfare Agents

Three broad classes of warfare agents are considered: organophosphate (OP) nerve agents (Munro et al., 1994; Newmark, 2007); the cyanides (Baskin et al., 2009); and a third general category that is less weaponized but whose effects are important as potential terror agents, the arsenics and ricin.

Respiratory tract irritants such as phosgene and vesicants, including sulfur mustard, have subsidiary cardiac impact but the mode of disability is acute lung injury.

The classes of nerve agents of primary interest are the "G" agents, first synthesized in Germany. Denser than air, these agents hug the ground and represent a vapor hazard due to their volatility. Tabun (GA), sarin (GB), and primarily soman (GD) are the ones most widely used. "V" (venomous) agents were developed in the United Kingdom. VX is more toxic than the G agents. Novichok, developed in the Soviet Union, is the most potent nerve agent, but there is little public information available. Cyanides are less weaponized but are of concern. Arsenic and ricin are considered terror threats rather than battlefield weapons.

Background

Chemically induced cardiac failure has been the subject of a number of works. Baskin (1991) and Acosta (2001) provided an overview of the subject. Hypoxia is one of the effects of the decreased availability of ATP that decreases contraction in the muscle. The energy that is supplied by the phosphate bonds is possible only as long as aerobic glycolysis and oxidative phosphorylation are maintained. Under anoxic conditions this is no longer possible, and the adrenergic stimulus continuing calcium accumulation in the mitochondria leads to impairment of function, eventually causing dose-related lesions (Suzuki, 1968).

Signatures of Toxicity

Warfare agents disturb cardiac homeostasis. The main changes of concern include the following:

- Morphological tissue changes
- Enhanced neural stimulation
- Release of neuropeptides
- Activation of usually dormant cardiac currents
- Alteration in the storing and movement of ions
- Disturbances in energy use and storage

Ferrans et al. (1969) noted ultrastructural changes in myofibrils, including mitochondrial swelling and disorganization. Catecholamines also produce changes in the activity of oxidative enzymes. The decline in the activity progresses to the point at which necrosis is evident. Ancillary effects include loss of myocardial potassium and an increase in interstitial fluid. Intracellular calcium overload also develops as noted by Fleckenstein et al. (1974). It has been hypothesized that necrosis due to catecholamine overload may be caused by a defect in energy supply needed for the maintenance of cellular processes.

At low concentrations, the catecholamines, (epinephrine and norepinephrine) exert positive inotropic effects on the myocardium. High concentrations, however, can cause cardiac lesions (Inoue et al., 1998). Even physiologic concentrations, when extended over time, lead to cardiac damage, as shown by Szakacs and Mellman (1960). The LD_{50} of norepinephrine in rats is 680 mg/kg, but focal necrotic lesions are produced at doses as low as 0.02 mg/kg.

The oxidation product of catecholamine is adrenochrome, whose accumulation has also been linked to myocardial necrosis and morphological and subcellular alteration (Yates and Dhalla, 1975). Administration of adrenochrome to rats also induced heart arrhythmias. Free radicals may also contribute to these processes. It has been suggested that catecholamines may activate β-adrenergic receptors, stimulating adenylate cyclase, and thus elevating cAMP. This, in turn, activates protein kinase, increasing the phosphorylation of slow calcium channels and possibly resulting in overflow of calcium, leading to necrosis preceded by swollen sarcoplasmic reticulum (SR), altered enzymatic activities, and lower ATP.

Cyanide primarily blocks oxidative phosphorylation and ATP production. Every heart beat uses up to 2% of the energy available to the cell. Arsenic primarily causes long QT (LQT) interval on the ECG by blocking the fast potassium current, an action that is a precursor to VF.

The activation of dormant currents by the presence of xenobiotics, including cell swelling, radically changes the electrical homeostasis of the tissue (Zoltani and Baskin, 2007). Toxicities of major weaponized agents are given in Table 37.2.

Nerve Agents

There is a difference in distribution of nerve agents for different organs of the body as well as different locations within the heart. Roth et al. (1993) detail effects on the heart. Also, soman is deposited in "depots" where time-release effects cause unsuspected difficulties in treatment.

TABLE 37.2 Toxicities of Weaponized Agents

Agent	LCt_{50} (mg min/m^3)	ICt_{50} (mg min/m^3)	LD_{50} (mg/kg)
GA	400	300	14.28
GB	100	75	24.28
GD	70		5.0
VX	50	35	0.1428
Cyanide	2,500–5,000		1.1

Mechanism of Action

Nerve agents are OP compounds that irreversibly inhibit AChE, leading to ACh accumulation, and cause overstimulation of muscarinic and nicotinic ACh receptors. The effect at the SA node, the primary site of heart control, is inhibitory and bradycardia results. VX primarily affects neurotransmitter receptors, those of norepinephrine, and also affects the central nervous system not related to AChE inhibition.

Toxicants change the homeostatic distribution and timing of the presence of electrolytes, enzymes, and other constituents of myocytes and their environment. These changes affect and determine the current flow, in turn affecting the electrical state and possibly signaling the onset of electrical instability. Table 37.2 shows toxicities of weaponized nerve agents.

Electrocardiographic Signature of OPs

Disturbances in the electrical activity of the heart caused by xenobiotics are readily discernible in a surface ECG (Dalvi et al., 1986; Chuang et al., 1996; Yurumez et al., 2009).

OPs cause QT prolongation on the ECG that subsequently can degenerate into TdP. In one reported OP case, 79.7% had QT prolongations with ST-segment and T-wave abnormalities (Rubinshtein et al., 2002; Karki et al., 2004). Changes are ultimately expressed in arrhythmia, VF, and TdP, and severe disturbance of the energy homeostasis of the heart. Second-degree atrioventricular heart block as well as ST–T-wave alterations (Balali-Mood and Saber, 2012) have also been observed.

An initial indicator of an impending arrhythmia is prolongation of the QT interval. The lengthening can be caused by reduction in the outward currents or increased inward currents, for example, imbalance of inward and outward currents during the second and third phases of the cardiac cycle, such as the I_{Kr}, I_{Ks}, the $I_{Ca(L)}$, and I_{Na} currents. These currents can generate EAD and trigger activity at the end of repolarization. Primary sites are the Purkinje fibers and the mid-myocardial M cells. In the Purkinje fibers, at higher positive resting potentials than the ventricles, blockade is voltage-dependent, with increased block in the depolarized tissue. The failure of complete repolarization leads to dispersion of refractoriness and enhanced arrhythmogenicity. Acquired LQT interval involves pause-dependent or short–long–short RR interval sequences on the ECG and enhancement of sympathetic nervous system tone. LQT is favored in cases of severe bradycardia, hypokalemia, and conditions that lead to EAD. LQT may not be a sufficient condition for TdP. Stimulation of adrenergic receptors plays a significant role, but it can enhance or inhibit after-depolarization.

The electrical activity of the heart is modulated by hormones and neurotransmitters. Xenobiotics disturb their balance. The parasympathetic system releases ACh and the sympathetic system releases norepinephrine and epinephrine. These bind to α and β types of receptors. The α_1-receptors are present on the postsynaptic member of the organ and mediate vasoconstriction and stimulation of Na^+/K^+-ATPase, the Na^+/Ca^{2+} exchanger, and the Na^+/H^+ exchanger. This affects the I_{KATP} and inhibits the $I_{Na}{}^+$ and I_{to}. The α-receptor stimulation thus effectuates depolarization, and the α_2-receptor inhibits norepinephrine release.

Cardiac β_1-receptors and atrial and ventricular β2-receptors take part in positive inotropic response. Inhibition of catecholamines at β-adrenergic receptor sites interrupts the production of cAMP and inhibits calcium influx, producing a negative inotropic effect that yields a reduction in the heart rate.

The main effect of adrenergic stimulation is to enhance the intracellular adenylyl cyclase activity. This, in turn, increases cyclic adenosine monophosphate levels. Protein kinase A is activated, thus modulating phosphorylates, calcium, and potassium channels. Phosphorylation of the calcium channel increases the inward current leading to EAD.

β_1-Adrenergic stimulation increases the activity of the Na^+/K^+ pump and inhibits EAD. Hyperpolarization of resting membrane potential counteracts automaticity, leading to a decrease in heart rate. α_1-Adrenergic stimulation hyperpolarizes membranes and enables EAD and TdP by blocking potassium channels. Release of norepinephrine enhances α_1-adrenergic stimulation, facilitating EAD (Schomig et al., 1995). In LQT, increased sympathetic discharge may induce both bradycardia and TdP. Increased adenosine increases ventricular refractoriness and sympathetic tone setting the conditions for re-entrant arrhythmias. Hypokalemia prolongs cardiac action potential and may be the precursor of EAD. The net outward potassium currents are reduced and the inward calcium current is increased.

Toxic Effects of OPs on the Heart

The anticholinesterase effects of nerve agents depend on the sites where they act. The muscarinic effects in the heart act through the parasympathetic system, whereas the nicotinic effects act through the sympathetic system. Agents like tabun, sarin, and soman, analogous to ACh, are capable of changing the receptor sites, which increases the conductance of electrophysiological signals related to the enhancement of neuromuscular function. VX, however, counteracts the effect of ACh, interrupting neuromuscular function.

ACh is a neurotransmitter that is present in the parasympathetic nervous system and is stored in vesicles. Its function, under normal circumstances, is terminated

by AChE that is present in both the presynaptic and postsynaptic membranes. OPs inhibit AChE by electrophilic (a reactant that accepts an electron pair from a molecule with which it forms a covalent bond) attack of the enzyme. The neurotoxicity of OPs was exploited in the development of nerve gases. The key to the understanding of OP inhibition of AChE is the enzyme's serine hydroxyl group behavior. Normally, it attacks ACh at the carboxyl carbon, yielding a covalent bond between the enzyme and the ACh substrate. The transiently acetylated enzyme is next hydrolyzed and the active enzyme site is regenerated. The active serine hydroxyl group is attacked by the electrophilic phosphorus of the OP instead of the ACh. A covalent nonhydrolyzable bond is formed between the enzyme and the OP, leaving the enzyme in an inactivated form. Release of an alkyl chain further strengthens the phosphorus–enzyme bond. With AChE inactivated, the amount of ACh present increases, resulting in overstimulation of the tissue. The pathophysiological effects that result can be explained by the overabundance of the ACh. Overstimulation of muscarinic receptors in the heart leads to bradycardia.

SPECIFIC WARFARE AGENTS OF CONCERN REGARDING THE HEART

Nerve Agents

The heart may be affected by both muscarinic and nicotinic effects. Stimulation of the parasympathetic nerve endings (in the former) and excess ACh on the nicotinic receptors (in the latter) are of importance. The cardiovascular effects are tachycardia caused by the overstimulation of the sympathetic system, bradyarrhythmias, atrioventricular block, hypotension and QT prolongation, VF, and TdP (Grmec et al., 2004).

OP intoxication manifests itself in three phases. First, nicotinic phase hypertension and sinus tachycardia occur. Second, sinus bradycardia and parasympathetic overstimulation and ST-segment changes on the ECG and rhythm disturbances occur. During the last phase, TdP and sudden cardiac death occur. According to Ludomirsky et al. (1982), a QTc (QT corrected for heart rate) of 580 ms signals high probability of sudden cardiac death. Roth et al. (1993) provided further insight from actual cases.

A breakdown of actual cardiac symptoms for OP poisoning in hospital admissions is given by Karki et al. (2004). Sixty-seven percent of the acute OP cases had QT prolongation, 24% experienced ST-segment elevation, and 17% had inverted T-waves. Nine percent had atrial tachycardia, 9% had ventricular tachycardia, and 4% had VF. Sinus tachycardia was observed in 35% of admissions, whereas sinus bradycardia was noted in 28%. Noting that acidosis and electrolyte derangement play a major role in the development of cardiac events, they recommend "atropine in adequate doses very early in the course of the illness" as the strategy to be implemented. Table 37.3 summarizes the effect of OP on the electrophysiology of cardiac tissue.

OPs are known to induce time-delayed neurotoxicity. This is due to the inhibition of an esterase in nerve tissue, neuropathy target esterase (NTE), that is also found in muscle and blood cells. The NTE level in the blood is an indicator of the inhibition of the enzyme. Inhibition of NTE, aging, and the process of following the OP binding to an active esterase site that prevents the reactivation of the site are important for selection of an antidote against certain OP nerve agents. It is of primary concern for the Novichok agent. There is little information available regarding its neurotoxicity and cardiac toxicity caused by OP.

VX

The most toxic nerve agent available in the West is VX. It is an inhibitor of AChE, which acts by increasing the ACh at the nerve synapses. Toxicity sets in when more than 50% of the AChE enzyme is inhibited. The $AChE_{50}$ value for VX is taken as 0.023 mg/kg in an oral dose (Sidell, 1974). Activities of RBC-AChE and plasma butyrylcholinesterase (BChE) are other markers of toxicity of OPs. In tests on human volunteers, 1 μg of VX/kg by intravenous infusion showed a decrease in AChE activity of more than 50%, but for these tests no cardiac symptoms were recorded. VX produces intense stimulation of nicotinic ACh receptor ion channels and muscarinic ACh receptors. Although not recorded in human volunteer tests, cardiac effects are known to take place based on animal studies. VX exposure produces positive inotropic effects. Arrhythmia was noted in rats and dogs (Robineau and Guittin, 1987). In guinea pigs treated with VX, delayed after-depolarization was found (Corbier and Robineau, 1989). The effect of VX is ascribed to inhibition of the rat cardiac Na^+/K^+-ATPase α_1 isoform. At a concentration of 1 μM, the inhibition is 35% (Robineau et al., 1991). Physostigmine, hyoscine, and HI-6 are undergoing investigation as antidotes (Munro et al., 1994; Wetherell et al., 2007).

Tabun (GA)

GA, a unitary chemical munition, inhibits AChE, the enzyme responsible for the breakdown of the neurotransmitter ACh. When inhaled, its toxicity is half that of sarin. It decreases plasma and RBC-AChE activities significantly in the blood. At 20–25% of RBC-AChE baseline, the effect of the nerve agent becomes noticeable. There is no evidence of systemic toxicity other than the cholinesterase activity (Parker et al., 1990; Munro

TABLE 37.3 Effect of OP on the Electrophysiology of Cardiac Tissue

	Lesions				
AChE	**Anoxia**	**Acidosis**	**Modulated Ion Concentration**	**Release of Catecholamines**	**Second Messenger (VIP, Others)**
ACh overload causes bradycardia, slows conduction in AV, prevents hydrolysis $[Ca^{2+}]\uparrow \rightarrow I_{K,ACh}\uparrow$	Lowers ATP, cAMP; ATPase inhibition $I_{K,ATP}\uparrow$ (activated), AP shortened	pH↓, Na^+/H^+ exchange↑, [ATP]↓, $[K^+]_o\uparrow$, reduces I_{Kr} by increasing rate of deactivation, shifts voltage dependence of activation to more positive potentials, $g_K\uparrow$	$[K^+]_o\uparrow$ effect on velocity of propagation, inexcitability	Prolongs AP, $[Na^+]\uparrow$, Na^+/K^+-ATPase antagonized DAD enhanced difference for α, β receptors	Adenylate cyclase activation
Antagonizes adenylyl cyclase		Cytoplasmic $[Ca^{2+}]\uparrow$ slows repolarization, reduces max. diastolic potential	$[Na^+]\uparrow$ Na^+/K^+ pump inhibition, Na^+/Ca^{2+} exchanger $\rightarrow Ca^{2+}$ influx	α stimulation: reperfusion arrhythmia, calcium overload, gap junction conductance↓, exchanger stimulation, activitates Na^+/K^+ pump	$I_f\uparrow$, cAMP↑, →HR↑ OP reduces cAMP $\rightarrow Ca^{2+}$ influx, inhibits adenylate cyclase, stimulates ATP, $I_K\uparrow$, affects $I_{Ca(L)}$, EAD, DAD→arrhythmia
Arrests cAMP synthesis, depresses I_f (pacemaker current)		$I_{Ca(L)}\downarrow$, $I_{Na}\downarrow$ (inactivation of fast Na^+ channel), decreased excitability, CO_2 accumulation	$[Ca^{2+}]\uparrow$ Na^+/Ca^{2+} exchanger↓ reduced SR uptake		
ACh inhibits adenylate cyclase			$[Mg^{2+}]\uparrow$ (hydrolysis of ATP), activates enzymes, reduces $I_{Ca(L)}$, I_{K1}, $I_{K,ACh}$, I_{KATP}, I_{Ks}	β stimulation: adenylate cyclase, elevates cAMP, increases calcium influx, I_f activation, triggered activity improves modal conduction	
Intermediate stage					
AF, VF					

g, conductivity of the tissue; [..], concentration; $I_{(.)}$, ionic current with the subscript denoting the channel type; ↑, increase; ↓, decrease; →, yields.

et al., 1994). GA has not been shown to produce organophosphorus-induced delayed neurotoxicity except at extremely high doses. The cardiac effect of GA conforms to OP-caused arrhythmias and AV block.

Sarin (GB)

Sarin was involved in terrorist attacks in Japan. The increase in sympathetic and parasympathetic tone results in tachycardia, ST-segment modulation (Abraham et al., 2001), and arrhythmia. Inhibition of cholinesterase within the neuroeffector junction also affects nerve impulse transmission by direct action. Direct action on muscarinic or nicotinic ACh receptors (Somani et al., 1992) is observed when the blood level of sarin exceeds the micromolar level. Sarin inhibits RBC-AChE 80–100% and plasma-BChE between 30% and 50% (Grob and Harvey, 1958). It also binds to aliesterase, an enzyme that contributes to ester-link hydrolysis.

Sarin exhibits OP-delayed cardiotoxicity. Allon et al. (2005) suggest epinephrine-induced arrhythmias as a possible cause in cases of sarin intoxication. The hypothesis is supported by Khositseth et al. (2005), who showed that epinephrine changes T-waves in the ECG where AT prolongation already exists.

Soman (GD)

The most widely used nerve agent, soman (GD), exerts a defining effect on cardiovascular function. Myocardial degeneration and necrosis were noted by Britt et al. (2000) in soman-exposed rhesus macaques. Generally, on contact, bradycardia and modulated cardiac output are followed by hypotension and changes in the ECG. Notably, AV conduction modulation, QT extension, T-wave, and ST-segment changes characteristic of myocardial infarction and ACh-induced coronary vasospasm are noted. For the cardiac effects of OP, important references include Sidell (1974), Kiss and Fazekas (1983), and Anastassiades and Ioannides (1984). McKenzie et al. (1996) also showed that in swine a dose of $2 \times LD_{50}$ soman intravenously increased coronary sinus plasma ACh by 314% and decreased coronary blood flow to 55% of control. The evidence indicates OP-caused deaths are due to ACh-caused coronary vasospasms that culminate in MI. This is seen as VF on the ECGs. Recently, the

Food and Drug Administration approved pyridostigmine bromide as a pretreatment for soman poisoning (Newmark, 2007).

Novichok

In literature, little is known about these agents developed in the Soviet Union. They are assessed to be 5-times to 10-times more toxic than VX (Smithson et al., 1995; Ellison, 2008). The toxicity of these binary agents does not rely primarily on the inhibition of AChE, but it is thought that they cause permanent neuropathy. Consequently, conventional nerve agent antidotes may not work. Reactive oximes such as potassium 2,3-butanedione monoximate may be useful in detoxification. No published information is available for cardiac pathologies caused by Novichok agents.

Antidotes for OP Nerve Agents

Enzymatic hydrolysis is a primary route for elimination of nerve agents. Specifically, treatment for OP intoxication includes atropine, a muscarinic receptor antagonist, an anticonvulsant such as diazepam, and a cholinesterase reactivator, an oxime. It has been found that drug-induced inhibition of ACh release and accumulation in the synaptic cleft, such as adenosine receptor antagonist early in the OP intoxication, improve the chances of survival. Some AChE reactivators, such as bispyridinum oximes, HI 6, and HLö 7 with atropine, are quite effective. A large number of other oximes are also undergoing investigation (Balali-Mood and Saber, 2012).

Cyanide

Classified as a blood agent, cyanide is usually deployed as hydrogen cyanide and cyanogen chloride. Considerable literature exists on the effects of cyanide (Suzuki, 1968; Baskin et al., 2009). Cyanide binds irreversibly to its target sites. In the human host, it preferentially accumulates in the hypothalamus and neural tissue. Its concentration in red blood cells is much greater than in plasma. Lethal dose is approximately 1 mg/kg or inhalation of 50 mL of hydrogen cyanide gas.

Toxicity

Cyanide binds to Fe^{3+} in heme-containing proteins. This inhibits the terminal cytochrome complex IV of the electron transport chain. The blocklock of complex IV by cyanide depletes ATP culminating in cell death. Oxygen is unable to reoxidize the reduced cytochrome a3. Thus, cellular respiration is inhibited, as well as ATP production, in essence depriving the cells, tissue, and, ultimately, the whole body of oxygen. Hypoxia evolves into metabolic acidosis and decreased oxygen saturation. The extent of lactic acidosis indicates the severity of the cyanide poisoning. In a collapsed individual, plasma lactate is an indicator of cyanide poisoning. In severe cyanide poisonings, up to 98% of the cyanide in the bloodstream is tightly bound to red blood cells. The ancillary response is myocardial depression and decline in cardiac output. Bradycardia, hypotension, and cardiac arrhythmia then develop into VF and cardiovascular collapse.

In severe cyanide poisoning, autonomic shock due to the release of biogenic amines plays a role. The coronary arterial vasoconstriction, resulting in an increase in central venous pressure, leads to the observed shock-like state that is not attributable to inhibition of cytochrome oxidase. In the liver, CN is metabolized by rhodanese to thiocyanate, which is excreted in the urine.

Hypoxia is one of the signatures of cyanide poisoning. In CN poisoning, as in ischemia, oxidative metabolism is blocked and acidosis is enhanced. Acidosis decreases contractility and metabolism while sparing ATP supplies.

A dose of 0.54 mg of hydrogen cyanide per kg of body weight is fatal (average of 1.4 mg). Data indicate that the heart absorbs the second most amount of cyanide per organ weight. However, in sublethal exposure, cyanide-fed rabbits (Okolie and Osagie, 2000) do not show hemorrhaging in the cardiac tissue that was noted by Suzuki (1968). Cyanide also causes decline in $[K^+]_i$ (i.e., significant hypokalemia) and an increase in $[Na^+]_i$. These changes were not reflected in the skeletal muscle. Cyanide caused decline in ATP, the energy source of the cell, to less than 10% of the normal value, which activates the otherwise dormant potassium channel and the outward current I_{KATP}. These changes result in shortening of the AP and decrease in the contraction.

Cyanide also causes endogenous catecholamine release (Schomig et al., 1995; Inoue et al., 1998). Inoue et al. (1998) also point out that cyanide-produced depolarization increases intracellular calcium due to the suppression of the potassium channels and activation of the voltage-dependent calcium channel. Anoxia induces suppression of the sodium pump and activates cation channels due to the decrease in ATP. A further consequence of the presence of cyanide in the tissue is inhibition of the Na^+/Ca^{2+} exchanger (NCX) (Ju and Allen, 2005). NCX is important for the pacemaker currents. Metabolic inhibition of NCX reduces the firing rate of pacemaker cells.

Cyanide poisoning is marked by metabolic acidosis and a large anion gap. The latter is a consequence of the blocked oxidative phosphorylation and the increased rate of glycolysis. Maduh et al. (1990) showed that cyanide also affects H^+ and, thus, the pH of the tissues. In turn, the Ca^{2+} transport process is disrupted, leading to an increase in cystolic $[Ca^{2+}]$. Acidification depolarizes the cell membrane and changes the potassium conductance.

Cyanide also exerts a strong influence on the vagus nerve, and thus on the VIP that under normal conditions exerts a strong inotropic and chronotropic effect.

TABLE 37.4 Effect of CN on the Electrophysiology of Cardiac Tissue

P	QRS	ST	T	Remarks	Reference
Disappearance (auricular arrest)	Change in amplitude, right axis deviation	Shortening, disappear	Incr. amplitude, origin of T-waves on QRS	0.11–0.20 mg/kg NaCN, inhalation, AV dissociation, HR↓, BBB, initial bradycardia, AV block, asystole	Wexler et al. (1947) (man)
	Lengthened	Absent or depressed, elevated after 20 s	Increased, decreased over time, T starts on top of R, diphasic, negative	0.4–0.8 mg/kg NaCNHR↓, bradycardia, Wenckebach, heart block, V-flutter, VF	Leimdorfer (1950) (cats)
		Short, shifted toward T-wave	Tall T, surpassing R, sometimes inverted	0.1 mg/kg NaCN, bradycardia, incomplete AV blockade, VF	Paulet (1955) (dog)
	Abnormal	Elevated, shortened	T-wave beginning high on QRS	0.3–0.6 sodium nitrite as antidote with sodium thiosulfate, atrial fibrillation	De Busk and Seidl (1969) (man)
	Narrow QRS	Nonspecific ST-T changes in anterior chest leads, shortened ST-segment	Fusion of T-wave into QRS, sharp rise of T-wave	Sinus tachycardia, acidosis with high anion gap	Chin and Calderon (2000) (man)
	Q-wave present in lead III, persisted after treatment of acidosis	Deviation of 4 mm in leads II, III, aVF, 2 mm in V_6, ST depression of 2–4 mm in V_1–V_4, *I*, aVL leads, normalization of ST-segment in precordial leads	T-wave inversion in II, III, aVF, V_5 and V_6 leads	Acidosis, CN level of 3 μg/mL, anion gap present	Sanchez et al. (2001) (man)

↑, increase; ↓, decrease.

VIP stimulates adenylyl cyclase activity. In ventricular myocytes, VIP potentiates voltage-gated Ca^{2+} channel currents and also acts on pacemaker currents.

As shown by Goldhaber et al. (1991), contractile failure (i.e., twitch shortening) is caused by cyanide. It is said to be due to failure of activation of the Ca^{2+} current. Electrocardiographic manifestations of CN poisoning have been shown by Wexler et al. (1947) and Katzman and Penney (1993), described by Zoltani et al. (2004), and are summarized in Table 37.4.

The ECG of an individual (Wexler et al., 1947) executed by inhalation of cyanic acid revealed that initially, between the first and third minutes, slowing of the heart rate was discernible with the disappearance of the P-wave. Later, the heart rate increased slightly. T-waves showed an increase in amplitude and a marked shortening of the ST-segment. One subject, unlike some others in this cohort, showed normal AV conduction until ventricular tachycardia and VF developed.

Antidotes for CN Poisoning

Bhattacharya et al. (1995) and Cummings (2004) described the following approaches:

1. Use of a nitrite (oxidizing agent such as sodium nitrite) to change the ferrous ion of hemoglobin to a ferric ion. The created methemoglobin binds cyanide, forming cyanmethemoglobin. One drawback, however, is that impairment of oxygen transport occurs (i.e., the amount of hemoglobin available is reduced). Amyl nitrite to generate methemoglobin is no longer preferred because it does not bind enough cyanide.
2. Sulfur donors for conversion of cyanide to thiocyanate by rhodanese or other sulfur transferases that are a source of sulfur. For moderate poisoning, sodium thiosulfate is the usual choice.
3. Use of cobalt chemistry that chelates the cyanide directly, such as hydroxycobalamin.
4. Hydroxocobalamins, precursors to vitamin B_{12}, are preferred in France and elsewhere in Europe. Hydroxocobalamin binds cyanide to form cyanocobalamin. It does not interfere with tissue oxygenation, but large doses are required to be effective.
5. In the United Kingdom, dicobalt edentate, which chelates the cyanide directly, is preferred, but assurance that cyanide poisoning is present is needed because this antidote contains cobalt, which can be toxic.

OTHER TERROR AGENTS

Arsenic

Arsine blood agents were first developed for battlefield use during World War I. Due to difficulties with dispersion, they were never used. Arsenic, however, has potential use as a terror agent (Sidell et al., 1997; Ellison, 2008).

Arsine is the simplest compound of arsenic. It is colorless and 2.5-times denser than air, with an odor resembling garlic. Arsine binds to hemoglobin of the red blood cells, destroying them. Poisoning kills by allosteric inhibition of metabolic enzymes. Arsenic disrupts ATP production. Arsenic inhibits pyruvate dehydrogenese, uncoupling oxidation phosphorylation. Arsenic poisoning also occurs through arsenic–oxygen compounds, especially arsenic trioxide, As_2O_3 (ATO), which is 500-times more toxic than pure arsenic.

ATO has been effectively used as a remedy for relapsed acute promyeloctic leukemia, but with the side effect that it causes QT interval prolongation, possibly heralding ventricular arrhythmia (Chiang et al., 2002). Abnormalities in $I_{Ca(L)}$ in myocytes were also noted (Sun et al., 2006). The direct effect of ATO on cardiac repolarization with its effect on I_{Kr} was noted by Haverkamp et al. (2000). ATO also causes cellular Ca^{2+} overload and augments action potential duration (Yamazaki et al., 2006). Chronic arsenic exposure leads to QT prolongation blockage of I_{Kr}, TdP, and T-U alternans, and to changes in the T-wave result (Little et al., 1990; Ficker et al., 2004).

It has been suggested that potassium ion channel alteration induced by arsenic may be related to hERG trafficking defects. ECG changes in arsenic poisonings have been reported by Fennel (1981). The T-waves are domed. ECG changes, especially when arsenic involvement is not severe, are reversible. Hemodialysis and BAL (dimercaprol) therapy (to remove excess lead) has been found to be effective. Sun et al. (2006) in a recent publication suggested that choline can normalize QT interval abnormality by inhibiting $[Ca^{2+}]_i$ and $I_{Ca(L)}$ in ventricular myocytes when ATO is present. Arsenic intoxication results in widened QRS by 0.06 s and prolonged QT (Ahmad et al., 2006). Ventricular tachycardia and VF have been reported by St Petery et al. (1970).

Ricin

Ricin, a toxic glycoprotein derived from the castor bean, causes hypotension and myocardial hemorrhage. The Center for Disease Control and Prevention (CDC) lists it as a category B agent because of its easy availability as a terrorist weapon. Only a limited amount of information is available in the open literature on the effect of ricin on the heart. The medical files of Georgi Markov, the Bulgarian journalist assassinated in London with what was assumed to be ricin, are not publicly available (Crompton, 1980).

Ricin is a glycoprotein comprising two chains linked by a disulfide bond. Its toxicity results from one of the chains inhibiting protein synthesis by irreversibly inactivating eukaryotic ribosomes. The lethal dose of ricin has been set at 1–20 mg/kg of body weight (Bradberry et al., 2003). Christiansen et al. (1994) performed extensive experiments on rabbits. Their main findings include the following:

1. Ricin caused vasodilatation and increased endothelial-dependent vascular relaxation, resulting in hypotension.
2. Ricin disturbed calcium homeostasis, leading to cell necrosis.
3. Ricin reduced both systolic and diastolic left ventricular function.
4. Ricin caused myocardial hemorrhage.

Balint (1974) and, later, Zhang et al. (1994) found that at the lethal dose in rabbits, ricin caused hemorrhage and necrosis. Christiansen et al. (1994) found that the release of norepinephrine from sympathetic nerves in the vasculature is not impaired by ricin. The CDC cite cardiovascular collapse (hypovolemic shock) with signs and symptoms of ricin poisoning that may be encountered.

ECG abnormalities in children who ingested castor beans have been noted by Kaszas and Papp (1960). These include QT interval lengthening, repolarization changes, and intraventricular conduction disturbances. Crompton (1980) later reported on experiments in pigs that experienced hemorrhagic lesions and an abnormal ECG due to ricin. Genes of ricin toxicity have been identified, suggesting therapeutic targets (Moreau et al., 2011). At the present time, no antidotes or effective therapy are available to counteract the effects of ricin.

THERAPEUTICS UNDERGOING DEVELOPMENT

Chemical warfare agents almost instantaneously affect the cardiac system. miRNA signatures give rapid and conclusive evidence of the affected site and degree of involvement. Predominantly, although not exclusively, the injury turns off genes expressed through miRNAs. Thus modulating genetic response to overcome dysregulation of miRNAs therapy aims to normalize miRNA expression, silence those that are overexpressed, and replace those that are downregulated.

The heart expresses a large number of miRNAs (Wang, 2011) that have been correlated with the genes that affect the heart's varied functions, including the

TABLE 37.5 Warfare Agent Induced Cardiac Pathologies

Cardiac Syndrome	Physical Symptom	Dysregulated miRNA	Cellular Expression	Reference
Heart failure	Inability of heart to pump sufficient blood	miR-423-5p miR-18b-3p miR-129-5p miR-1254 miR-675 miR-622	Siderophages generated	Sahu (2013); Mittmann et al. (1998)
Ischemia	Restricted blood flow to tissues	miR-1 miR-133a miR-208a miR-499	ATP level drop, surge in reactive oxygen, level necrosis, intracellular Na^+ up, pH decline	Gidlof et al. (2013); Vickers et al. (2014)
Modulated ion flow	ECG changes, arrhythmia	miR-1 miR-133	Membrane excitability, conduction changes, modulated repolarization	Grant (2009)
Vascular	Inflammation, vasodilation, hemorrhage	mir-126 miR-17 miR-146a	Endothelial cells promote vascular homeostasis, release factors including NO	Yamakuchi (2012)

electrophysiology. Many miRNAs have a large number of target genes. The change in the value of the particular miRNA from normal reflects not only the injured organ but also the degree of injury. Thus, for example, changes in the ECG can be related to particular miRNAs.

A common sign of cardiac injury is upregulation of miRNAs. An effective counter-measure is inhibition of the affected miRNAs. AMOs (anti-miRNA oligonucleotides), single-stranded 2′-*O* methyl–modified oligonucleotides fragments that are antisense to its target miRNA, have been used. The methyl group improves the binding to RNA. AMO acts by base-pairing producing loss-of-function of the miRNAs. Multi-target AMO (MT-AMO) enables a single AMO fragment to have the capability of targeting multiple miRNAs. miRNA sponges are similar but contain multiple binding sites for an miRNA seed family.

miRNA inhibitors bind to complementary mRNA sequences that result in post-transcriptional gene silencing. Engineered oligonucleotides, antogomirs, and miR erasers and sponges are currently preferred. Anti-miRNAs contain the reverse sequence of a mature miRNA that is able to reduce the endogenous levels of the miRNA. In addition, it must be cell-permeable, stable, and have high probability to be able to bind to the selected miRNA.

miRNAs play a role in cancer pathogenesis. miRNAs that function as tumor suppressors may be downregulated by disease whereas oncogenes are upregulated. mRNA targets of these miRNAs have been identified. This suggests that genes involved with atrial and VF can also be modulated. In fact, miR-1 changes in the myocardium of normal hearts have induced arrhythmias. In view of quantifiable association of miRNA and gene expression, cardiac injury caused by warfare agents may be amenable to therapies based on miRNAs.

Heart failure, ischemia, and vascular inflammation have specific associated miRNAs (Table 37.5). A pathological condition caused by an injury may reflect several conditions; thus, the miRNA expression profile may not be unique.

Ischemia upregulates several miRNAs, including the miR-15 family. As shown by Hullinger et al. (2012), oligonucleotides can effectively suppress expression of miR-15 and reduce infarct size and remodeling after ischemic injury.

Tissue-specific regulation of miRNAs has been achieved by adeno-associated viruses enabling continuous replacement. It has also been successfully used in gene therapy to overcome tumor genesis. Considerable details regarding miRNA therapy are provided by van Rooij (2011) and Thum (2011).

In replacement therapy, to restore loss of function caused by a warfare agent, miRNA mimics can be used to reintroduce miRNAs into affected cells that also reactivate pathways and target silenced genes. Lipoproteins would be the preferred means to introduce the encapsulated miRNA to achieve these ends.

For resolution of cardiotoxicity caused by warfare agents, miRNA approaches with specificity and timeliness offer myriad opportunities.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Per organ weight, the heart is the second most preferred depository for several of the warfare agents used.

Disturbance of the electrical homeostasis, the cellular energy production, and breakdown of the electrical control network within the tissue are mostly affected.

Areas that need further insight include the following:

- Chemical warfare agents cause miRNA concentration changes in body fluids. Tissue specificity and time of appearance of the changes after exposure need to be correlated with conventional biomarkers and electrophysiological markers, such as ECG changes.
- Means of reactivation, such as overcoming "aging" caused by OP-based agents.
- Nanoparticles that carry medicines show promising results. Their use as counteracting agents for xenobiotic poisoning needs to be examined.
- Does neuropathy target esterase (NTE) contribute to cardiac toxicity?

Human data for various cardiotoxic scenarios are unavailable. The means of prompt identification of the particular xenobiotic causing poisoning remains an urgent task. Progress in metabolomics and the identification biomarkers that result from metabolic changes caused by the presence of xenobiotics will enable the development of chip-based rapid-responding assaying devices.

Currently, PubMed does not give any hits for miRNA associated with warfare agents, including soman, tabun, or VX. Hopefully, this chapter will be an incentive to follow-up the approach outlined here, which has tremendous potential for the understanding and treatment of cardiac toxicity.

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CHAPTER

38

Ocular Toxicity of Chemical Warfare Agents

Patrick M. McNutt and Tracey L. Hamilton

INTRODUCTION

The eye is highly sensitive to injury by a wide array of chemical warfare agents (CWAs). The most significant ocular injuries are caused by the vesicants, which can produce injuries ranging from conjunctival irritation and lacrimation at very low doses to corneal ulceration and loss of vision at high doses, with a permanent progressive corneal degeneration occurring in a small percentage of victims. Even CWAs that manifest with short-term and reversible ocular symptoms can result in incapacitation. In some cases, ocular symptoms may be a prodromic marker of exposure and indicative of future pathophysiologies. Thus, understanding the toxicological mechanisms underlying the ocular responses to a CWA is critical to evaluating their impact on the visual apparatus as well as to developing therapeutic strategies.

Traditionally, CWAs are divided into seven general categories based on their toxicological properties: vesicants, biological toxins, blood agents, tear agents, nerve agents, incapacitating agents, and choking agents (Table 38.1; Romano et al., 2008). In reviewing the toxicopathogenic processes by which these agents elicit ocular symptoms, it became apparent that they can alternatively be grouped into three categories based instead on the specific mechanisms of ocular toxicity: (i) those that cause acute ocular irritation via non-specific, cytotoxic mechanisms; (ii) those that cause ocular irritation via non-specific noncytotoxic mechanisms; and (iii) those that specifically target and disrupt neuro-ophthalmic behaviors (Table 38.2). It is proposed that this novel grouping may prove useful in considering the toxidromic progression (and treatments thereof) associated with each group of agents. For example, ocular exposure to the group 1 cytotoxic agents involves a common set of symptoms, including a pro-inflammatory response, chemosis, corneal lesions, corneal edema, limbal dysfunction, and the possibility of corneal ulceration at very high doses. Independent of the mechanism of toxicity, the clinical management of these injuries is essentially the same. In contrast, the group 3 neuro-ophthalmic modulators manifest by modulating either the activities of innervating ocular neurons or the ability of effector tissues to respond to neuronal signals. This modulation

TABLE 38.1 Representative CWAs of Each Major Category, Including Biotoxins and Incapacitating Agents

Vesicants		**Blood Agents**	
Mustard gas	HD	Hydrogen cyanide	AC
Nitrogen mustard	HN	Cyanogen chloride	CK
Lewisite	L	**Tear Agents**	
Phosgene oxime	CX	Mace	CN
Nerve Agents		CS gas	CS
Tabun	GA	CR gas	CR
Sarin	GB	OC gas	OC
Soman	GD	**Psychomimetic Incapacitating Agents**	
Cyclosarin	GF	3-Quinuclidinyl BZ	BZ
VX	VX	**Biological Toxins**	
VR	VR	Botulinum Toxin A	X
Choking Agents		Ricin toxin	W
Phosgene	CG	Staphyloccocal Enterotoxin B	UC
Diphosgene	DP	Saxitoxin	TZ
Chlorpicrin	PS	Tetrodotoxin	PP

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TABLE 38.2 Agents Grouped by Nature of Ocular Manifestations. Italicized Items Represent Entire Agent Categories.

Ocular irritants, cytotoxic
Vesicants
Blood agents, aerosol delivery
Choking agents
Ricin
staphylococcal enterotoxin B
Ocular irritants, noncytotoxic
Tear agents
Ocular neuromodulation
Nerve agents
Tetrodotoxin
Saxitoxin
Botulinum
Blood agents, systemic
BZ

can be specific to particular neuronal subtypes, such as with cholinergic agonists or antagonists, or can be nonspecific, such as with blood agents. Nearly all of the CWAs discussed in this chapter can be distributed into one of these three groups based on their principal mode of toxicity. The blood agents are an interesting exception. Not only can the blood agents elicit neuro-ophthalmic responses to systemic exposure, but they can also be absorbed into the eye to produce a nonspecific ocular cytotoxicity. Thus, when the blood agent injury is caused by systemic distribution, they are primarily considered to be neuro-ophthalmic modulators, but when the exposure is due to ocular absorption, they are considered to be primarily cytotoxic ocular irritants.

Each of the agents listed in Table 38.1 manifests with some degree of ocular injury. Overall, however, the vesicants pose the most significant threat to the integrity and function of the eye. Vesicants are a class of highly reactive alkylating compounds that catastrophically disrupt cellular functions by forming covalent adducts with proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA). In contrast, the organophosphorus-like nerve agents (OPNAs) block acetylcholinesterase (AChE) activity, causing the synaptic accumulation of acetylcholine (ACh) and the overstimulation of cholinoreceptive neurons. The resulting cholinergic toxidrome potentiates sympathetic signaling, resulting in distinctive but reversible changes in the pupillary apparatus, the lens, and possibly the retina. Incapacitating agents such as 3-quinuclidinyl benzilate (BZ) elicits an anticholinergic toxidrome, producing effects that are the inverse of the OPNAs, including mydriasis and paresis of accommodation. Blood agents interfere with oxygen delivery and oxygen utilization, causing acute cytotoxicity due to progressive tissue hypoxia and necrosis. The tear agents are nonlethal, lacrimating agents that rapidly irritate mucosal membranes, peripheral ocular nerves, or both, causing significant acute discomfort that is typically transient and reversible. The conjunctiva is particularly susceptible to blood agents and tear agents because of its exposed location and extensive innervation. Finally ocular toxicities caused by exposure to three representative categories of exceedingly potent biotoxins will also be discussed: botulinum neurotoxins (BoNTs), a family of potent exotoxins produced by the *Clostridium* family of bacteria; ricin, a poison extracted from the castor bean; and *Streptococcus* enterotoxin B (SEB), a bacterial superantigen.

The list of CWAs, CWA derivatives, and potential CWAs with ocular toxicity is lengthy. In order to refine this discussion, representative CWAs were chosen from (i) traditional chemical and biotoxin threat agents identified by the US Department of Defense (DOD) and the Centers for Disease Control and Prevention (CDC); (ii) Schedule 1A–3A substances, as described in the 1993 United Nations Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on their Destruction, also known as the 1993 Chemical Weapons Convention (CWC); and (iii) representative chemicals of toxidromic families. This chapter will not address agents that are no longer considered operationally relevant, such as chlorine or agents that are currently considered classified by the US government.

BACKGROUND

The Structure of the Eye

Understanding the effects of CWAs on vision requires a basic awareness of ocular structure, including tissue-specific aspects of injury response and regenerative capacities. Rather than providing a comprehensive description of the eye, this chapter will briefly address ocular structures that are specifically involved in the clinical manifestation of CWA toxicity. The eye can be considered to be constructed of three layers (or tunics), which provide the biochemical and structural foundations for the refraction and detection of light. Each layer plays a key role in enabling the entry and processing of light that enables appropriate visual perception. These layers are separated by fluid-filled chambers, which provide nourishment as well as structural support (Figure 38.1).

The outer layer of the eye includes the sclera, conjunctiva, cornea, and corneal limbus. As a result of their exposed locations, these tissues are primary targets for CWAs. The white, opaque sclera is a tough fibrous connective tissue layer containing interlacing type I collagen bundles and elastic fibers that gives the eye its shape. The anterior surface of the sclera is covered by the conjunctiva,

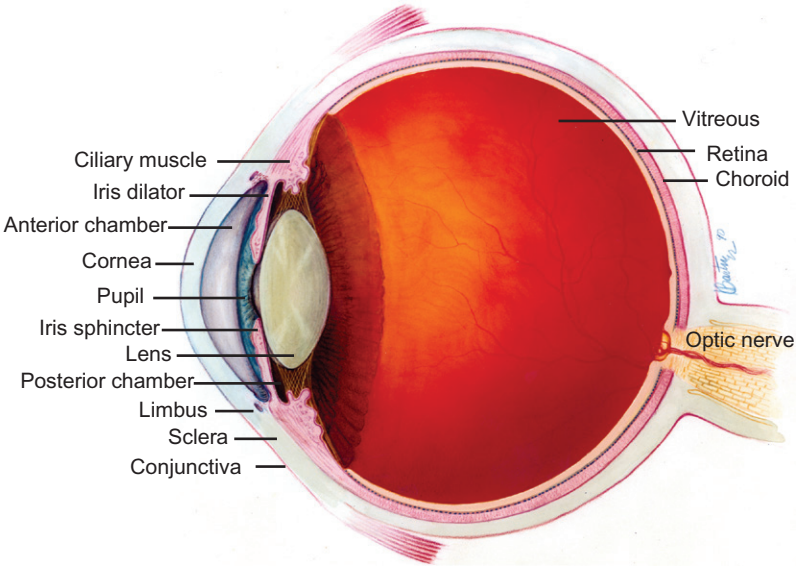


FIGURE 38.1 Anatomy of the eye. Diagram of the human eye, labeled with the structures involved in ocular toxicity following exposure to CWAs. *Source: Image modified with permission from National Eye Institute, National Institutes of Health, reference number NEA04.*

a layer of stratified columnar epithelial cells that produce mucus to help lubricate the eye. The conjunctiva is richly supplied with blood vessels and is highly susceptible to inflammation. At the front of the eye is a transparent, avascular, highly innervated tissue called the *cornea.* The cornea is responsible for protecting the eye against insults such as injury and infection. It also provides roughly two-thirds of the total refractive power of the eye and is therefore the major refracting lens (Meek et al., 2003). The corneal limbus is located at the junction between the conjunctiva and cornea and plays an essential role in sustaining and repairing the corneal epithelium. Limbal cytotoxicity, therefore, has severe consequences to corneal function, as discussed in the next section.

Between the cornea and the uveal tissues is the anterior chamber, which contains a watery fluid called the aqueous humor (AH). The anterior chamber exerts positive pressure against the corneal endothelium to help shape the cornea (a.k.a., intraocular pressure), and together the cornea and the AH form an outer lens that plays the most significant role in refracting light towards the center of the eye. The AH also provides nourishment for the tissues lining the anterior chamber, and consequently it is rich in metabolites (Goel et al., 2010). The AH is continuously secreted by the ciliary epithelium, flowing from the posterior chamber, through the iris into the anterior chamber and exiting the eye primarily via the trabecular meshwork, where it is absorbed into the bloodstream. Humans produce AH at approximately 3 μL/min, with the entire volume being replaced every 90 min (Goel et al., 2010). Consequently, CWAs that penetrate through the cornea to the anterior chamber may result in intracameral biomarkers that can subsequently be detected within the plasma or even result in systemic toxicity (Romano et al., 2008; McNutt et al., 2012a,b). AH production and drainage are critical to normal ocular function, and functional disruption results in long-term consequences to corneal integrity and vision.

The middle layer of tissues includes the choroid, iris, lens pupillary apparatus and ciliary body. General inflammation of these tissues is called *uveitis* or *iritis.* The choroid is the inner vascular layer of the eye, underlying the sclera and providing oxygen and nutrients to the retina. Like the conjunctiva, the choroid is prone to inflammation. However, due to its deep intraocular location, it is isolated from exposure to most CWAs. The iris is a contractile diaphragm that controls the size of the pupillary aperture, and therefore the amount of light that enters the eye. Pupil size is controlled by two iris muscles: the pupillary sphincter, which is responsible for constriction of the pupil, and the pupillary dilator, which is responsible for dilation of the pupil. These muscles are innervated by sympathetic and parasympathetic nerves that control constriction and dilation, respectively. Behind the iris is the posterior chamber, a narrow space delineated by the iris, lens, and ciliary structures through which AH flows. The lens is a crystalline structure that focuses light on the retina. The ciliary muscle controls focus (accommodation) by changing the shape of the lens under the control of the autonomic nervous system. Since changes in pupil size and accommodation are principal ocular symptoms associated with exposure to many CWAs, the function of the neuro-ophthalmic tissues will be described in greater detail in the section below.

The majority of the volume of the eye consists of the vitreous chamber, which is filled with the vitreous humor, a transparent, gelatinous mass of extracellular matrix material similar to the corneal stroma which is expressed during development. The vitreous humor holds the neural layer of the eye—the retina—in position. The retina is comprised of 10 layers of nervous tissue made up of millions of hierarchically organized light receptors that are connected to the brain by the optic nerve. The neural layer is not believed to exhibit specific responses to most agents, except in the cases where systemic distribution of neurotoxic CWAs may have a pathophysiological effect on retinal function and information processing.

Finally, there is the ocular adnexa (not shown), which are tissues that provide a supportive environment to the globe, such as the lacrimal glands, which are located underneath the outer portion of the upper eyelid, the eyelids, and the extraocular muscles. The lacrimal glands produce tears that help lubricate and moisten the eye, as well as flush away any foreign matter that may enter the eye. The extraocular muscles control convergence, allowing the eyes to synchronize and provide binocular vision.

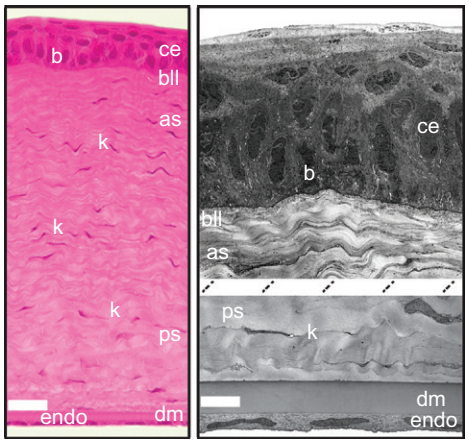


FIGURE 38.2 **Structure of the cornea.** Left: Representative histology of a rabbit corneal section. Scale bar is 40 μm. Right: Transmission electron microscopy of a rabbit eye, demonstrating the ultrastructure of different corneal layers. Scale bar is 4 μm. Labels: ce: corneal epithelium; b: basal epithelial cell; bll: Bowman's-like layer; as: anterior stroma; k: keratocytes; ps: posterior stroma; dm: Descemet's membrane; endo: corneal endothelium. Unpublished data.

Neuromodulation of Ocular Tissues

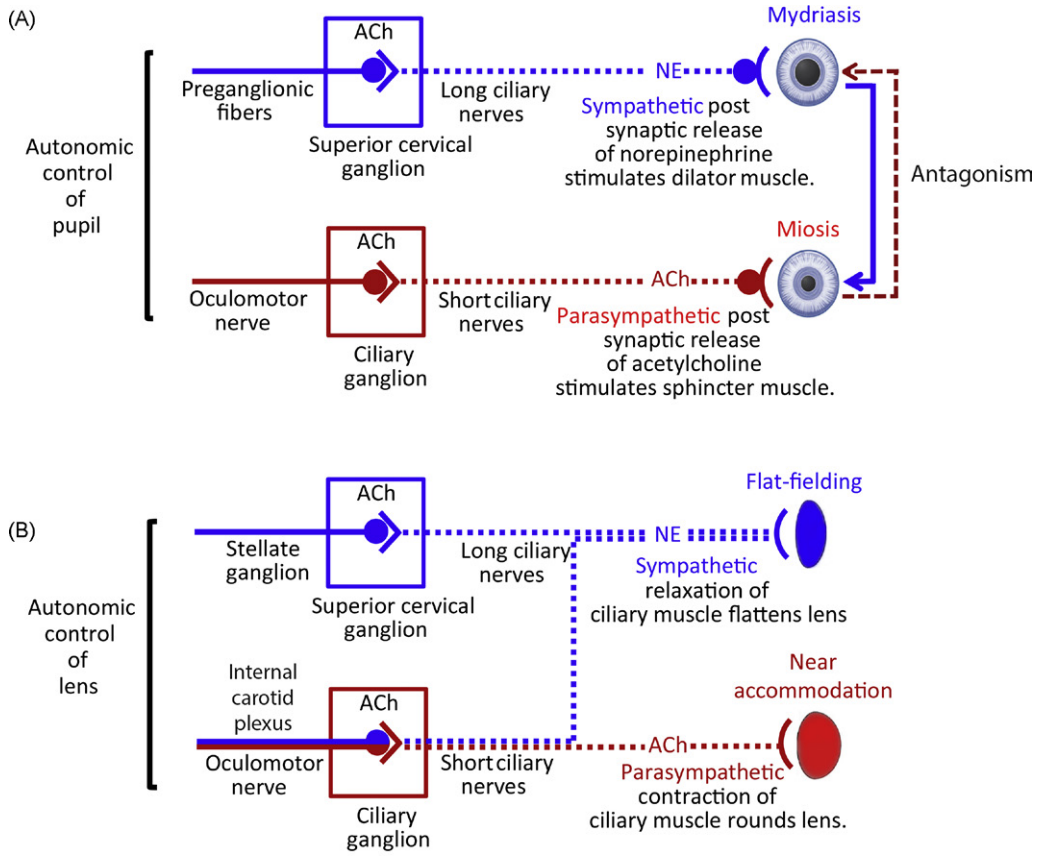
A number of CWAs exert their effects by modulating neuronal control over ocular function (Table 38.2). Autonomic neurons provide input to the intrinsic ocular muscles (the sphincter pupillae, the dilator pupillae, and the ciliary muscle) and the lacrimal glands. Neuronal afferents in the eye include sensory neurons from the conjunctiva and cornea, reflexive contributions to the iris, ciliary muscle, and eyelids, and the densely innervated retina. Finally, the extraocular muscles and eyelids are controlled by cholinergic motor neuron inputs.

Under normal conditions, pupil size and focal accommodation result from a balance between sympathetic (adrenergic) and parasympathetic (cholinergic) nerve inputs (Figure 38.2) (Levin and Kaufman, 2011). Autonomic control over pupil dilation is mediated by sympathetic postganglionic neurons originating from the superior cervical ganglion and synapsing with the iris dilator muscle. Stimulation of the sympathetic pathway results in the release of norepinephrine onto α-adrenergic receptors on the sphincter dilator, dilating the pupil and increasing the activation of light receptors in the retina. The short ciliary parasympathetic nerves project from the ciliary ganglion to form cholinergic synapses with the iris sphincter. Stimulation of the parasympathetic pathway results in pupillary constriction (miosis), reducing light input and causing tunnel vision. The dilator and sphincter muscles are also antagonistic, such that inhibiting one pathway is sufficient to allow the other pathway to become dominant. The system is further complicated by the differential expression of stimulatory and inhibitory receptors in the uveal muscle tissues, presumably coordinating the complex interplay of sympathetic and parasympathetic signaling (Whikehart, 2003).

Control over lens accommodation by the ciliary muscle is coordinated with pupil size and binocular convergence to optimize focus. The ciliary muscle receives both parasympathetic and sympathetic inputs from the ciliary ganglion via the short ciliary nerves. Postganglionic sympathetic signals result in the release of norepinephrine onto the ciliary muscle, activating β-adrenergic receptors and relaxing the muscle, which in turn optimizes refractive power for far vision. In contrast, parasympathetic activation activates muscarinic receptors, causing ciliary muscle contraction and rendering the lens more spherical for near-focus.

Structure and Regenerative Capacity of the Corneal Tissues

The degree of CWA absorption, penetration, and reactivity with conjunctival or corneal tissues is a major component of ocular toxicity. Irreversible ocular injuries are caused by those agents that disrupt the integrity of the cornea, causing deep keratitis, stromal scarring, endothelial toxicity and possible corneal perforation. Corneal function is critically dependent on the ability of the corneal epithelium and corneal endothelium to maintain stromal deturgescence by controlling fluid ingress at the anterior and posterior margins of the cornea, respectively. Disruption of either barrier results in



(C) Representative effects of neuro-ophthalmic agents on autonomic nervous system balance

- Organophosphorus nerve agents potential cholinergic signaling, shifting the balance towards Parasympathetic dominance.
- BZ block cholinergic signaling, shifting the balance towards Sympathetic dominance.
- Low-doses of systemic botulinum block cholinergic release, shifting the balance towards Sympathetic dominance.
- High-doses of systemic botulinum block cholinergic and adrenergic release, producing tonic and poorly responsive pupils that are not dominated by either autonomic pathway.

FIGURE 38.3 **Autonomic nervous system inputs to the pupil and the lens.** Diagram depicting the balance between sympathetic and parasympathetic signaling to the pupil (A) and lens (B), and summarizing how agents causing neuro-ophthalmic toxicity can alter this balance (C).

corneal edema, increased production of pro-inflammatory mediators, and entry of matrix-active enzymes into the stroma. Persistent corneal edema may then progress to corneal degeneration, with severe effects on vision that can require corneal transplants (Eagle et al., 1989; Alomar et al., 2011). Given the susceptibility of the cornea to chemical and mechanical injury, the preservation of vision depends on the regenerative capacity of the cornea.

The cornea is composed of five histologically distinct layers with different regenerative capacity: corneal epithelium, Bowman's or Bowman's-like layer, stroma, Descemet's membrane (DM), and corneal endothelium (Figure 38.3). The corneal epithelium is a stratified epithelium with 5–7 cell layers that provides a dynamic physical barrier between the external environment and intraocular space. Tight junctions among the different layers of corneal epithelial cells form a highly impermeable barrier to tear film and microorganisms (Klyce, 1972). The basal epithelial cells have several important functions, including the secretion of matrix factors, establishment of hemidesmosomal attachments to focal adhesion complexes in the anterior stroma, and proliferation to generate new epithelial cells. Anterior to the basal epithelial cells are the wing-like suprabasal cells. Over time, the suprabasal cells move superficially and terminally differentiate into the outer layers of squamous epithelial cells. These cells are shed from the ocular surface during normal wear and tear and are replaced by the steady superficial migration from underlying epithelium. Thus,

homeostasis of the corneal epithelium is critically dependent on mitotic renewal.

Regeneration of the corneal epithelium primarily relies on a small population of limbal epithelial stem cells (LESCs) located in the basal region of the limbus. Although LESCs slowly cycle during homeostasis, they can become highly proliferative in response to corneal injury, undergoing asymmetric division to produce daughter transit amplifying cells (TACs) that migrate into the basal layer of the corneal epithelium and undergo limited proliferation to replace corneal epithelial cells lost during normal exfoliation or due to injury (Ordonez and Di Girolamo, 2012). The limbal niche is highly vascularized, and factors that regulate niche function are delivered via multiple sources, including systemic circulation, tear film, AH, support cells in the LESC niche, keratocytes in the stroma, and corneal epithelial cells. Adverse conditions, such as chemical injury or persistent inflammation, can disrupt niche function, leading to a constellation of symptoms known as *limbal stem cell deficiency (LSCD*; Ahmad, 2012; Osei-Bempong et al., 2013). Dysregulation of the LESC niche has severe consequences for the corneal epithelium, which in turn evokes symptoms of corneal failure, such as invasion of goblet cells from the conjunctiva into the cornea, neovascularization, chronic inflammation, recurrent corneal erosions, keratitis, corneal ulceration, and stromal scarring.

Beneath the corneal epithelium is the corneal stroma, which encompasses about 90% of the corneal volume. The stroma is a highly ordered tissue composed of tightly packed collagen fibrils that are structured by keratin sulfate proteoglycans into lamella (Hassell and Birk, 2010). The uniform packing is critical in rendering the stroma transparent to light and is highly dependent on maintenance of the stroma in a deturgescent state by the barrier functions of the corneal endothelium and corneal epithelium. Persistent corneal edema caused by failure of these barriers ultimately results in corneal degeneration, with possible loss of vision (Eagle et al., 1989). Under normal conditions, the stroma is sparsely populated by keratocytes, a quiescent population of mesenchymal cells that secretes transparent corneal material. Keratocytes exhibit variable responses to corneal injury, depending on their proximity to the injury (West-Mays and Dwivedi, 2006; Wilson, 2012). After a penetrating injury to the cornea, nearby keratocytes undergo apoptosis, prompted by the stromal influx of signaling molecules from the tear film or AH. More distant keratocytes undergo a fibroblastic transformation, become active, begin to proliferate, and start synthesizing matrix metalloproteinases that assist in tissue remodeling and scar formation. If the entry of pro-inflammatory mediators into the stroma is reduced by re-establishment of barrier activity, the myofibroblasts disappear and the activated keratocytes continue to slowly repair the stroma.

The corneal endothelium is a monolayer of cells on the DM that regulate corneal nutrition and hydration by balancing a semipermeable barrier activity with active ion transport mechanisms. Since adult human corneal endothelial cells (CECs) do not appear to proliferate *in vivo*, the corneal endothelium has a limited ability to recover from cytotoxic injury (Joyce et al., 1996; Senoo and Joyce, 2000). Under normal conditions, gaps in the CEC monolayer are rapidly filled by the spreading of proximal cells, driven by morphologic changes that compensate for endothelial cell loss. Once the focal CEC density falls below the threshold level required for endothelial function (estimated to be 1,000–2,000 cell/cm^2) (Joyce et al., 1996) the corneal endothelium can no longer keep the stroma in a dehydrated condition, resulting in a persistent corneal edema that, in turn, causes secondary keratopathies such as anterior segment inflammation, epithelial bullae, and LSCD (Eagle et al., 1989; Petroll et al., 1995). Thus, while endothelial function can be restored after a mild injury by CEC spreading, more severe injuries may exceed the repair capacity of the human endothelium.

VESICANTS

Sulfur mustard, nitrogen mustard, and lewisite are considered Schedule 1A substances according to the CWC. These substances are defined as having few legitimate applications, if any. The largest section of this chapter will focus on the vesicant class of CWAs for three reasons. First, the vesicants pose the most significant threat to the eyes, with the development of irreversible, vision-threatening injuries following severe exposures. Second, the clinical, biological, and functional consequences of ocular exposure to vesicants have been extensively researched. Third, the vesicants have a high potency against ocular tissues and ocular injury can occur at doses that produce minimal systemic or cutaneous effects. Given the importance of eyesight in the sensory armature, ocular injury can not only incapacitate victims for days to weeks, but also incurs a significant degree of associated psychological distress.

The Mustard Gases

Sulfur mustard (HD; $C_4H_8Cl_2S$) and nitrogen mustard (HN-1; $C_6H_{13}Cl_2N$) are highly reactive adducting compounds that exert their cytotoxic effects through a combination of genotoxicity, production of reactive oxygen species (ROS), direct interference with protein function, and disruption of metabolism. Although the mustards are well known for their cutaneous blistering effects, the ocular injury is clinically distinct from the cutaneous injury, influenced by a variety of factors unique to

FIGURE 38.4 *Gassed*, by John Singer Sargent. *Source: Reprinted with permission from the John Singer Sargent Gallery.*

the eye, including structure, biochemical composition, and regenerative mechanisms. The tactical implications of HD use on troop morale and combat capability is beautifully captured in the John Singer Sargent painting *Gassed*, based on the artist's firsthand observations of a German mustard gas attack on British troops on August 21, 1918, in which he depicted lines of vesicant-blinded troops moving slowly toward casualty stations (Figure 38.4). Harry L. Gilchrist, the medical director of the Gas Service, US Army Expeditionary Force, described a similar phenomenon following a mustard attack on US soldiers (Gilchrist and Matz, 1933):

> At first the troops didn't notice the gas and were not uncomfortable, but in the course of an hour or so, there was marked inflammation of their eyes. … by the time the gassed cases reached the casualty clearing station, the men were virtually blind and had to be led about, each man holding on to the man in front with an orderly in the lead.

A combination of the physical properties of HD and the clinical consequences of HD exposure made it the most effective CWA during World War I, earning HD the moniker of "king of battlefield gases" (Fitzgerald, 2008; Ganesan et al., 2010). HD was responsible for 77% of all gas injuries in World War I (Hughes, 1942) and large-scale deployment in both World War I and during the Iran–Iraq war (1980–1988) resulted in well over 600,000 casualties, with estimates of 75–90% developing ocular injuries (Papirmeister et al., 1991). HD has also been used in a large number of smaller conflicts between 1917 and 1997, including by Egypt against North Yemen during 1963–1967, Italy against Ethiopia from 1935 to 1940, and the Soviet Union against China in 1934 and again in 1936–1937 (Tuorinsky, 2008).

Toxicokinetics of the Acute Ocular Mustard Injury in Human Victims

Due to the large aqueous–mucous interface between the corneal surface and the tear film and the high metabolic rate of corneal epithelial cells, eyes are roughly tenfold more sensitive to HD injury than skin or pulmonary tissue (Pickard, 1919; Solberg et al., 1997; Safarinejad et al., 2001). Therefore, it is not surprising that approximately 75–90% of HD casualties develop ocular symptoms, with 10% presenting with severe ocular damage (Pickard, 1919; Pechura and Rall, 1993). For example, of 998 victims exposed during the production of sulfur mustard at Edgewood Arsenal from September 1941 to March 1943, 78% developed acute ocular injuries (Uhde, 1946). A similar ratio held true for battlefield casualties during World War I and the Iran–Iraq war (Tuorinsky, 2008).

The spectrum of ocular injury among sulfur mustard victims is primarily determined by exposure time and concentration, with symptoms ranging from mild conjunctivitis to chronic advanced corneal disease (Pechura and Rall, 1993). A remarkable series of experiments involving live human military volunteers has provided rigorous concentration:time (Ct) data for the acute component of ocular mustard injury (Reed et al., 1918; Walker et al., 1928; Guild et al., 1941; Anderson, 1942; Smith and Dunn, 1991). Based on these experiments, exposure to 5–30 $mg\,min/m^3$ is the lowest detectable dose, producing a mild conjunctival injection without corneal involvement that resolves within days. Doses of 60–75 $mg\,min/m^3$ cause conjunctivitis, conjunctival and corneal irritation, and photophobia (Mandel and Gibson, 1917; Mann and Pullinger, 1944; Gates and Moore, 1946; Balali-Mood and Hefazi, 2005). In some cases, ocular injuries are sufficiently severe at 60 $mg\,min/m^3$ to render soldiers combat-ineffective and require medical treatment for 1–2 weeks. Severe intraocular injuries developed at exposures that exceeded 100 $mg\,min/m^3$, characterized by corneal edema, keratitis, ocular pain, blepharospasm, uveitis, neovascularization, and corneal perforation. The resulting corneal epithelial vesication and corneal edema require weeks to months to heal. Doses of 200 $mg\,min/m^3$ cause corneal edema and clouding, eyelid edema, and severe blepharospasm and

TABLE 38.3 Summary of Ocular Manifestations and Healing Times Following Exposure to SM Vapor

Injury Class	Estimated Dose (mg min/m^3)	Characteristic Ocular Manifestations	Symptomatic Recovery
Class I	12–70	Conjunctival injection and ocular irritation, without lacrimation, blepharospasm, or photophobia	1–2 weeks
Class II	100–200	Above symptoms plus mild corneal involvement, such as corneal edema and epithelial vesication. Symptoms appear 6–12 h after exposure, including edema and epithelial erosions.	6–12 weeks
Class III	>200	Corneal swelling, edema and epithelial lesions, with destruction of limbal blood vessels at higher doses. MGK may develop. At very high doses, penetrating corneal ulcers occur.	Months to never

Source: Adapted from Solberg et al. (1997).

require months to heal. At doses exceeding 400 mg min/m^3, severe corneal damage develops that requires months of hospitalization. At these doses, blindness can rapidly develop due to corneal scarring and corneal ulceration. Based on these experiments, the minimum incapacitating vapor dose was estimated to be 60 mg min/m^3 and the median incapacitating dose (ICt_{50}) was estimated to be 100–200 mg min/m^3 (Project Coordination Staff, 1946; Reutter and Wade, 1994). At this dose, 50% of soldiers exposed under battlefield conditions will be incapacitated by the resulting ocular injury. Estimates of battlefield air concentrations of mustard gas during World War I were 19–33 mg/m^3: thus, within 2 min, victims would exceed the minimum incapacitating dose, and a 5 to 10 min exposure would incapacitate 50% of soldiers (Solberg et al., 1997). Note that ocular exposure to liquid HD dramatically increases the risk of corneal ulceration and vision loss and therefore is far more dangerous than vapor-induced injury (Papirmeister et al., 1991).

Approximately 90% of soldiers and workers exposed to sulfur mustard in World War I initially presented with symptoms of conjunctivitis, photophobia, and blepharospasm. The subsequent injury progression was observed to follow one of three clinical trajectories (Classes I–III), which differed based on the nature of the symptoms and the recovery time (Table 38.3) (Hughes, 1945b). Class I injuries occurred in 75% of those with ocular presentation, involving mild symptoms without corneal involvement or significant chemosis that resolved within 1–2 weeks. Based on the Ct data presented here, Class I injuries are caused by doses ranging from 5–60 mg min/m^3. Class II injuries occurred in 15% of the victims, and correspond to a dose of 100–200 mg min/m^3. Class II injuries involve eyelid, conjunctival, and corneal lesions and result in incapacitation lasting 4–6 weeks. Signs of the Class II injury include chemosis, corneal edema, epithelial erosions, severe ocular pain, blepharospasm, and photophobia. Class III injuries occurred in 10% of victims and are caused by exposure to more than 200 mg min/m^3. Class III injuries are characterized by severely affected eyes, with significant corneal involvement. Symptoms include severe ocular pain, reduced vision, blepharospasm, uveitis, edematous eyelids, limbal necrosis, chemosis, corneal erosions, and corneal edema. Class III was further subdivided into Class IIIa and Class IIIb. Class IIIa victims presented with moderate corneal symptoms, with a prognosis of 6 weeks to 3 months before soldiers could return to duty. In contrast, Class IIIb victims exhibited severe corneal changes resulting in disability of more than 3 months, and possibly causing complete loss of vision. Of the 939 ocular casualties caused by accidental exposures in mustard gas production factories in England, 10.4% presented with Class III symptoms, but only 1% developed Class IIIb corneal lesions (Hughes, 1945a).

Evidence for a Delayed Ocular Mustard Injury in Human Victims

Starting in the late 1920s, reports appeared of a late-onset complication in survivors of severe HD ocular injuries, in which ostensibly healed eyes developed a "delayed keratitis" from 8 to 25 years after exposure (Pechura and Rall, 1993). It was initially noted that following recovery from the acute lesion, which took 4–6 months, patients remained largely asymptomatic for a decade or longer. However, approximately 1% of survivors subsequently developed a progressive ulcerative corneal disease that did not heal, characterized by photophobia, lacrimation, recurrent corneal erosions, idiotypic keratitis, decreased corneal sensitivity, and progressive corneal degeneration. As of 1939, approximately 300 victims of the severe form of mustard gas keratopathy (MGK) had been described in the literature (Phillips, 1940). In 1944, it was reported that this keratitis developed intermittently in Class III–injured veterans over a period of 8–17 years after initial exposure, with a sudden increase in incidence from 17 to 25 years (Mann, 1944). Strikingly, of the 84 patients with delayed-onset HD injuries that were involved in that study, 76 believed that they had fully recovered from the acute injury prior to clinical reappearance.

Additional evidence for the late-onset injury accumulated among survivors of the Iran–Iraq war, which represented the first instance of the prolonged deployment of

HD munitions during the modern era, producing 50,000–100,000 military and civilian casualties (Willems, 1989; Khateri et al., 2003). Medical follow-up of this population using modern clinical methods has been illuminating with regard to the scope and progression of the acute and late injuries. In the largest available study, 34,000 survivors of HD injury were screened 13–20 years after exposure (Khateri et al., 2003). While 60.7% of survivors had no ocular symptoms, 35% were classified as having mild symptoms, involving persistent conjunctival irritation; 3.6% as having moderate symptoms, involving corneal opacities, mild corneal edema, and band keratopathy; and 0.7% as having severe symptoms, which included corneal melting and neovascularization. In a separate, nonoverlapping study involving 134 patients examined 17–22 years after exposure, 83% of survivors presented with ocular complications, which included burning (69%), photophobia (64%), blepharitis (28%), tearing (12%), corneal ulceration (12%), and retinal and conjunctival complications (4.5%) (Namazi et al., 2009). In another study specifically evaluating ocular injury in 40 veterans from 16–20 years after a single, high-dose exposure, 39 reported persistent ocular sequelae, including chronic conjunctivitis (17.5%), corneal thinning (15%), limbal ischemia (12.5%), corneal opacity (10%), corneal vascularization (7.5%), and corneal epithelial defects (5%) (Balali-Mood et al., 2005). A total of 6 patients exhibited severe corneal involvement and were diagnosed with the severe form of MGK.

This delayed ocular HD injury is predominantly associated with the Class III survivors with latencies ranging from 1 to 40 years after exposure (Solberg et al., 1997; Balali-Mood and Hefazi, 2005). Onset is typically abrupt, characterized by the appearance of photophobia, tearing, and corneal and limbal lesions. However, the etiology of the late HD injury is still unknown. Corneal tissues removed from MGK victims during surgical interventions or postmortem exhibit signs of chronic inflammation, corneal thinning and ulceration, neovascularization, and corneal degeneration, suggesting a persistent injury that is beyond the healing capacity of the cornea (Richter et al., 2006; Javadi et al., 2007; Kanavi et al., 2010). Without a clear understanding of the etiopathogenesis of chronic HD injury, treatments have been mostly palliative in nature. Surgical interventions such as corneal keratoplasty and limbal stem cell transplants have had mixed outcomes, with successes largely restricted to mild cases of MGK (Richter et al., 2006; Javadi et al., 2007, 2011; Baradaran-Rafii et al., 2013).

Toxicokinetics of the Acute and Late-Onset Ocular Mustard Injuries

The acute symptomatic progression of the ocular HD injury has been well described (Mann and Pullinger, 1944; Tuorinsky, 2008). The duration of the latent period between exposure and the appearance of clinical symptoms is inversely correlated to the severity of exposure (Tuorinsky, 2008). At vapor doses that cause Class I injury, the latent period is 4–12 h. At moderate or high vapor doses that cause Class II or III injuries, the latent period may be as short as 1–3 h. Symptoms typically appear within an hour after exposure to liquid sulfur mustard. Following the latent period, the earliest symptoms of injury are sensations of grit in the eyes, ocular soreness, lacrimation, conjunctival injection, chemosis and corneal injection. In Class II or III injuries, the corneal epithelium sloughs from the basement membrane within 6–12 h, leading to severe pain, photophobia, corneal edema and impaired vision. By 24 h, corneal edema has increased the thickness of the cornea up to 300%, disrupting vision and causing corneal pain. After a week, corneas begin to show clinical improvement with subsiding edema. Class II injuries subsequently develop superficial corneal vascularization, secondary corneal edema, and recurrent corneal symptoms that persist for several weeks before ultimately resolving.

The progression of healing in more severely injured eyes is less straightforward. A review of dose response data and case studies has enabled clinicians to identify three clinical trajectories among Class III–injured eyes: (i) injury resolution similar to Class II, but over a period of months and without the subsequent reoccurrence of corneal symptoms; (ii) a chronic injury that develops immediately after the acute injury and fails to heal; and (iii) delayed-onset lesions that appear 1–40 years after exposure (Duke-Elder, 1972; Papirmeister et al., 1991; Javadi et al., 2005, 2007). The latter two trajectories are collectively referred to as MGK. Although the relationship between the chronic and delayed-onset forms of MGK is unclear, the severe form of each involves an idiotypic, noninfectious keratitis with secondary keratopathies such as persistent epithelial lesions, corneal neovascularization, and progressive corneal degeneration (Khateri et al., 2003; Mousavi et al., 2009). Since both the chronic and delayed-onset forms of MGK result from a severe corneal exposure and share similar symptoms, it may be that a common etiology is involved, despite temporal differences in clinical onset.

The pathogenic mechanisms responsible for MGK are unknown, but the most common clinical sequelae include recurring corneal epithelial lesions, corneal neovascularization, a progressive corneal degeneration and frequent impairment or loss of vision (Solberg et al., 1997; Javadi et al., 2005; Balali-Mood and Hefazi, 2006). In a study restricted to 48 veterans suffering chronic ocular symptoms, 31 (64.6%) developed MGK directly from acute injury (i.e., the chronic form), whereas the delayed-onset form developed in 17 (35.4%), with latencies ranging from 1 to 15 years (Javadi et al., 2005). Limbal

lesions were present in 81% of the MGK eyes, suggesting that development of limbal stem cell disorder is a common sequelae of MGK. Corneal signs included scarring (87.5%), neovascularization (70.8%), thinning (58.3%; resulting in corneal perforation in 4.2% of patients), and recurring epithelial defects (31.3%). In a separate study involving 149 seriously eye-wounded veterans, 90% of casualties with chronic ocular symptoms who were followed for 10–15 years showed no improvement, and in many cases, the injury became progressively worse (Ghasemi et al., 2009). According to these studies and many others, the delayed HD injury is usually progressive and difficult to clinically manage (Safarinejad et al., 2001). Not surprisingly, chemical warfare survivors with ophthalmologic complications suffer from a significantly lower quality of life as a consequence of their chronic injuries (Mousavi et al., 2009).

Mechanistic Studies of HD Toxicity

Animal models have been used to study the pathogenesis of acute and chronic HD ocular injuries for over 70 years (Mann and Pollinger, 1944; Pechura and Rall, 1993; Ruff et al., 2013). One of the most productive models has been an *in vivo* vapor exposure model in rabbits. The rabbit cornea is structurally similar to that of the human, and at functionally equivalent doses, rabbit and human eyes exhibit nearly identical lesions (Mann and Pullinger, 1944; Gates and Moore, 1946). Recently, a rabbit vapor HD exposure model has been shown to produce dose-dependent, multiphasic corneal injuries with sequelae similar to those observed in human victims (Kadar et al., 2001; Milhorn et al., 2010; McNutt et al., 2012a,b).

Using the rabbit vapor exposure model, the progression of structural, biochemical, and molecular changes during acute injury and the transition to MGK have been elucidated. Briefly, exposed corneas develop an acute lesion within 1 day, characterized by vesication of the corneal epithelium, stromal keratocytosis, corneal edema, and CEC loss. The corneal epithelium then undergoes a robust healing response, regenerating an intact, stratified epithelium with rudimentary hemidesmosomal attachments to adhesion plaques in the anterior stroma by 7 days after the exposure. Grossly, corneas appear to be healing from 1 to 2 weeks (the quiescent phase), with few clinical symptoms other than lingering edema. Resolving corneas subsequently undergo a rapid decrease in corneal thickness, reaching baseline levels by 6 weeks; a time frame that is similar to Class II or IIIa injuries in humans (Figure 38.5) (Kadar et al., 2001; McNutt et al., 2012a,b). In contrast, the failure of corneal edema to resolve at 3 weeks and beyond is the earliest clinical marker of MGK onset. Histopathological markers of MGK include recurring basal epithelial cell cytotoxicity, basement membrane zone degeneration, loss of LESCs, inflammatory cell infiltration, elevation of cytokines, persistent endothelial failure, delayed inmigration of keratocytes, stromal degeneration, and redundant deposition of basement membrane components caused by cyclical attempts to regenerate the epithelium (Kadar et al., 2001; McNutt et al., 2012a,b). Secondary pathologies, such as epithelial bullae formation, recurring corneal erosions, neovascularization, and LSCD, subsequently develop, further interfering with stable repair of the ocular surface. Postexposure, steroid-based anti-inflammatory protocols reduce and postpone the appearance of late injury. However, they do not prevent late injury, suggesting that anti-inflammatories fail to treat the central pathology response for MGK onset (Amir et al., 2000; Kadar et al., 2009; Gordon et al., 2010). The distinct pathophysiology of MGK revealed in these studies suggests the involvement of pathophysiologies that operate on different time scales and in different corneal compartments than during the acute injury. However, all studies agree that persistent edema is a primary causative factor underlying MGK onset.

Etiogenesis of the Delayed Ocular HD Injury: Current Theories

Damages to the corneal limbus and the corneal endothelium have emerged as pathophysiologies that may be critically involved in MGK onset, progression, or both. Both pathologies are consistent with clinical observations in human MGK corneas and are spatiotemporally expressed in such a way to contribute to the long-term corneal HD injury (Baradaran-Rafii et al., 2010; Jafarinasab et al., 2010).

In a clinical study of 35 Iranian patients diagnosed with MGK, all MGK eyes were demonstrated to exhibit LSCD (Baradaran-Rafii et al., 2010). Similar symptoms were observed in rabbit eyes expressing MGK symptoms, including goblet cell invasion (an indicator of limbal destruction) and a significant reduction of LESCs (Kadar et al., 2011, 2012). Interestingly, LESC loss did not appear to occur during the acute phase following HD exposure; rather, it developed concurrent with the appearance of MGK symptoms. These data have been interpreted to suggest that dysfunction of the LESC niche is a delayed phenomenon that occurs after the acute injury. A proinflammatory response and nerve damage has been found in the corneal limbus of both rabbits and humans during MGK onset, implicating their involvement in the initiation of the late ocular injury (Javadi et al., 2005; Kanavi et al., 2010; Kadar et al., 2011). Regardless of the causal relationship between LSCD and MGK, LSCD is a clinically important aspect of MGK and the delayed loss of LESCs raises the possibility of a postexposure therapeutic window during which limbotrophic treatments could preserve LESCs.

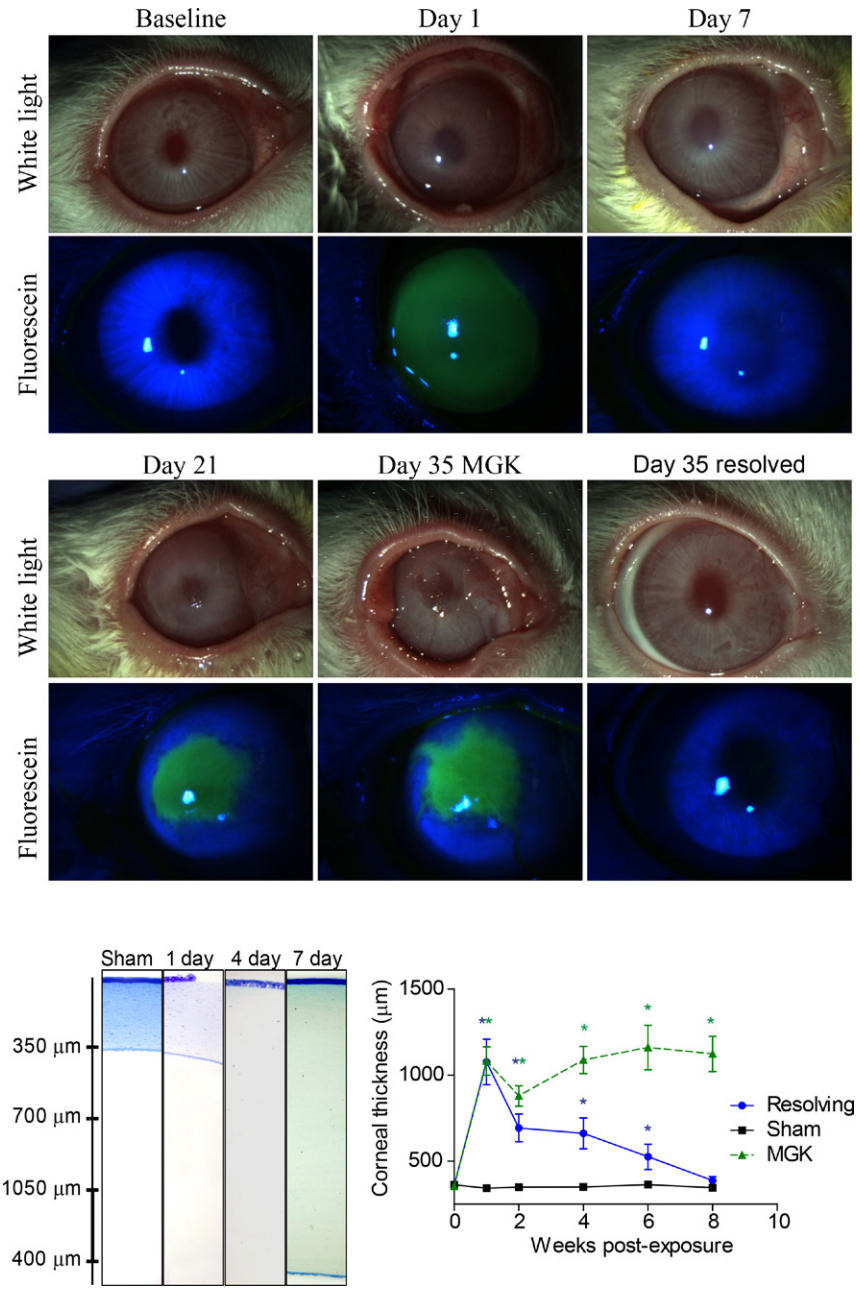


FIGURE 38.5 Development of acute and MGK injuries in HD-vapor exposed rabbit eyes. (Top panels) Rabbit eyes were exposed to HD vapor for 2.5 min and evaluated by slit-lamp examination using fluorescein uptake as a measure of epithelial vesication. All panels are from the same animal. Note the transient recovery of an impermeable epithelial cap by 7 days, only to be followed by a recurring epithelial lesion by 21 days in an MGK cornea. Middle right represents a resolved eye, which appeared to have fully healed. (Bottom left) Histology demonstrating massive increase in corneal edema during the first week, a result of cytotoxicity in the corneal epithelium and endothelium. Note: lesion margin in the epithelium of the 1 d cornea. (Bottom right) Longitudinal changes in corneal edema in resolving versus MGK eyes. Note the biphasic transition at 2 weeks in MGK corneas.

A second important pathology that was recently described is failure of the corneal endothelial barrier due to CEC loss. Early reports of HD toxicity included endothelial cell toxicity and uveitis in Class III injuries, suggesting that HD can penetrate through the cornea to the anterior chamber (Mann and Pullinger, 1944; Hughes, 1945b). Evidence of endothelial toxicity has also been identified in human MGK corneas, including reduced CEC density with increased variability in size and morphology (Jafarinasab et al., 2010). More recently techniques such as transmission electron microscopy, *in vivo* confocal microscopy, functional assessment of barrier,

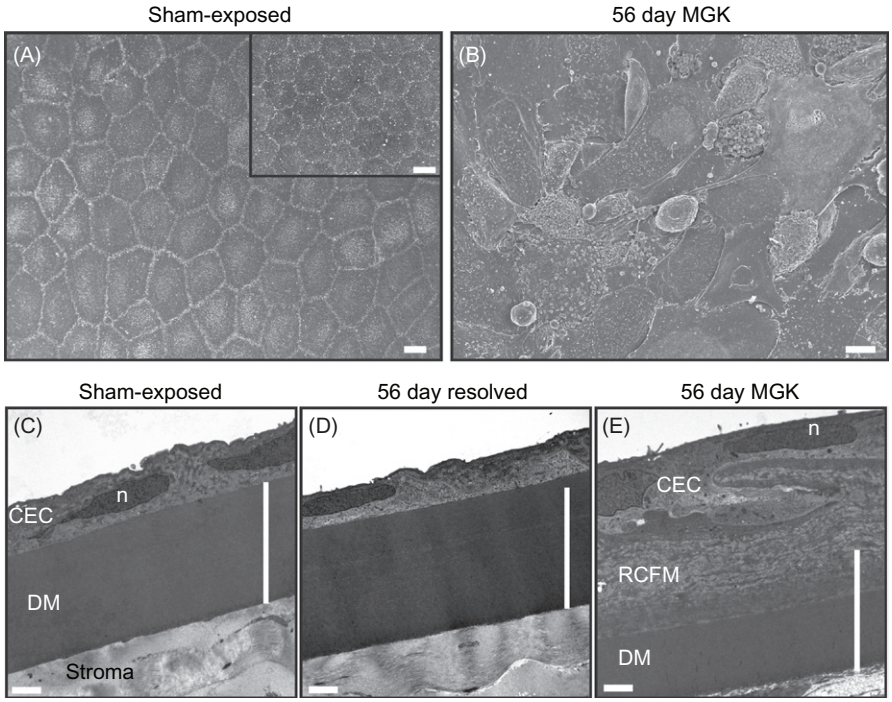


FIGURE 38.6 **Ultrastructural evidence of endothelial cytotoxicity.** (A–B): Scanning micrographs showing CEC morphologies in resolved (A) and MGK corneas (B–D) 8 weeks after SM exposure. Inset in A is a sham-exposed cornea at same magnification, highlighting the increased size of CECs in resolved corneas. Note evidence of ongoing cytotoxicity and a delayed healing response in B. Scale bars = 10 µm. Transmission micrographs of posterior cornea comparing ultrastructure of sham-exposed (C), resolved (D), and MGK (E) corneas 8 weeks after SM exposure. Note the extensive thickening of the posterior DM and formation of a retrocorneal fibrous membrane in the MGK cornea. For comparison, the vertical white lines represent the full thickness of the DM in sham-exposed controls. Labels: corneal endothelial cell (CEC); nucleus (n); Descemet's membrane (DM); retrocorneal fibrous membrane (RCFM). Scale bars = 2 µm.

and immunocytochemistry have been used to demonstrate in vapor-exposed rabbit eyes that corneal endothelial toxicity is associated with acute injury and MGK corneas, but not resolved corneas (McNutt et al., 2013). MGK corneas exhibited an idiotypic endothelial injury characterized by focal CEC loss, abnormal CEC morphologies, and a diffusively thickened DM (Figure 38.6). In contrast, resolved corneas exhibited a normal appearing corneal endothelium. Notably, other clinical endotheliopathies involving the rapid loss of large quantities of CECs, such as aphakic bullous keratopathy, produce secondary keratopathies that are strikingly similar to MGK, including epithelial bullae, delayed LSCD, and corneal inflammation (Taylor et al., 1983; Eagle et al., 1989; Alomar et al., 2011).

The potential involvement of endothelial toxicity in the late HD injury is intriguing for several reasons. First, it provides a mechanism to explicate the dose dependence of MGK onset, based on the permeation of sufficient doses of HD through the cornea to cause injury to the corneal endothelium. Second, it proposes that the clinically biphasic injury progression observed during the acute phase is an epiphenomenon arising from the differential healing capacities of the corneal epithelium and corneal endothelium. Furthermore, the limited ability to heal large endothelial lesions may contribute to the prolonged recovery time following Class III ocular injuries. Third, it provides a single pathology to explain both the chronic and delayed-onset forms of MGK. For example, in this model, the chronic form of MGK would be caused by the inability to restore an intact endothelial barrier, resulting in untreatable corneal edema. Alternatively, in the delayed-onset form of MGK, the delayed loss of CECs (i.e., as a consequence of aging, or due to delayed cytotoxic mechanisms such as genotoxicity) from an endothelium that is already diminished due to HD injury eventually results in endothelial failure and corneal decompensation. Additional implications of this hypothesis include the possibilities that therapies to restore the endothelium may mitigate the likelihood of MGK and that quantitation of endothelial loss may provide a diagnostic to identify corneas likely to develop MGK. Although there are still a large number of questions regarding the extent to which endothelial cytotoxicity and LSCD contribute to the acute injury and to MGK pathologies, it is encouraging that mechanistic studies have moved beyond the corneal epithelium to study other ocular tissues.

Lewisite

Lewisite was first synthesized in 1904 and stockpiled by the United States, Germany, and Japan during World War II. Although Lewisite is considerably more potent than HD, it produces immediate symptoms of physical discomfort, which rendered it relatively ineffective as a stand-alone chemical warfare agent. However Lewisite was found to increase the environmental persistence of HD by depressing its freezing point at doses that had no apparent physiological effect, and therefore Lewisite stockpiles were retained for several decades.

The toxicological effects of ocular exposure to lewisite (L; $C_2H_2AsCl_3$) are mediated by the interaction of inorganic arsenite (AsO_3^{3-}) with thiol groups of biologically active proteins, including dihydrolipoic acid (DHA). DHA is a co-factor in several critical enzyme systems critically involved in energy production, and disruption of DHA inactivates these enzyme complexes. For example, As^{3+} interaction with the E3 component of the pyruvate dehydrogenase complex prevents the conversion of pyruvate to acetyl-CoA, impairing ATP production and resulting in energy depletion, metabolic failure, and cytotoxicity (Young, 1999). Though lewisite injury progression can be mitigated by treatment with sulfhydryl-containing competitive antagonists of lewisite, such as 2,3-dimercaptopropanal (aka, British anti-lewisite; BAL), an ocular formulation of BAL is not currently available (Vilensky and Redman, 2003). Ocular neutralization of lewisite is very time-critical; unless BAL is topically administered with 2–5 min, lewisite eye injuries are irreversible.

Toxicokinetics of Ocular Lewisite Injuries

The distinctive toxicokinetic effects of lewisite exposure on ocular tissues produces an injury progression that is very different from the mustards (Ottinger et al., 1973; Romano et al., 2008; Tuorinsky, 2008). Unlike the latency associated with mustard exposure, lewisite causes rapid ocular irritation, lacrimation, blepharospasm, and chemosis. Eye pain occurs immediately, reaching its peak in 4–8 h. Corneal vesication, perforation, and blindness are observed following instillation of as little as 1 µL of neat lewisite to the corneal surface (Pechura and Rall, 1993). Vapor administration of 0.15 mg min/m^3 causes significant conjunctival injection and swelling of the eyelids, whereas corneal injuries occur at concentrations as low as 2.5 mg min/m^3. Permanent eye damage occurs at concentrations as low as 15.2 mg min/m^3. Since lewisite can be detected by smell at about 14–23 mg/m^3, permanent ocular injury is likely within 1 min of lewisite being detected that way (Gates et al., 1946). At high vapor doses or following liquid droplet exposure, delayed effects develop within 6–24 h, including vesication of the corneal endothelium, full-thickness keratocytosis, corneal edema, inflammatory cell infiltration, neovascularization, corneal perforation, and blindness.

Despite the twentyfold to thirtyfold increase in the toxicity of lewisite compared to the mustards, the ocular injuries caused by vapor exposure to lewisite are predicted to be less significant than HD under field conditions (Gates et al., 1946). Ocular irritation is almost immediate even at low concentrations, such that exposed personnel would be alerted to the presence of an irritant and able to rapidly take protective action. Second, the rapid onset of blepharospasm, ocular pain, and edema at low concentrations causes the eyes to close involuntarily, reducing the total ocular exposure.

Lewisite is very lipophilic, and ocular absorption has been reported to elicit toxicological responses in the ciliary body and iris (Young, 1999). At similar doses, lewisite is significantly more efficient than HD at evoking corneal edema (Pechura and Rall, 1993). At high doses, lewisite-exposed eyes rapidly develop miosis and severe uveitis, suggesting the trans-corneal permeation of lewisite to the inner chambers of the eye (Pechura and Rall, 1993). Since lewisite efficiently permeates the cornea to produce acute toxicity in posterior ocular tissues, it is also likely to cause CEC toxicity. This is consistent with a 1947 report that first described the symptomatic similarities between the mustards and lewisite, despite their distinctive modes of actions (Adler et al., 1947). Notably, this study also reported the focal loss of CECs following liquid instillation of Lewisite directly to the corneas of rabbits. It is currently unknown if lewisite exposure produces the equivalent of a late HD injury. While delayed-onset ocular effects analogous to severe HD-exposed eyes were not reported in lewisite-exposed rabbits, the rabbits were only followed for 30 days after exposure (Mann et al., 1946).

Phosgene Oxime

Although technically considered a vesicant, phosgene oxime (CX; Cl_2CNOH) does not produce blisters, and thus it is more appropriately considered an urticant, or nettle agent. Phosgene exposure causes significant pain on exposed skin and eyes through an unknown mechanism. Eyes rapidly develop conjunctivitis, lacrimation, lid edema, and blepharospasm after mild exposures, while more severe exposures can result in keratitis, iritis, corneal perforation, and blindness (Romano et al., 2008). In general, eye lesions are fairly similar to those caused by lewisite. Phosgene oxime injury has not been well studied, and long-term consequences of exposures are unknown.

Inhalational injury studies conducted in humans are limited to a controlled study with informed volunteers (Malatesta et al., 1983). This study reported that a vapor concentration of 1 mg/m^3 was the limit of physiological

detection. Exposure to 3 mg min/m^3 was defined as the minimum effective concentration causing an unpleasant or irritating sensation on the conjunctiva, nose, or skin. In the same study, mice, guinea pigs, and rabbits exposed to 3,000–15,000 mg min/m^3 phosgene oxime displayed agitation, respiratory difficulty, and intense lacrimation within 30 min. These symptoms appeared to resolve within 72 h, although no supportive histology or histopathology data were published.

NERVE AGENTS

The OPNAs include the G-series agents and the V-series agents, which are designated Schedule 1A substances (Table 38.1). The OPNAs inactivate AChE by alkyl phosphorylation of a serine hydroxyl group at the esteratic site of the enzyme (Romano et al., 2008). Once conjugated to AChE, the nerve agent eventually loses an alkyl side chain by hydrolysis in a process known as *aging*, further enhancing the stability of the enzyme–nerve agent complex. Inactivation of AChE by ageing is currently irreversible *in vivo*, and recovery of cholinergic function mainly depends on synthesis of new enzyme. Functionally, OPNA inhibition of AchE prolongs the residency of ACh neurotransmitter in the synapse, thereby resulting in the excessive stimulation of postsynaptic receptors. In the peripheral nervous system (PNS), the toxidromic progression is mediated by cholinergic overstimulation of several types of receptors, including nicotinic receptors at neuromuscular junctions; nicotinic receptors at autonomic ganglia; muscarinic receptors at parasympathetic efferents onto smooth muscles; and finally, muscarinic receptors on adrenal glands and sweat glands. Unlike the severe pathologies resulting from ocular exposure to the cytotoxic ocular irritants, the neuro-ophthalmic effects of exposure to OPNAs is comparatively mild and fully reversible.

Ocular symptoms are among the earliest and most sensitive indications of the cholinergic toxidrome caused by exposure to nerve agent vapor, particularly tearing, pupillary constriction (miosis) and loss of accommodation. Ocular symptoms of a cholinergic toxidrome can also be expressed at doses that do not cause other apparent evidence of toxicity, and therefore can serve as an early physiological marker of exposure to nerve agents (Baker and Sedgwick, 1996). For example, the most reliable indicator of exposure after the Tokyo sarin attack was miosis, which was observed in 90% of victims (Wiener and Hoffman, 2004). Ocular toxicity by OPNAs is specifically mediated by overstimulation of muscarinic receptors and excessive excitation of parasympathetic signaling within the eye, causing contraction of the pupillary sphincter and ciliary muscle and overstimulation of the lacrimal glands, which produces the toxidromic symptoms of miosis, blurred vision and tearing (Romano et al., 2008).

TABLE 38.4 Comparison of OPNA Lethal Ct_{50} and Miosis Ct_{50} Values

Agent	Lethal Ct_{50} (mg min/m^3)	Miotic Ct_{50} (mg min/m^3)
GA	400	2–3
GB	100	3
GD	70	<1
GF	Unknown	<1
VX	50	0.04

Source: Modified from Hurst et al. (2007) and Romano et al. (2008).

Miosis develops within seconds to minutes after the onset of ocular exposure to the agent or liquid vapor. This effect is a consequence of increased neurotransmission in the parasympathetic nervous system and is a result of the parasympathetic use of ACh as a neurotransmitter. The first intraocular target of nerve agents is the persistent overstimulation of parasympathetic muscarinic receptors on the pupillary sphincter muscle, causing the iris to contract and reduce light input into the eye (see Figure 38.2). This is corroborated by multiple findings. First, it was shown that nerve agent–induced miosis is inversely proportional to AChE activity in the iris (Soli et al., 1980). Second, pretreatment with the muscarinic receptor antagonist atropine prevents the miotic response (Dabisch et al., 2005). The second intraocular target of nerve agents is the ciliary muscles that control lens accommodation. In the presence of a nerve agent, overstimulation of the ciliary muscle alters the lens shape, increasing accommodation and thereby producing the blurred vision that is also characteristic of nerve agent exposure. While the dim and blurred vision reported following ocular exposure to OPNAs has been attributed to the reduced entry of light into the eye due to miosis, the presence of several subpopulations of cholinergic neurons within the retina raises the possibility that overstimulation of cholinergic terminals in the retina may contribute to the cholinergic toxidrome (Voigt, 1986). The final ocular target of the OPNAs is the parasympathetic inputs to the lacrimal glands, which causes increased tearing.

Notably, the miotic effect of OPNAs is mainly due to ocular absorption rather than systemic distribution, since liquid agent on the skin will not cause miosis until near-lethal doses (Table 38.4; Romano et al., 2008). In contrast, a droplet of liquid in or near the eye will cause miosis at very low concentrations (Hurst et al., 2007). Together, these suggest that the relatively privileged environment of the inner eye is more susceptible to direct absorption of nerve agent and drugs than to systemically circulated compounds. Furthermore, they indicate that the onset

of tearing with miosis, in the absence of pain, is a prodromic indicator of exposure to OPNAs.

PSYCHOMIMETIC INCAPACITATING AGENTS

Incapacitating agents are defined by the Department of Defense as "an agent that produces temporary physiological or mental effects, or both, which will render individuals incapable of concerted effort in the performance of their assigned duties" and are not intended to be lethal (Romano et al., 2008).

The development and use of psychomimetics as incapacitating agents have produced a variety of apocryphal stories, driven in part by the testing of LSD, PCP and BZ on military volunteers at Aberdeen Proving Ground (McFarling, 1980, National Research Council, 1984). Collectively, the incapacitating agents are an unusual category of agents, with varied physicochemical characteristics and psychopathological effects. The incapacitating agents include compounds with a wide spectrum of psychotropic effects resulting from psychomimetic activities, including agents that act as stimulants, depressants, psychedelics and deliriants. Psychomimetic agents that have been described in a military context include muscarinic antagonists, cannabinoids, indoles and anxiogenics.

The only psychomimetic chemical explicitly listed in the CWC is BZ, a Schedule 2A agent. BZ is an anticholinergic glycolate related to atropine that acts as a competitive antagonist of the postjunctional muscarinic receptors at ganglia and parasympathetic innervations onto smooth muscle and exocrine glands. By blocking the ability of muscarinic receptors to respond to the synaptic release of acetylcholine, BZ inhibits parasympathetic signaling and drives the ocular nerves toward sympathetic dominance. This shifts the pupillary balance toward mydriasis, failure of accommodation, and lacrimal paralysis. Thus, BZ effects on the eye are the opposite of the OPNAs, resulting in mydriasis, the loss of near-focus, and dry eye. Like nerve agents, once normal cholinergic signaling is restored, the ocular effects of BZ are fully reversed.

Unlike the prodromic effects of many agents on the ocular system, ocular symptoms of BZ inhalation occur secondary to more general signs of intoxication, such as incoordination, confusion, and slurred speech (Sidell et al., 1997). Although minimal human dose–response data are available for ocular symptoms of BZ exposure, relevant studies have been conducted in other species. The effective species-specific concentration:time values for mydriasis following inhalational exposure of BZ were less than 130 mg min/m^3 in dogs, 70 mg min/m^3 in monkeys, and 40 mg min/m^3 in rabbits (Ketchum, 1963; McNamara, 1963). At incapacitating doses (estimated to be 8 µg/kg), central effects persist in humans for 3–4 days; however, the persistence of ocular effects after such treatments were not described.

BLOOD AGENTS

The principal blood agents are hydrogen cyanide (AC; HCN) and cyanogen chloride (CK; NCCl). These agents are Schedule 3 toxic chemicals according to the CWC. Schedule 3A substances have legitimate, large-scale industrial uses; therefore, large stockpiles are likely to be available for repurposing as CWAs.

Cyanide is one of the least toxic of the lethal CWAs. The inhalational LCt_{50} values for AC and CK have been estimated to be 2,500–5,000 and 11,000 mg min/m^3, respectively (Simeonova, 2004). The cyanide ion (CN^-) is the toxic moiety, mediated primarily by its great affinity for the heme a_3 moiety of cytochrome c-oxidase in mitochondria, a key component in oxidative respiration. This interaction blocks the last stage in the electron transfer chain, resulting in cellular hypoxia and a shift of aerobic to anaerobic cellular respiration, leading to cellular ATP depletion and lactic acidosis. Therefore, tissues with high metabolic demands, such as neurons and cardiac cells, are key targets for toxicity. At lethal doses, death occurs within 6–8 min (Sidell et al., 1997).

Exposure to the blood agents can have two distinct effects on the eye, depending on the route of exposure. The retina and optic nerve are principal symptomatic targets of acute systemic cyanide exposure, with mydriasis commonly occurring at sublethal cyanide exposure and vision failure developing at higher doses. This is due to the metabolic inhibition of highly active neurons secondary to vascular distribution of the CN^- ion. The appearance of fixed and dilated pupils is common late in the toxidromic progression, but this is likely to result from the general loss of autonomic neuronal function rather than ocular-specific toxicity (Grant and Schuman, 1993).

Alternatively, topical administration of blood agents to the ocular surface results in local absorption and toxicity, primarily concentrated in anterior ocular tissues such as the conjunctiva and lacrimal glands. Thus, topical exposure to the blood agents elicits mild ocular symptoms, primarily characterized by lacrimation and conjunctival irritation. In animal studies, the administration of sodium cyanide (1.7–5.3 mg/kg/day) to the inferior conjunctival sacs of rabbits resulted in immediate conjunctival irritation and lacrimation (Ballantyne, 1983b). In a separate study, rabbits that were administered 0.9 mg/kg of hydrogen cyanide to the conjunctival sacs were reported to develop general keratitis (Ballantyne, 1983a). These two experiments involved the administration of

lethal doses of cyanide in liquid form directly to the conjunctiva, which is likely to lead to the very rapid cytotoxicity of local nerves, blood vessels, and epithelial cells. Exposure to sublethal concentrations of cyanogen chloride vapor similarly caused intense conjunctival irritation, severe blepharospasm, and lacrimation.

CHOKING AGENTS

The choking agents are chloropicrin (PS; CCl_3NO_2), phosgene (CG; $COCl_2$), and diphosgene (DP; $ClCO_2CCl_3$). These agents injure the nose, throat, and lungs, causing pulmonary edema and respiratory distress (Dembek, 2007). Although diphosgene has very similar physiochemical properties to phosgene, it readily condenses to a liquid and is therefore easier to handle and store. The ocular irritation caused by all three agents occurs at doses that are equivalent to the limit of olfactory detection. The acute symptoms of ocular exposure to phosgene and diphosgene (and possibly chloropicrin) are due to their hydrolysis to hydrogen chloride (HCl), creating what is essentially a chemical burn (Dembek, 2007). The longer-term respiratory symptoms are primarily due to hydrolysis as well as acylation, resulting from reaction of phosgene with nucleophilic moieties on macromolecules, such amino, hydroxyl, and thiol groups. Acylation denatures lipid and protein and disrupts enzymatic function and appears to play a more significant role than hydrolysis in longer-term pathophysiologies. In contrast, the mechanism of action for chloropicrin is not well understood, but may involve an oxidative reaction with biological thiols, such as glutathione and hemoglobin.

Although there is a paucity of acute human data containing ocular pathogenesis and toxicokinetics caused by phosgene or diphosgene, ocular irritation involving burning, conjunctivitis and lacrimation has been reported to develop within 20 min of exposure to 12 mg/m^3 phosgene (Bast and Bress, 2002). At concentrations of 30-40 mg/m^3, severe ocular irritation develops. Although phosgene's poor water solubility means that minimal hydrogen chloride is produced by hydrolysis, even small quantities are sufficient to elicit an initial irritation to the eyes, nasopharynx, and respiratory tract. Contact with liquid phosgene may result in corneal opacities and delayed perforation, consistent with a chemical burn injury and presumably resulting from the effects of HCl on the cornea.

Human exposure concentrations and times are better described for chloropicrin, which is roughly one-third as lethal as phosgene with an estimated median lethal dose of approximately 20,000 mg min/m^3. At low concentrations chloropicrin causes profuse lacrimation and conjunctivitis. At high concentrations it is severely irritating to the lungs, eyes, and skin. Thus the primary effects observed with short and long-term exposure to chloropicrin are sensory and respiratory irritation. The most likely route of ocular exposure is conjunctival and corneal absorption. Ocular irritation occurs prior to other symptoms of exposure, including respiratory irritation, and at doses that are below the limit of olfactory detection. Studies in human volunteers indicate that lacrimation and blepharospasm is apparent at 5.0 mg min/m^3, conjunctival irritation is apparent at 10–100 mg min/m^3, and intolerable ocular irritation developed at 500 mg min/m^3 (Fries and West, 1921, Prentiss and Fisher, 1937). These studies are consistent with more recent findings following an industrial accident in California that produced a plume of chloropicrin with estimated air concentrations between 0.17–1.0 mg/m^3 (Barry et al., 2010). Of the 324 residents who reported symptoms consistent with chloropicrin exposure, 302 (93.2%) presented with ocular symptoms such as lacrimation, ocular pain and burning.

RIOT CONTROL AGENTS

Riot control agents (RCAs), also called *irritants*, *lacrimators*, and *tear gas*, produce transient discomfort and eye closure that render the recipient temporarily incapable of fighting or resisting. The RCAs in use today are CS (2-chlorobenzalmalononitrile; $C_{10}H_5ClN_2$), CN (mace; C_8H_7ClO), CR (dibenzoxazepine; C_8H_7ClO), OC (oleoresin capsicum; $C_{18}H_{27}NO_3$), and various combinations of these agents. The RCAs share several characteristics that make them very effective as nonlethal agents, including rapid onset, with symptoms developing within seconds of exposure; a short duration of effect, with recovery occurring within 30 min after the end of exposure; and a high median lethal concentration with a low effective concentration meaning that they cause acute incapacitation without long-term effects or disabilities (Table 38.5) (Olajos, 2004). The eye is the most sensitive organ to RCAs, and although each RCA has slightly different effects, they all produce sensations of conjunctival and corneal burning, tearing, blepharospasm, and conjunctival injection. Barring direct application of solid RCAs to the eye or mechanical injury from the deployment of RCAs, the ocular symptoms are transient and fully reversible. Although RCAs are not considered to be CWAs according to the 1925 Geneva Convention, President Gerald Ford signed Executive Order 11850, banning the use of RCAs in war, except in defensive actions, without the advance approval of the president. Thus, although RCAs are incapacitating agents, they are not considered *military* incapacitating agents. However since RCAs are increasingly being deployed in other contexts and can theoretically be deployed in a military capacity, we will discuss them here.

TABLE 38.5 Toxicological Parameters of RCAs

Compound	Ocular Irritancy	Rate of Action	Irritancy Threshold (mg/m^3)	Intolerable Concentration (mg/m^3)	Lethal Concentration ($mg\,min/m^3$)
CS	Profound	Instantaneous	0.004	5	25,000
CN	Profound	Instantaneous	0.3	35	14,000
CR	Profound	Instantaneous	0.002	1	100,000
OC	Profound	Instantaneous	–	–	–
Chloropicrin	High	Rapid	2–9	50	20,000

Source: Modified from Olajos (2004).

RCAs can be categorized based on two general mechanisms of action. The C agents (CS, CN, and CR) are SN_2-alkylating agents that react readily with sulfhydryl-containing enzymes, such as lactate dehydrogenase. For example, CS reacts rapidly with the disulfhydryl form of lipoic acid, a coenzyme in the pyruvate decarboxylase system. These agents act as peripheral sensory irritants, which act primarily upon the eyes, respiratory tract, and skin. Despite small differences in potency, the C agents are similar in that exposure results in the near-instant ocular irritation, burning, and swelling of the conjunctiva, with uncontrolled lacrimation, blepharospasm, and increased intraocular pressure. The use of CS has largely replaced CN due to being significantly more effective as a sensory irritant, but much less toxic. Ocular irritancy studies for the C agents have been conducted in various animal species and in human volunteers (Punte et al., 1963; Ballantyne and Swanston, 1978). Generally, these studies were consistent in showing that exposure to low concentrations of CS was immediately irritating and rapidly became incapacitating at higher doses, but significant corneal toxicity did not occur until very high concentrations were achieved. For example, instillation of 5–10% CS to the corneal surface caused acute conjunctivitis, chemosis, keratitis, neovascularization, epithelial lesions, and corneal inflammation. The mechanism of injury at high concentrations is unknown, although it has been speculated that the alkylating properties of the C agents could disrupt protein structure. The poor water solubility of C agents limits their principal mode of action to the corneal epithelium. The potential for ocular irritation from CS is less than with CN; however, prolonged exposures to both will result in conjunctivitis and photophobia (Ballantyne et al., 1974).

The second class of RCAs is the capsaicins, represented by OC and its derivatives. The capsaicins are naturally occurring compounds of the capsicum plants, which include chili peppers and jalapenos. Capsaicin binds TRPV1, a vallinoid type I receptor that is activated by elevated temperature (43°C) or abrasion. TRPV1 is expressed by nociceptor neurons in the eye, and TRPV1 activation elicits symptoms of pain. Clinical signs of exposure to OC include lacrimation, transient conjunctivitis, redness, burning, pain, swelling, and blepharospasm. If applied directly to the eye, OC can cause neurogenic inflammation, insensitivity to chemical and mechanical stimuli, and loss of the blink reflex (Olajos and Salem, 2001). Generally, not much is known about the general toxicity of OC, but because it is a widely used food product, it is believed to be relatively nontoxic.

BIOLOGICAL TOXINS

Although there is ongoing disagreement over whether biotoxins should be classified as biological warfare agents (BWAs) or CWAs, they are clearly more similar to CWAs in their deployment, clinical manifestation, and medical management. Three categories of biotoxins will be discussed next, each of which exhibits a distinctive mechanism of action: neurotoxins, metabolic toxins, and bacterial superantigens.

Biological Neurotoxins

The BoNTs are highly lethal bacterial toxins produced by the *Clostridium* species, a family of bacteria that lives in the soil and in low-oxygen conditions. Clinical manifestations of botulism can occur following the ingestion, inhalation or injection of preformed toxin or by productive infection by toxin-expressing strains. There are currently eight known BoNT serotypes (/A–/H), of which /A, /B, /E, /F have been directly associated with human disease (Simpson, 2004; Dover et al., 2013). The toxin is expressed as a single 150 kDa peptide, which is post-translationally nicked to produce a dichain composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond (reviewed in Simpson, 2004). The HC mediates binding to presynaptic receptors and entry of toxin into the neuron via synaptic endocytosis. Acidification of the endosome triggers the HC to form a pore in the endosome membrane allowing the translocation of the LC from the lumen to the neuronal cytosol.

The LC then targets and cleaves one or more of three soluble NSF attachment protein receptor (SNARE) proteins with exquisite specificity: synaptosomal-associated protein 25 (SNAP-25; BoNT/A, /C, /E); vesicle associated membrane protein 1-3 (VAMP1-3; BoNT-/B, /D, /F, /G); or syntaxin (BoNT/C). The SNARE proteins are essential components of the synaptic exocytosis mechanism, and their cleavage prevents functional assembly of the ternary complex, thereby blocking neurotransmitter release. The combination of efficient neuronal targeting and presynaptic activation renders BoNTs the most potent substances known, with estimated human lethal doses as low as 0.1–1 ng/kg.

BoNTs interfere with the synaptic release of ACh from several types of cholinergic neurons, including motor neurons, preganglionic neurons, parasympathetic neurons, and some sympathetic neurons, such as those that innervate sweat glands. Furthermore, although there is a partial selectivity for cholinergic synapses, BoNT can also impair neurotransmission at other types of synapses, including adrenergic and noradrenergic sympathetic synapses (MacKenzie et al., 1982). Chemical denervation of autonomic signaling can occur at ganglia, which are cholinergic; at the synaptic fasciculation, where the neuron releases neurotransmitter onto the smooth muscle cells; or in both locations. Thus, the effects of intoxication on ocular behavior can vary based on dose and time after intoxication. Regardless, prominent neurologic findings in all forms of botulism include diplopia, blurred vision, and dilated pupils with weak pupillary responses. Tear production may be reduced because of peripheral parasympathetic cholinergic blockade, and ptosis is common.

The speed that nerve terminals are intoxicated is closely related to neuronal activity, since higher rates of neurotransmitter release correspond to increased toxin uptake. Consequently, ophthalmic manifestations are among the earliest signs of clinical presentation (Konig et al., 1975; Levy et al., 1991). Common findings include accommodative paresis (59%), blurred vision (89%), mydriasis (52%), and photophobia due to paralysis of the cholinergic terminals of the parasympathetic nerves. Impairment of tear production results from paralysis of the autonomic nerves innervating the lacrimal glands, leading to dry eye. Paralysis of the extraocular muscles is also an early symptom, producing double vision (diplopia, 59%), fixed gaze (36%), nystagmus (56%), and drooping eyelids (blepharoptosis, 80%). Manifestations at very high doses include tonic pupils that are poorly responsive to light, suggesting significant paralysis of both autonomic pathways. Supersensitivity of the iris sphincter muscle to muscarinic agonists is a characteristic in severely intoxicated eyes, suggesting that antagonistic signaling via the sympathetic pathway is also impaired (Caya, 2001).

Given the ocular BoNT toxidrome, it is obvious why the botulinum toxins are included in the neuro-ophthalmic modulators. Unlike nerve agent, which stimulates the parasympathetic pathway, or BZ, which blocks the parasympathetic pathway, botulinum activity can chemically denervate both signaling pathways at high doses, leading to poorly responsive or tonic pupils. In contrast, at lower systemic doses BoNT preferentially blocks cholinergic neurotransmitter release, resulting in the dominance of the sympathetic signaling pathway.

The shellfish poison saxitoxin (STX) and the pufferfish poison tetrodotoxin (TTX) are also biological neurotoxins. Both toxins antagonize the voltage-gated sodium channels responsible for the rising phase of an action potential in neurons, thereby preventing action potential propagation and reducing or eliminating neurotransmitter release. Both toxins are exceedingly potent, with intravenous LD_{50} values of 10 µg/kg and 8 µg/kg, respectively. The inhalational LD_{50} value for STX is estimated to be 5 mg min/m^3 (the equivalent value for TTX is not available). Although there is minimal data on toxic effects following topical exposure in healthy eyes, in principle the conjunctiva and corneal epithelium should provide an effective barrier to ocular absorption. Interestingly, topical administration of STX or TTX to deepithelialized corneas or by subconjunctival injection produces a long-lasting anesthetic effect, without causing ocular irritation or corneal edema (Duncan et al., 2001). In contrast, systemic administration can paralyze the ocular neurons, blocking pupillary light responses, spontaneous or reflexive eye openings and gaze (Lan et al., 1999). At intermediate doses, patients may experience blurred vision, darkened vision, and loss of accommodation, with temporary blindness occurring at higher doses. There do not appear to be any chronic complications from ocular paralysis resulting from exposure to TTX or STX, and upon recovery, oculomotor coordination, pupillary responses, and visual acuity were reported to be physiologically normal. These data suggest that the corneal epithelium and endothelium act as effective barriers to prevent the transcorneal absorption of TTX or STX, and therefore, ocular responses to aerosol exposures are more likely to result from inhalation and systemic distribution than from direct corneal absorption.

Ricin

Ricin is a heterodimer glycoprotein that is isolated from the castor bean. After nonspecific internalization into a wide variety of cells, ricin cleaves a glycosidic bond on the 60S subunit of eukaryotic ribosomes, preventing protein synthesis and causing cytotoxicity within 8 h. The median LD_{50} of ricin is around 22 µg/kg in humans from injection or inhalation. There is little information available on the ophthalmic manifestations

of ocular exposure to ricin; however, topical administration to the eye is severely irritating, causing chemosis and conjunctivitis (Hunt, 1918; Grant and Schuman, 1993). Ocular instillation of 100 µg to rabbits, guinea pigs, or mice causes extensive ocular inflammation that may lead to permanent corneal damage and blindness. It is unlikely that ricin can penetrate into the globe, and therefore the direct action of ricin must occur principally at the ocular surface (Strocchi et al., 2005).

Staphylococcus Enterotoxin B

SEB is a 23- to 29-kDa polypeptide in the bacterial superantigen family that cross-links the major histocompatibility class II receptor and T-cell receptors. This short-circuits the antigen processing and presenting mechanism, stimulating the release of pathologic levels of proinflammatory cytokines while failing to activate regulatory feedback signaling (Ahanotu et al., 2006). Prior to the 1975 Biological Warfare Convention (the biological equivalent of the CWC), SEB was studied by the United States as a biological agent that could be used to incapacitate soldiers in the battlefield. SEB was an attractive potential BWA because it can easily be aerosolized, is stable, and at low doses can cause widespread systemic damage when inhaled. In most circumstances, aerosol exposure results in a profoundly incapacitating illness lasting as long as 2 weeks, but it does not cause death (Sidell et al., 1997). SEB-induced ocular injuries have been described only three times in literature, following exposures where workers placed contaminated materials close to their eyes in the laboratory. Symptoms appear within 1–6 h, and included significant eyelid edema, periocular swelling, and acute conjunctivitis with discharge. Symptoms resolved within a week, although at least one patient appeared to exhibit continued hypersensitivity. These suggest that casualties resulting from an inhalational exposure may present with ocular toxicities that can be acutely incapacitating although, as with ricin, primarily localized to the ocular surface (Rusnak et al., 2004).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A wide array of CWAs and biological toxins exhibit acute ocular toxicities. The mode of action of these agents can be broken into three general categories: those that exhibit acute cytotoxic activity; those that stimulate symptomatic distress without directly causing cytotoxicity; and those that modulate neuro-ophthalmic signaling, thereby causing incapacitation, without directly injuring corneal tissues. The exquisite sensitivity of the eye to many of these agents enables the rapid appearance of ocular manifestations to serve as prodromic indicators of exposures. In the case of the CWAs that are most injurious to the eye—namely, vesicants—considerable research is still needed to identify therapeutic modalities that protect against long-term, progressive sequelae that can result in loss of vision. For most other agents, the ocular toxicity is mild or reversible at reasonable exposure estimates. However, one cannot discount the acute psychological distress of ocular pain or impaired vision, particularly if the causative agent is unknown and the prognosis is unclear. Until comprehensive treatments are identified for the cytotoxic CWAs, vision will always be a primary target of CWA deployment.

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CHAPTER

39

Dermal Toxicity of Sulfur Mustard

Joshua P. Gray[1], *Michael P. Shakarjian*, *Donald R. Gerecke and Robert P. Casillas*

INTRODUCTION

Skin integrity is in part determined by the interaction of a number of proteins that form a continuum of molecules linking together to ensure the epidermis and dermis are tightly attached to one another. These macromolecules include the keratins that form the intermediate filaments in the cytoplasm of keratinocytes, integrins that are made of two subunits and found in the hemidesmosomes of the basal keratinocyte membrane, and a variety of laminins, collagens, and their receptors, situated both in the basement membrane that separates the epidermis from the dermis and in the dermis itself. Any chemical agent that disrupts this continuum of linking proteins will result in a breach in skin integrity and ensuing histopathology (Uitto et al., 2007). One such compound is the alkylating agent sulfur mustard [bis(2-chloroethyl) sulfide; HD; SM], which causes detachment of the epidermis from the dermis (this chapter uses the abbreviation SM for sulfur mustard because, in civilian dermatological research, HD is the abbreviation for the hemidesmosome, an important morphological structure found in the cell membrane of keratinocytes). Skin exposure to SM starts a complex series of events with a host of normal skin responses to wounding that interact with, influence, and regulate each other to result in cutaneous toxicity. Various mediators of injury that regulate inflammation, immune responses, cell death, and a number of signaling pathways have been implicated in the process. This chapter describes our current knowledge of the cutaneous actions of SM by discussing the basic mechanism of action and mediators involved to provide for the reader a comprehensive understanding of the histopathology of SM-exposed skin. The injury process is described and SM-induced injury is compared with other types of wound injury. Additionally, various vesicant injury models are described and potential therapeutic countermeasures are discussed.

BACKGROUND

Military Use

SM, a vesicant or blistering agent, has been used intermittently as a chemical warfare agent since 1917, when Germany first introduced it as a weapon against British soldiers in Ypres, Belgium. Subsequently, in the 1930s, it was reported to be used by Italy against Abyssinia (Ethiopia), by Poland against Germany, and by Japan against the Chinese. During the 1960s, SM was allegedly used by Egypt against Yemen. The last well-documented use in the 1980s was by Iraq against Iran and the Kurds (Papirmeister et al., 1991a,b,c). As recent as 2013, Syria, working with the Organization for the Prohibition of Chemical Weapons (OPCW) and the United Nations, has begun destroying its chemical weapon stockpile that included both nerve agents and SM. Also overseen by the OPCW, Libya completed destruction of its SM stockpile in 2013.

Wound Repair

Even repair of the simplest skin wound requires the coordination of a number of physiological processes and events. The tissue injury causes blood vessel disruption

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and an activation of the inflammatory pathway that releases a battery of mediators. Re-epithelialization requires activation of additional pathways and the initiation of specific events at specific times, such as extracellular matrix remodeling and the proliferation and migration of keratinocytes. If these events are not precisely coordinated, then the end result can be delayed healing and/or scar formation. Because the repair process is complicated, a review of the wound healing events in dermal injury might be helpful. There are at least three phases to wound healing after dermal injury (Gurtner et al., 2008). Inflammation (first 24–48 h) is the first. This phase occurs early and involves activation of the coagulation cascade, inflammatory pathways, and immune system. These biological processes involve an attempt to maintain homeostasis by preventing blood and fluid loss, removing dead tissue, and preventing infection. The second is the wound repair phase (2–10 days after injury). This phase involves the proliferation and migration of several cell types. Keratinocytes migrate over the injured dermis, endothelial cells proliferate to form new blood vessels, and fibroblasts differentiate into myofibroblasts to initiate wound contraction. Third is the remodeling phase (2–3 weeks after injury). This phase results in the final wound repair. All the activated cellular processes turn off. Many cell types undergo apoptosis or leave the wound site. The extracellular matrix is remodeled and resumes normal functions.

These same basic phases are applicable to the injury caused by SM, but the pathology frequently is more severe than other types of dermal wounds and the basic timing of the various phases may be extended in SM injury (Papirmeister et al., 1991a,b,c; Dacre and Goldman, 1996; Balali-Mood and Hefazi, 2006). Normal clothing provides little protection to the skin from a liquid or vapor exposure to SM. Once in contact with the skin, the lipophilic properties of SM allow it to rapidly penetrate the epidermal barrier, while its high reactivity and bifunctional nature lead to alkylation of a wide range of molecules (Figure 39.1). The ultimate injury caused by SM is highly dependent on the dose and length of exposure to the agent, resulting in a wide range of histopathology. Erythema, resembling sunburn, may be the mildest and earliest form of skin injury, occurring 2–48 h after SM exposure (USAMRICD, 2007). The erythema may be accompanied by pruritus or burning pain and small vesicles may eventually develop, which coalesce to form bullae (Figure 39.2). Fluid-filled blisters increase in size, their color ranging from yellow to tan. The fluid itself does not contain active alkylating agent, nor does it have vesicating properties. When SM is applied as a liquid, the exposure concentration is even higher than that of a vapor application and severe lesions are more likely. The resultant wounds are prone to secondary infections and may cause chronic ulcers that are resistant to wound repair. There may be permanent pigmentation changes in the skin after exposure to SM. Clearly, the effects of SM on skin are complex and involve many systems and pathways. The following sections attempt to address the mechanism of action of SM and the systems and pathways involved in an organized way.

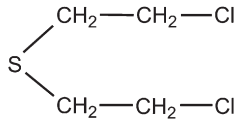

FIGURE 39.1 Chemical structure of SM [bis(2-chloroethyl) sulfide].

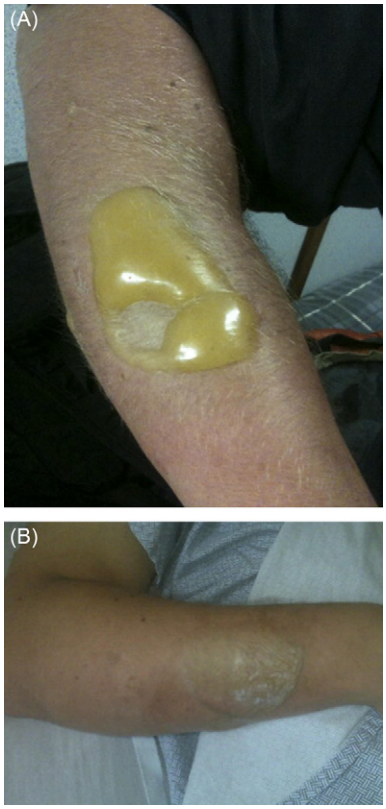

FIGURE 39.2 Fluid-filled bullous blister of a clam fisherman accidentally exposed to SM, shown 24 h (A) and 6 days (B) after exposure. The patient was exposed when handling discarded WWI munitions trawled from the sea bed off the coast of New England in 2010 (Weibrecht et al., 2012). *Source: Reproduced with permission from Elsevier.*

PATHOGENESIS

The bulk of what is known about human skin injury from SM has been gathered from victims of the military use of the compound (Requena et al., 1988; Balali-Mood and Hefazi, 2006; Hefazi et al., 2006). Nevertheless, there

are several reports in which the cutaneous vesicating effects of SM were studied experimentally in human subjects (Papirmeister et al., 1991a,b,c; Dacre and Goldman, 1996). In one comparative study (Daily et al., 1944), 12 volunteers had forearm vapor cup applications of SM, trifunctional nitrogen mustard (HN3), and lewisite. The conclusions were that SM exposure caused the largest vesicles formed, the earliest peak vesication, the longest healing time, and the most severe final scar. Lewisite was the second most potent agent and HN3 was the least potent, although the HN3 lesions had the most edema of the three vesicants examined. The results confirmed the previously published studies that followed the development of skin pathogenesis after topical exposure to SM.

Clinical injury by SM has been extensively reviewed. SM injury to human skin begins almost immediately after exposure, when the highly reactive bifunctional SM directly alkylates resident proteins. However, recognizable skin pathology does not usually occur for several hours to a day after exposure. The very first physical sign of SM exposure is usually erythema, which may or may not be associated with itching (Papirmeister et al., 1991a,b,c). This is often followed later by distinct fluid-filled blisters (Figure 39.2) that break open and become covered with a scab. Many researchers have noted that the blisters resemble those formed by epidermolysis bullosa (EB), a genetic or acquired skin pathology that results in a separation of the epidermis from the dermis (Figure 39.3). In both SM exposure and EB, there may be multiple rounds of blistering and healing in an individual (Balali-Mood and Hefazi, 2005; Pillay, 2008). In fact, some of the therapeutic agents for EB are being tested as potential medical countermeasures against SM injury. These are addressed later.

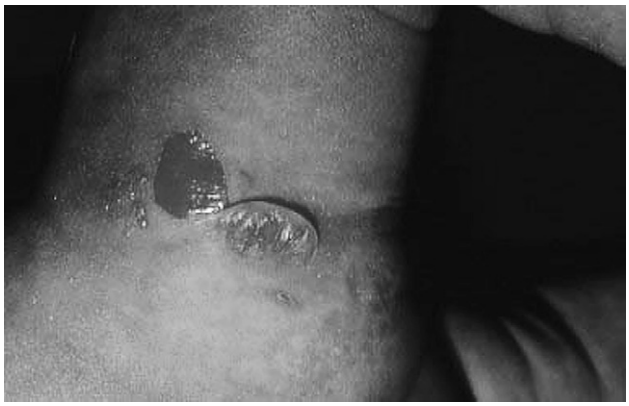

FIGURE 39.3 Fluid-filled bullous of patient with EB caused by truncated gamma-2 polypeptide chain of laminin-332 (formerly laminin 5, a heterotrimeric glycoprotein).

Cytotoxicity of SM

Although stable in lipophilic solvents, SM has a half-life of only 24 min at room temperature in aqueous physiological solutions because it rapidly reacts with water to form thiodiglycol and HCl. This rapid activation of SM in an aqueous environment also allows it to react with small molecules of biological interest, as well as proteins, carbohydrates, lipids, RNA, and DNA (Bartlett and Swain, 1949; Papirmeister et al., 1991a,b,c; Debouzy et al., 2002; Noort and van der Schans, 2002). The initial reaction involves the formation of a cyclic ethylene sulfonium ion that readily targets reactive groups on skin components, including sulfhydryls, phosphates, ring nitrogens, and carboxyl groups. As a bifunctional alkylating agent, SM forms monofunctional adducts and intramolecular and intermolecular cross-links. Because it is so reactive and indiscriminate in its molecular targets, SM affects many pathways and is cytotoxic on many levels. There are at least three independent mechanisms of cytotoxicity that have been proposed and each is addressed in separate sections.

Alkylation of DNA/Poly(ADP-Ribose) Polymerase Activation

One of the major targets of SM alkylation is DNA that can form both monofunctional adducts and bifunctional cross-links (Papirmeister et al., 1991a,b,c; Debiak et al., 2009), which can persist for at least 21 days (Batal et al., 2013). Complementary DNA strand and intrastrand cross-links occur after SM exposure (Walker, 1971). The cross-linking of cellular DNA underscores the importance of DNA repair pathways in preventing cytotoxicity of SM-exposed cells. These repair pathways include both the nucleotide and base excision pathways (Matijasevic and Volkert, 2007; Jowsey et al., 2009). However, recent evidence suggests that the homologous recombination is perhaps the most important mechanism for SM exposure, with nucleotide and excision pathways playing a supportive role (Jowsey et al., 2012).

DNA alkylation can also lead to single-strand and double-strand DNA breaks. These breaks trigger activation of a family of nuclear repair enzymes called poly(ADP-ribose) polymerases (PARP) (Papirmeister et al., 1985; Shall and de Murcia, 2000). Although low levels of PARP activation may signal repair, excessive activity can deplete cells of PARP's major substrate, NAD^+. Depletion of NAD^+, in turn, inhibits ATP production, which is essential for metabolism (Martens and Smith, 2008). Apoptosis or necrosis may result, depending on level of ATP depletion, cell type, and other factors (Rosenthal et al., 2001). Preventing the depletion of NAD^+ by interfering with PARP activation has been the rationale for testing PARP inhibitors as therapeutic countermeasures (Debiak et al., 2009). To date, the literature suggests this strategy has been unsuccessful *in vivo* (Casillas et al., 2000a,b).

Reactions with Glutathione/Oxidative Stress

It is also hypothesized that oxidative stress plays a key role in the cutaneous vesicating actions of SM. Under homeostatic conditions, a net reducing environment is maintained in tissues by the presence of glutathione (GSH), which serves as a buffer against cytotoxic electrophiles and reactive oxygen species (ROS). GSH peroxidase catalyzes the reaction of reduced GSH with hydrogen peroxide. Depletion of intracellular GSH allows the accumulation of oxidants such as H_2O_2, which become abundant and actively contribute to lipid peroxidation and other types of cellular damage. Dermatotoxic agents such as ultraviolet radiation (UVA) and psoralens have been shown to deplete intracellular GSH, resulting in cellular toxicity (Wheeler et al., 1986). The propensity of SM to react with sulfhydryls is thought to lead to a concentration-dependent depletion of reducing equivalents within cells. Recent findings also suggest that SM and related vesicants can interact with key intracellular reductases to generate mustard-free radicals (Brimfield et al., 2009). In other tissues, SM exposure has been shown to reduce GSH peroxidase and GSH reductase activity, potentially contributing to the depletion of reduced GSH (Husain et al., 1996; Jafari, 2007). In addition, inflammatory cells, which infiltrate into the skin in response to SM-induced injury, generate additional ROS that contribute to oxidative stress (Droge, 2002). This raises the possibility that the vesicating actions of mustard alkylating agents involve oxidative stress.

Recent evidence indicates that GSH depletion and oxidative stress are important mechanisms of cutaneous toxicity induced by SM and its vesicating analogues. Cutaneous GSH peroxidase gene expression is increased after SM treatment in the mouse ear vesicant model (MEVM), suggesting that this enzyme is important in reducing oxidative stress due to SM exposure (Buxton et al., 2001).Treatment with the monovalent mustard analogue, chloroethyl ethyl sulfide (CEES), has been shown to significantly deplete GSH in mouse and human keratinocyte cell lines (Tewari-Singh et al., 2011). Furthermore, depletion of intracellular-reduced GSH by buthionine sulfoxamine sensitizes keratinocytes treated *in vitro* with SM (Simpson and Lindsay, 2005) or CEES (Tewari-Singh et al., 2011).

There is more direct evidence from both *in vivo* and *in vitro* studies that CEES produces oxidative stress. CEES application to the dorsal skin of SKH-1 hairless mice led to time-dependent and dose-dependent increases in oxidative damage to tissue proteins as evidenced by modification by lipid peroxides, radical adduction, and the formation of protein carbonyls (Pal et al., 2009). Increased DNA oxidation, detected as the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG), was also a consequence of CEES exposure. Subsequent studies with the JB6 mouse keratinocyte cell line and primary fibroblast in culture confirmed the ability of CEES to increase 8-OHdG and also increase both mitochondrial and cytosolic superoxide generation (Inturi et al., 2011). A report using mouse PAM212 keratinocytes in three-dimensional air–liquid interface cultures demonstrated concentration-dependent increases in hydrogen peroxide and protein carbonyl formation after topical application of CEES (Black et al., 2010a,b). These changes were accompanied by increases in the relative mRNA of Cu,Zn-SOD, catalase, thioredoxin reductase, and several glutathione-*S*-transferases (GSTs), suggesting a compensatory cellular response to oxidative stress. Although the role of GSTs in modulating mustard vesicant–induced cutaneous toxicity is unclear, their levels have been documented to increase overall in response to SM (Gross et al., 2006). In summary, there is now compelling evidence that the loss of cellular reducing equivalents and the increase in oxidative stress are key contributors to cutaneous toxicity by SM and its vesicating analogues.

Reactions with GSH/Calcium Homeostasis

Depletion of GSH also affects calcium homeostasis; treatment of primary human epidermal keratinocytes with buthionine sulfoxamine decreased the level of reduced GSH but increased intracellular Ca^{2+} (Ray et al., 1993). Neuroblastoma cells treated *in vitro* with 0.3 mM SM maintained high cell viability for nearly 9 h, which then decreased with time. The decrease in cell viability was prefaced by an increase in free intracellular calcium that occurred between 2 and 6 h after exposure (Ray et al., 1995). Intracellular-free calcium is a well-recognized marker of cell stress (Ruff and Dillman, 2007). Induction of intracellular calcium is thought to contribute to apoptosis induced by SM exposure in keratinocytes *in vitro* (Rosenthal et al., 2003). Despite these findings, Sawyer's group has shown that the sensitivity of primary human keratinocytes to SM was unaffected when calcium was reduced using chelators or increased using the membrane ionophore ionomycin (Sawyer and Hamilton, 2000). These findings suggest that although calcium levels are significantly altered by SM treatment, modulation of calcium alone is neither necessary nor sufficient for SM-induced apoptosis.

Inflammation

Inflammation is likely one of the major driving forces of the skin pathology caused by SM exposure. The contribution of cytokines to the inflammatory events is well established and their signaling pathways are important targets of medical intervention for potential countermeasures of vesicant injury. The literature linking inflammation and wounding is so extensive as to fill a book by itself. This section therefore only presents an overview

of the inflammatory response to dermal skin injury. In short, cytokine signaling involves several Janus kinase (Jak)-signal transducer and activator of transcription (Stat) pathways (O'Shea and Murray, 2008). These pathways are the major regulators of all the cell types involved in inflammation and they can be either positive or negative mediators to the inflammatory process. Both pro-inflammatory and anti-inflammatory events are under the influence of the Jak–Stat pathways and hundreds of cytokine receptors are regulated via these pathways including more than 40 type I and type II cytokine receptors and receptors for interleukins, interferons, growth-stimulating factors, leptin, erythropoietin, and many more.

As for vesicant-injured skin, the inflammatory response appears to be biphasic. This is based on our years of experience in addition to that of others who reported that inflammation played a minor role in the initial events of SM-induced cutaneous injury but had much greater importance at later stages (Papirmeister et al., 1991a,b,c). In another examination, the authors argued that inflammation may be more significantly involved in the vesication event than previously believed, with inflammatory cells and mediators contributing directly to the formation of the primary lesion (Cowan and Broomfield, 1993). In a recent study, Joseph et al. (2011), documented a correlation between markers of inflammation and DNA damage with structural changes in skin after SM exposure (Joseph et al., 2011).

Numerous accounts, from both *in vivo* and *in vitro* studies, have now documented an increase in a number of inflammatory cytokines in response to SM. Applications of SM in the MEVM have resulted in induction of IL-1β, IL-6, TNFα, and GM-CSF within 6 h (Sabourin et al., 2000; Wormser et al., 2005). Although IL-1α was not detected in these studies, its inducibility by SM vapor was confirmed in experiments in which the backs of hairless mice were exposed (Ricketts et al., 2000). Further, relative mRNA levels of IL-1β, IL-6, IL-8, and TNFα have been demonstrated to significantly increase within 24 h of SM vapor treatment in full-thickness skin biopsy samples from weanling pigs (WP) (Sabourin et al., 2002). Studies with cultured human keratinocytes have shown that these cells respond directly to SM with the production of cytokines. IL-1β, IL-6, IL-8, and TNFα were detectable in culture media from these cells 24 h after treatment with 100–300 μM SM (Arroyo et al., 2000). Cultured skin fibroblasts have also been shown to express TNFα in response to SM. The chemotactic activity of keratinocyte-derived IL-8 could initiate the transmigration of circulating granulocytes into SM-exposed tissues where they could contribute to the primary lesion. Other cytokines may be involved in priming and activation of the recruited immune cells. Although cytokine induction may occur via several means, there is now ample evidence that the inflammatory transcription factor NF-κB is one such pathway activated by SM (Atkins et al., 2000; Minsavage and Dillman, 2007; Rebholz et al., 2008). In fact, enhanced synthesis of the aforementioned cytokines in resident skin cells may occur as a result of this NF-κB activation (Ghosh et al., 1998).

Additional inflammatory mediators have been detected in cutaneous tissues as a consequence of SM treatment. These include free arachidonic acid (Lefkowitz and Smith, 2002) and its cyclooxygenase (Rikimaru et al., 1991; Dachir et al., 2004a,b) and lipoxygenase products (Tanaka et al., 1997). Furthermore, the increased capillary permeability observed would allow a variety of circulating inflammatory participants, such as complement components and kininogens, to enter the dermal interstitium (Rikimaru et al., 1991). Clearly, vesicant injury involves a host of inflammatory mediators similar to those seen in other types of wounds, where individual cytokines have been singled out as potential therapeutic targets.

Protease Activation

Although there may be protease involvement in the initial SM injury, most studies have viewed the involvement of serine and matrix metalloproteases (MMPs) in SM injury as a downstream event because MMPs in particular regulate various inflammatory and repair processes (Parks et al., 2004). The regulatory role confounds the potential use of MMP inhibitors as therapeutic agents because they play a beneficial role in wound repair and skin degradation. Increased protease activity after SM exposure has been reported *in vitro* in human peripheral blood lymphocytes (PBL) (Cowan and Broomfield, 1993) and human epidermal keratinocytes (Smith et al., 1991), *ex vivo* in rabbit skin organ cultures (Higuchi et al., 1988; Woessner et al., 1990) and human skin explants (Rikimaru et al., 1991; Lindsay and Rice, 1996), *in vivo* in hairless guinea pig (HGP) skin (Cowan et al., 1993, 1994; Kam et al., 1997), and *in vivo* in the mouse ear (Powers et al., 2000) and hairless mouse (Casillas et al., 2000a,b). The continued assessment of proteolytic activity in animal models is useful for characterizing specific proteases important to SM injury and for identifying effective protease inhibitors with therapeutic use in reducing or eliminating tissue injury caused by SM cutaneous exposure. The relationship between SM-increased protease activity and the subsequent vesication (blistering) that occurs in SM lesions remains unclear, but emerging literature implicating protease involvement is consistent with the known ability of proteases to degrade basement membrane components *in vitro* and *in vivo*. Inhibition of these proteases should reduce the extent of the injury, promote a more rapid recovery, and provide a useful adjunct to other therapeutic strategies aimed at

preventing SM-induced degenerative pathophysiological events (Cowan et al., 1993).

Apoptosis

Mechanisms underlying SM-induced apoptosis have been carefully explored using primary cultures of human keratinocytes. Treatment of keratinocytes with 100–300 μM SM resulted in activation of caspase 8, which initiates the Fas-dependent death receptor pathway, and caspase 9, which initiates the mitochondrial apoptotic pathway (Rosenthal et al., 2003). Fas and Fas ligand were upregulated in a concentration-dependent manner by SM leading to activation of caspase 3, the central executioner protease. Transfection of immortalized keratinocytes with a dominant-negative Fas-activated death domain resulted in a blunted caspase response to SM. Microvesication and tissue injury produced *in vivo* by SM exposure of transfected cells after grafting onto athymic nude mice was also reduced by this treatment.

Changes in intracellular calcium levels are known to activate the mitochondrial pathway of apoptosis. A key regulator of Ca^{2+}-dependent proteins is calmodulin. SM has been shown to cause a time-dependent induction of calmodulin in keratinocytes (Simbulan-Rosenthal et al., 2006). Moreover, depletion of calmodulin using antisense probes attenuated SM-induced activation of caspases involved in the mitochondrial pathway of apoptosis. Both antisense and pharmacological inhibition of calmodulin prevented SM-induced nuclear fragmentation in the keratinocytes. Bad, a proapoptotic Bcl-2 family member present in an inactive phosphorylated form in viable cells, was also activated by SM. Furthermore, cyclosporine A, a selective inhibitor of calcineurin, a Bad phosphatase, inhibited SM-induced keratinocyte apoptosis. These results suggest that calcium-dependent activation of Bad may be a mechanism by which SM induces apoptosis in keratinocytes.

One form of cellular demise common to epithelial cells is detachment-initiated apoptosis, also referred to as anoikis (Frisch and Francis, 1994). Epidermal keratinocytes rely on signals derived from the surrounding extracellular matrix for survival. It is possible that loss of these signals plays a role in SM-induced epidermal cell injury, and that cell detachment from the basal lamina precedes cytotoxicity. Several lines of evidence support this possibility. First, SM can alter the dynamics of cytosolic proteins that exert control over the attachment of cells to the basement membrane. For example, SM can modify intracellular actin microfilaments and keratin intermediate filaments known to be important in maintaining epithelial cell connections with the basal lamina. Thus, Hinshaw et al. (1999) reported that SM causes changes in the actin microfilament architecture and morphology of human keratinocytes within 3 h of exposure (Hinshaw et al., 1999). This was associated with a significant decrease in keratinocyte adherence without evidence of cytotoxicity. In addition, Werrlein and Madren-Whalley (2000) found that SM caused rapid, significant decreases in immunodetection of keratins 5 and 14, an intermediate filament pair found in undifferentiated keratinocytes (Werrlein and Madren-Whalley, 2000). In both *in vivo* (Gunhan et al., 2004) and *in vitro* studies with human keratinocytes (Dillman et al., 2003), and with purified proteins (Hess and FitzGerald, 2007), keratins 5 and 14 have been found to be alkylated by SM as well as nitrogen mustard (mechlorethamine, HN2) and 2-CEES, the monofunctional analog of SM. Sites of alkylation may be similar to dominantly acting mutations in keratins 5 and 14 that are known to be responsible for the human blistering disorder, EB simplex, in which, like SM-induced blistering, basal epidermal cells are targeted (Fuchs, 1997). The keratin cytoskeleton of basal keratinocytes links to the hemidesmosome and makes connections, through plectin, with the β4 cytoplasmic tail of integrin α6β4, thereby strengthening adhesion to the basement membrane via laminin-332 (Giancotti and Tarone, 2003). Alkylation of keratins 5 and 14 could cause aggregation and loss of function of the intermediate filament network and serve as a prelude to basal cell separation from the basement membrane.

In addition to its actions on epidermal cells, SM can directly alkylate extracellular matrix proteins in the skin, a process that can also alter the ability of basal keratinocytes to maintain vital connections with the basement membrane. In support of this idea, Gentilhomme et al. (1998) showed that SM treatment of human dermal equivalents reduced the ability of naïve keratinocytes to deposit laminin at the dermal–epidermal interface (Gentilhomme et al., 1998). In addition, Zhang et al. (1995a,b) found that treatment of fibronectin and laminin with SM interfered with the ability of human epidermal keratinocytes to adhere to these matrix proteins (Zhang et al., 1995a,b). This inhibitory action was determined to be alkylation-dependent, because it could be prevented by co-treatment with SM scavengers. SM and nitrogen mustard also reduce cell and tissue immunoreactivity for laminin-332, as well as integrin α6β4 and collagen XVII (also known as bullous pemphigoid antigen), two hemidesmosomal components that are critical for keratinocyte adherence (Smith et al., 1997a,b,c, 1998; Zhang and Monteiro-Riviere, 1997; Werrlein and Madren-Whalley, 2000; Kan et al., 2003). Interestingly, each of these proteins, like keratins, has been implicated in human blistering disorders involving separation of the epidermis at the dermal–epidermal junction (Pulkkinen and Uitto, 1998; Yancey, 2005). These findings suggest that SM can alter the interaction of basal cells with matrix proteins critical for basement membrane detachment. The alteration may occur by reducing protein functionality, decreasing

protein expression, or increasing protein degradation. By whichever mechanism, a negative alteration in these critical anchoring components could lead to basal cell detachment and initiate anoikis.

Signal Transduction Pathways

SM exposure induces the activation of many molecular signaling pathways (reviewed in Ruff and Dillman, 2007). These pathways mediate many responses including inflammation, cell proliferation, cell differentiation, and apoptosis. Some that involve inflammation include the transcription factor NF-κB and the p38 MAP kinase. NF-κB is a transcription factor that is induced within 2–4 h after SM exposure (Minsavage and Dillman, 2007). Dillman has suggested that this delayed induction is due to a nontraditional pathway of stimulation, whereby p90RSK phosphorylates IκB or the p65 subunit of NF-κB (Ruff and Dillman, 2007). The MAP kinase p38 is activated in response to damaging stimuli, including heat, UVA, and pro-inflammatory stimuli. Dillman's group and others have noted an increase in activation of p38 by phosphorylation and demonstrated that inhibition of p38 phosphorylation resulted in a decrease in SM-induced pro-inflammatory cytokine production *in vitro* (Kehe et al., 2009). However, the importance of NF-κB or p38 activation in mediating SM cutaneous injury has not been demonstrated *in vivo*. Also, although important for understanding SM-mediated toxicity, these pathways are difficult to target pharmacologically.

MODELS OF DERMAL SM EXPOSURE

Introduction

Although there has been considerable investigation of SM therapies since the publication of Bruno Papirmeister's landmark compilation of *Medical Defense Against Mustard Gas* nearly 25 years ago, no suitable treatment for SM exposure to the skin has been developed (Papirmeister et al., 1991c). Since that time, there has been an increased focus on civilian research as the perceived risk of a terrorist-driven exposure to civilian populations has been recognized. The field has dramatically changed direction from strategies and therapies based on protection from SM exposure to therapies designed to subvert the blistering process and increase the rate of wound healing. New pharmaceutical strategies will be increasingly focused on combination therapies that target multiple processes in blister formation and the wound healing process, together with increased recognition of the importance of pharmaceutics.

Although many advances have been made in the production of skin barrier protection or postexposure skin decontaminants (such as skin exposure reduction paste against chemical warfare agents and reactive skin decontamination lotion (RSDL), respectively), drug countermeasures against vesicants remain a subject of intense investigation. The primary treatment strategy after exposure to vesicants such as mustard gas is decontamination. However, given the high reactivity of SM, there is a very short timeframe (3–5 min) in which decontamination can be effectively accomplished (reviewed in Vogt et al., 1984; Wormser, 1991); and some studies suggest that an extractable reservoir persists for much longer. Furthermore, because SM exposure does not cause immediate pain or noticeable effects, exposure is often not recognized until the effective window for decontamination has passed. Therefore, much research has focused on the identification of treatments that can be performed after exposure has occurred. These treatments can be directed at any stage during the progression of injury resulting from vesicant exposure, including before or after blister formation. This chapter focuses on the progress that has occurred since Papirmeister's publications (Papirmeister et al., 1991a,b).

Model Systems for Screening SM

The lack of definitive *in vitro* and *in vivo* models for SM skin injury provides an ongoing challenge. No animal model has produced a macroblister like that known to occur in humans and the major clinical target for SM treatments (extensively reviewed in Papirmeister et al., 1991c). Furthermore, experimental animal models have different skin characteristics, including a reduced barrier for chemical penetration as compared with human skin (Bartek et al., 1972). The reduced barrier results in greater systemic toxicity in animals, complicating other measures of cutaneous injury. Despite these difficulties, animal models have displayed great utility, and surrogate endpoints such as microblister (epidermal–dermal separation) formation or edema are acceptable biomarkers for measuring efficacy of candidate compounds.

A systematic characterization of animal models including the euthymic HGP, WP, the MEVM, and the hairless mouse showed that SM-induced subepidermal blister formation and epidermal cell death in all models tested (Smith et al., 1997a,b,c). Hairless mice are useful models of human skin; the absence of hair on the skin and increased skin thickness reduce the rapid penetration of toxicants (Walter and DeQuoy, 1980). Thus, the hairless mouse has emerged as an effective model for characterizing vesicant injury mechanisms and for early screening of candidate therapeutics (Blank et al., 2000; Casillas et al., 2000a,b; Ricketts et al., 2000; Sabourin et al., 2003; Pal et al., 2009; Tewari-Singh et al., 2009, 2010, 2011, 2012, 2013, 2014; Anumolu et al., 2011; Dorandeu et al., 2011; Jain et al., 2011; Vallet et al., 2012).

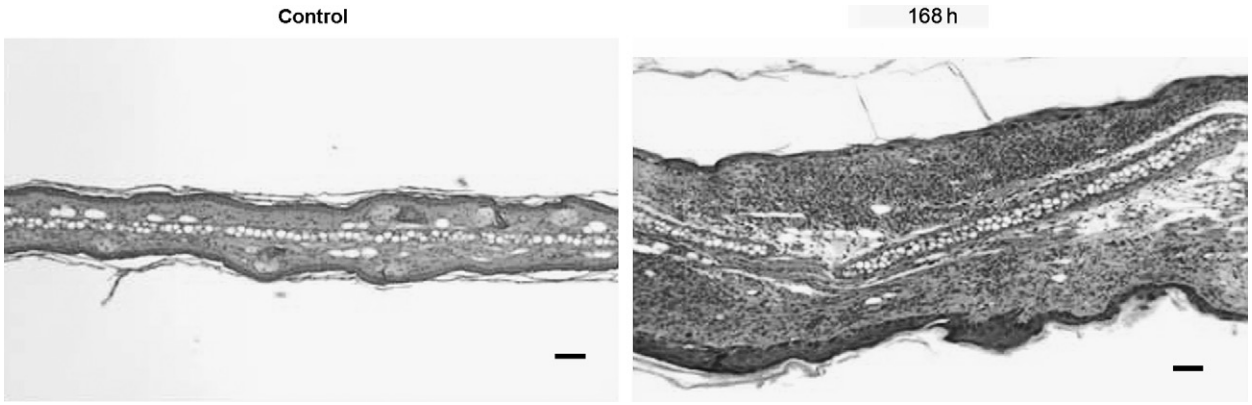

FIGURE 39.4 Biphasic inflammatory response in the MEVM: hematoxylin and eosin–stained paraffin sections of mouse ears with (right, 168 h after exposure) and without (left, carrier solvent, alone). Note the edema in the treated ear. The sample at 168 h after SM had a very large inflammatory cell infiltration.

First characterized in 1967, the HGP has been the most widely used model for vesicant exposure (Marlow et al., 1990a,b; Mershon et al., 1990; Yourick et al., 1991, 1992, 1993, 1995; Cowan et al., 1993, 1994; Graham et al., 1994; Smith et al., 1995; Kim et al., 1996; Kam et al., 1997; Millard et al., 1997; Dachir et al., 2010; Benson et al., 2011). Like human skin, SM-treated HGP experience defects in epithelial cell regeneration, basement membrane modifications, and increased necrosis and apoptosis, making them a good model for therapeutic testing (Kan et al., 2003; Dachir et al., 2006). There is one report utilizing the rabbit ear as a model of SM injury; however, we know of no reports further developing this model to screen candidate antivesicants (Zlotogorski et al., 1997).

The MEVM is performed by application of vesicant to the inner (medial) side of the ear (Casillas et al., 1997). Edema can be quantified by measuring 8-mm-diameter ear punch biopsy weights. Microscopic investigation shows clear histopathologic changes that include edema, epidermal–dermal separation, and necrosis. These effects occur in a dose-dependent manner (Figure 39.4). This model has been used to screen many compounds and remains the most cost-effective live animal screen available (Casillas et al., 2000a,b; Dachir et al., 2002, 2004a,b; Babin et al., 2003; Casbohm et al., 2004; Sabourin et al., 2004; Wormser et al., 2004a,b, 2005; Kiser et al., 2005; Amitai et al., 2006; Dillman et al., 2006). Furthermore, the benefits of a fully sequenced mouse genome and wide range of antibodies available make this model superior. Systemic effects do occur, as physiological and toxicological effects are detected in the contralateral (untreated) ear of animals (Babin et al., 2000).

WP and minipigs have also been used extensively for SM-induced skin injury (Lindsay and Rice, 1995; Brown and Rice, 1997; Graham et al., 1999; Chilcott et al., 2000; Graham et al., 2000a,b; Logan et al., 2000) (Figure 39.5) due to established similarities between human and pig skin (Graham et al., 2005). Erythema was found to peak at 24 h after a 15 min vapor exposure, with maximal edema occurring at 48 h after exposure (Smith et al., 1996, 1997a,b,c; Graham et al., 1999). The dermal–epidermal junction was also damaged at 48 h, with laminin 5 (renamed laminin 332) showing a progressive decrease in protein expression after SM injury (Smith et al., 1997a,b,c). Furthermore, inflammatory markers including IL-8, IL-6, IL-1β, and MMP-9 are induced within 72 h of treatment with SM (Sabourin et al., 2002). The WP is also used as a model of wound debridement to enhance repair (Graham et al., 2002a,b, 2006; Reid et al., 2007; Dalton et al., 2008), as discussed in greater detail later in this chapter.

The isolated perfused porcine skin flap (IPPSF) model is an effective *in vitro* model for SM exposure, characterized extensively by Monteiro-Riviere's laboratory (King and Monteiro-Riviere, 1990). Skin flaps consist of isolated, perfused skin with intact dermis and epidermis maintained by microcirculation in a system that mimics normal blood flow. Unlike other animal models, gross blisters are obtained, with blister-induced epidermal–dermal separation occurring at the upper lamina lucida (Monteiro-Riviere and Inman, 1995, 1997; Riviere et al., 1995). This model has allowed for several interesting experiments that could not have otherwise been performed. For example, the flow rate and composition of perfusing media can be altered: higher flow rate is associated with increased blister formation, whereas increased glucose in the perfusion medium has been shown to block the formation of microvesicles and blisters (Riviere et al., 1997). Whether increased glucose can protect against blister development in live animals has not been tested. No reports using this model have been published since 2004.

In addition to cell lines, artificial human skin has been used to test the effects of SM (Petrali et al., 1993). Human

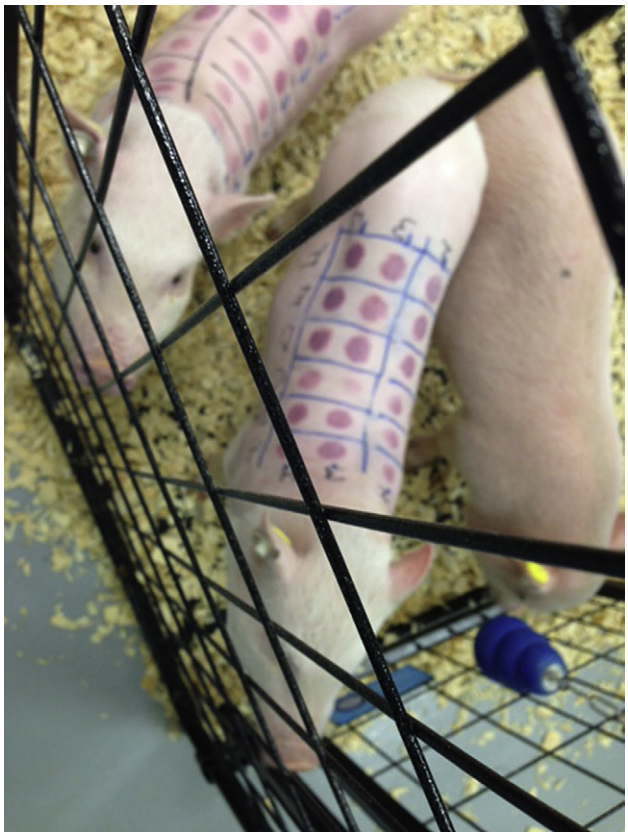

FIGURE 39.5 Gottingen minipig SM cutaneous injury 24 h after 30–120 min vapor cap exposures.

skin equivalent, commercially available as EpiDerm, is a fully differentiated artificial human skin with both a dermis and an epidermis (Monteiro-Riviere et al., 1997). Full-thickness models (EpiDerm-FT) have been evaluated for their potential use in SM models as well, in particular focusing on the dermal–epidermal junction and basement membrane components (Hayden et al., 2009). Studies by our laboratories demonstrated CEES-mediated gene induction in EpiDerm-FT and demonstrated the usefulness of this model in histological evaluation after vesicant exposure (Black et al., 2010a,b, 2011). EpiDerm-FT might also be used as an *in vitro* assay for screening of potential medical countermeasures against vesicants, such as was performed with liposome-encapsulated GSH (Paromov et al., 2011).

Decontamination

If accomplished early, skin decontamination can be an effective means of reducing injury from SM exposure (Sidell et al., 1997). Most SM decontamination strategies focus on its removal with solvents or adsorption of SM to inert substances. Even simple techniques such as washing with soap and water are surprisingly effective (Aasted et al., 1987). Washing with many other substances has been suggested, including kerosene, oil, gasoline, surgical spirits, and neutral sodium hypochlorite (Sollmann, 1919a,b; Chiesman, 1944; Jelenko, 1974; Gold et al., 1994; Wormser et al., 2002). Exposure to mustard liquid can also be treated with the use of binding agents, of which Fuller's earth is the best example. Fuller's earth is clay-rich soil commonly used to purify hydrophobic materials, including oils and greases (Chilcott et al., 2001). In SM injury, it is thought to function by binding in a near-irreversible manner to SM, preventing its absorption, association, and reaction with skin components. Fuller's earth is the standard to which other decontamination agents are tested; modifications of this compound and other related clay-like materials may be more efficacious (Lyle et al., 1984, 1986, 1987; Joiner et al., 1987). However, it is difficult to contain and uncomfortable to apply. Other items, including flour and talcum powder, were shown to be effective in reducing the progression of injury (Van Hooidonk et al., 1983). However, decontamination is ineffective in cases of vapor exposures to SM, which occur over a longer time period (McNamara, 1960). These results suggest that nearly anything that could adsorb a hydrophobic product would be effective in reducing SM injury.

Another strategy for decontamination is active chemical neutralization, whereby the SM undergoes a chemical reaction to prevent its reaction with outside components. There have already been successes with this type of approach, with the best example being RSDL, a product approved for military use by the United States and a number of other nations (FDA, 2003). RSDL has been demonstrated to be as effective as Fuller's earth in its decontamination properties (Taysse et al., 2007). The first generation of this product was composed of a solution of potassium 2,3-butanedione monooximate and its free oxime, diacetyl monooxime, in a mixture of monomethyl esters of polyethylene glycol. The solvent portion of the lotion is designed to solubilize chemical weapons away from the skin, while the oxime component readily reacts with mustards and with nerve agents, producing less toxic products (Sawyer et al., 1991a,b). In domestic pigs, treatment with RSDL 5 min after exposure to SM resulted in significantly less injury observed 3 days after exposure (Taysse et al., 2007).

Other approaches to chemical neutralization include the use of sodium thiosulfate as a reducing agent to function by reacting with activated SM (Hatiboglu, 1960; Owens and Hatiboglu, 1961; Bonadonna and Karnofsky, 1965; reviewed in McKinley et al., 1982). Original studies (WWI–1970) focused on its use as an injectable drug to prevent the bone marrow suppression seen with exposure to SM and related mustard agents. Systemic administration of this and other antioxidant molecules such as *N*-acetyl cysteine is discussed later in this chapter.

Another thiol, 2,3-dimercapto-propane sulfonic acid, showed protection against injury in mice exposed to SM vapor (Pant et al., 2000).

Decontamination can also be accomplished by the application of creams containing fluorinated cross-linker monomers (Liu et al., 1999). Deactivation occurs when substances within the cream actively react with and decontaminate the agents. Care should be taken with administration of these creams because they can trap chemical agents on the skin and prevent natural off-gassing. This may result in enhanced injury if inadvertently applied after exposure to a chemical agent. In some cases, perfluorinated creams have caused an 18-fold reduction in the rate of skin absorption (Chilcott et al., 2002).

After exposure to SM, a significant fraction of the agent remains unreacted in the skin, in what is often described as a skin reservoir. This reservoir is thought to contribute to ongoing injury by continually releasing SM, allowing further damage to occur. Recent studies demonstrated that application of ^{14}C-labeled SM was extractable from an *in vitro* pig skin model for up to 6 h (Dalton et al., 2004; Hattersley et al., 2008). Future work might focus on targeting this reservoir to prevent ongoing damage; no therapies are currently available for this purpose. Although still present in unreacted form, it seems that SM in this reservoir cannot be removed from the skin through the methods discussed.

Treatment of Blisters

The prolonged pathology observed in SM injury suggests two pathological mechanisms: an ongoing toxicity that contributes to a stepwise progression culminating in blister formation and a healing process that has been subverted. This is quite unlike the wound healing of thermal injuries. Aspiration (removal of the fluid within the blister) and deroofing (removal of the epidermal layer that constitutes the roof of the blister) are the main courses of action taken for larger, coalesced blisters to promote the healing process reviewed in Jenner and Graham (2013).

More recently, work has focused on the use of physical debridement of tissues, the surgical removal of tissue beyond the epidermal (roof) layer, to enhance the rate of wound healing (Graham et al., 2005) (Table 39.1). The slow rate of healing suggests that SM-modified proteins or other cellular components are preventing the normal healing process (Eldad et al., 1998). SM-mediated cross-linking of structural proteins such as laminins may not be easily repaired and may contribute to a delayed wound healing response (Zhang et al., 1995b). This could occur by promoting prolonged inflammation or by preventing the normal wound closure event that occurs as dermal cells migrate across and repair the blister area. Early studies demonstrated that SM-induced lesions in the skin of WP or Yucatan miniature pigs underwent faster wound healing when treating lesions by debridement after exposure to SM, whether the debridement was performed by CO2 laser or surgically (Graham et al., 1997, 2002a; Rice et al., 2000). Furthermore, when combined with skin grafting, debridement promotes wound repair (Graham et al., 2002b; Rice, 2003). Further investigations into different types of debridement in conjunction with other therapies are underway (Evison et al., 2006; Dalton et al., 2008).

THERAPEUTICS

Antioxidants

Chemical scavengers may be used to inactivate free radical forms of SM or the oxygen or nitrogen radicals thought to be formed as a consequence of SM activation. If given early enough after SM exposure, such scavengers might directly deactivate SM and perhaps reduce the reservoir of SM present in the skin. However, scavengers seem to have efficacy beyond the lifetime of SM itself in the skin, suggesting that other physiological mechanisms such as inflammation and oxidative stress might be reduced by these agents (reviewed in Papirmeister et al., 1991c). Scavengers have mostly been tested for their ability to reduce leukopenia and death due to the systemic effects of mustard exposure, rather than the reduction of skin injury (reviewed in Papirmeister et al., 1991c). Although most work has focused on scavengers as therapeutics in pulmonary exposures in which ongoing oxidative stress contributes to toxic outcomes such as pulmonary fibrosis or chronic obstructive pulmonary disease, there has been limited research of their efficacy in treating cutaneous injury.

Sodium thiosulfate, a potent antioxidant and scavenger, has been shown to be effective in reducing leukopenia and platelet decrease when given systemically in treatment for nitrogen mustard, particularly when given before exposure (Owens and Hatiboglu, 1961; Bonadonna and Karnofsky, 1965; McKinley et al., 1982). In the IPPSF model, perfusion with sodium thiosulfate modestly reduced microvesicle formation and attenuated the vascular response (Zhang et al., 1995a,b). However, it has limited efficacy against skin injury (Vojvodic et al., 1985; Zhang et al., 1995a,b).

GSH depletion has been shown to occur in several tissues and cell lines in response to mustard treatment (Omaye et al., 1991; Ray et al., 1995; Kulkarni et al., 2006). Depletion of GSH by buthionine sulfoximine treatment of isolated human leukocytes increased their sensitivity to SM toxicity (Gross et al., 1993). Given the critical role of GSH in maintaining the intracellular reducing state of the cell, restoration or pretreatment with GSH may

TABLE 39.1 Comparison of Dermabrasion (Debridement) Strategies for Enhancing the Rate of SM-Induced Skin Injury Repair

Therapy	Time of administration	Agent route	Species/System	Efficacy	Reference
Reactive skin decontamination lotion (RSDL)	Pretreatment	Topical	*In vitro*, human skin	18-fold reduction in skin absorption rate, may partially extract SM from the skin reservoir	Chilcott et al. (2002)
Dermabrasion	96 h posttreatment	Vapor, skin	Yucatan pigs (miniature, larger white)	Accelerated (≤3 ×) wound healing	Rice et al. (2000)
Debridement, CO_2 laser	6, 24, or 48 h posttreatment	Vapor, skin, 15 min	WP	3-fold fewer wounds. Improved histological skin structure	Graham et al. (1997)
Debridement, CO_2 laser	48 h posttreatment	Topical, liquid, 2 h	WP	Improved histological skin structure	Graham et al. (2000a,b)
Debridement, xeroform petrolatum	48 h posttreatment	Topical, liquid, 2 h	WP	Skin elasticity similar to sham (non-HD-treated) pig skin	Graham et al. (2006)
Debridement, Scarlet Red Ointment	48 h posttreatment	Topical, liquid, 2 h	WP	Skin elasticity similar to sham (non-HD-treated) pig skin	Graham et al. (2006)
Debridement, surgical tangential excision	48 h posttreatment	Topical, liquid, 2 h	WP	Mild improvement in healing	Graham et al. (2000a,b)
Debridement, surgical tangential excision and skin grafting	48 h posttreatment	Topical, liquid, 2 h	WP	Improved histological skin structure	Graham et al. (2000a,b)
Debridement, Versajet	48, 72, and 96 h posttreatment	Not specified	WP	No improvement at day 14	Dalton et al. (2008)
Debridement, Compound W	48, 72, and 96 h posttreatment	Not specified	WP	No improvement at day 14	Dalton et al. (2008)
Debridement, Collagenase Santyl	48, 72, and 96 h posttreatment	Not specified	WP	No improvement at day 14	Dalton et al. (2008)

WP, weanling pig.

protect against SM toxicity. Pretreatment of the basal epidermal keratinocyte cell line SVK-14, the upper respiratory tract cell line BEAS-2B, or the lower airway type II epithelial cell line A549 with GSH was shown to provide resistance to SM toxicity (Smith et al., 1997a,b,c; Andrew and Lindsay, 1998; Lindsay and Hambrook, 1998). Similar results were seen with other human cell lines, including G361, SVK-14, HaCaT, and NCTC human skin cells (Simpson and Lindsay, 2005). Also, stimulation of GSH concentration by pretreatment with GSH itself or the cysteine precursor 10 mM L-oxothiazolidine-4-carboxylate was shown to be protective against SM *in vitro* (Gross et al., 1993; Amir et al., 1998). However, these early successes did not translate effectively to animal model systems. Reduced GSH (400 mg/kg) given once before and twice after SM did not protect mice from SM toxicity (Kumar et al., 2001). Because GSH would be difficult to administer cutaneously, it is an unlikely therapeutic agent for the skin.

Cysteine is an amino acid with a reduced sulfur group that acts as an antioxidant. Cysteine residues are selectively alkylated in proteins by SM and the bifunctional nature of SM allows chemical cross-linking (Byrne et al., 1996). Early studies with nitrogen mustard in mice demonstrated that pretreatment with cysteine protected against toxicity (Contractor, 1963). However, microvesicle formation and dark basal cell formation were not protected in the IPPSF model (Zhang et al., 1995a). *N*-acetyl-L-cysteine (NAC) acts both as a scavenger and as an inducer of GSH synthesis, restoring the normal reducing status of the cell. Because SM lowers intracellular-reduced GSH, its restoration may contribute to increased tissue survival and repair. In addition, pretreatment with NAC may elevate GSH levels above normal and offer protection against low concentrations of SM (Atkins et al., 2000). *In vitro*, pretreatment with NAC protected PBL from 10 μM SM (Gross et al., 1993). Endothelial cells pretreated with NAC were resistant to loss of cell adherence and rounding after exposure to 250 μM SM (Dabrowska et al., 1996). Liposomes-containing NAC increased the viability of HaCaT keratinocytes in an *in vitro* study if given simultaneously

with CEES (Paromov et al., 2008). Although antioxidants have proven to be efficacious if given before SM exposure, their ability to ameliorate skin damage or enhance wound repair has not been shown.

Silibinin is the main active flavonone of silybin, an extract of the milk thistle plant (*Silybum marianum*). Silibinin or silybin has been tested in clinical trials as a chemotherapeutic agent (Singh and Agarwal, 2005; Deep and Agarwal, 2010) for hepatitis, for diabetic nephropathy, and other indications. The compound has diverse effects, including anti-inflammatory, antioxidant, and anti-metastatic effects (Gu et al., 2007; Singh and Agarwal, 2009; Deep and Agarwal, 2010). Topical silibinin (1 mg) attenuated CEES-mediated pathology in the skin of SKH-1 mice when applied 30 min after CEES exposure, including epidermal thickness, apoptosis, induction of pro-inflammatory genes, lipid peroxidation, and DNA oxidation (Tewari-Singh et al., 2012). Future studies must analyze longer time points than 24 h to determine the effects on wound healing.

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is an organo-selenium compound that functions as an anti-oxidant; it can directly scavenge hydrogen peroxide and peroxynitrite, mimics GSH peroxidase, and acts as an anti-inflammatory agent (Schewe, 1995). Ebselen itself reduced HN2-mediated inflammation at 24 h in the MEVM when applied in three treatments after exposure, with the first starting at 15 min. Although several ebselen derivatives are in development and have shown efficacy in *in vitro* studies in skin and lung cell lines, these have not yet been tested in animal models (Pino et al., 2013, 2014). Both ebselen and silibinin were noted to have diverse effects beyond those of antioxidants alone; future work should determine the relative importance of these functions versus their anti-oxidant properties.

Proteolytic Inhibitors

Proteases play a critical role in wound repair and remodeling, and therefore are likely important targets for the enhancement of wound repair (Mohan et al., 2002). In particular, damage to the basement membrane by MMPs is thought to be one mechanism responsible for dermal–epidermal separation seen in SM-induced blister formation. This could occur by upregulation of MMP expression, reduced competition for MMPs by adhesion molecules, or both (Mol, 1999; Danne et al., 2001; Shakarjian et al., 2006). MMP-9 expression is upregulated in WP skin (Sabourin et al., 2002) and the MEVM after SM exposure (Chang et al., 2006). Inhibition of proteases might therefore ameliorate damage caused by SM to structural components of the skin (Table 39.2). Explant cultures of SM-treated human skin co-treated with Ilomastat showed no epidermal–dermal separation (Schultz et al., 2004). SM-induced MMP-9 mRNA in the MEVM was partially inhibited by pretreatment with the MMP-9 inhibitor GM 1489 (Gerecke et al., 2005). The antibiotic doxycycline is also an MMP inhibitor that was shown to attenuate

TABLE 39.2 Summary of the Effects of Protease Inhibitors on SM Toxicity

Therapy	Time of administration	Therapy route	Therapy concentration	Agent route	Species/ system	Efficacy	Reference
Doxycycline	Co-treatment, then posttreatment	In media	500 μM	In media	HaCaT cells, *in vitro*	Reduced cellular detachment, but did not prevent apoptosis	Lindsay et al. (2007)
Doxycycline	1 h posttreatment	In media	100 μM	In media, 200 μM	HEK keratinocytes, *in vitro*	Reduction of SM-induced IL-8 production	Nicholson et al. (2004)
Doxycycline	Co-treatment	Topical	90 μM	Topical	*In vitro* human skin explants	No effect on SM-induced dermal–epidermal separation	Schultz et al. (2004)
GM 1489[a]	Pretreatment 15 min	Topical	20 μL of 25 mM	Liquid	Mouse	Reduced expression of MMP-9 mRNA	Gerecke et al. (2005)
Ilomastat	Pretreatment 15 min	Topical	20 μL of 25 mM	Liquid	Mouse	No effect	Gerecke and Sabourin (2005)
Povidone iodine	15 m and 24 h posttreatment	Topical	40 mg 10% povidone iodine	HN2, 0.5 mg	Haired guinea pig	>80% reduction in MMP-2 and MMP-9 activity in skin	Wormser et al. (2002)
Iodine	20 m posttreatment	Topical	1% w/v	Liquid, 1.27 mg	Pig	No effect	Margulis et al. (2007)

[a]*GM 1489—N-[(2R)-2,4-methylpentanoyl]-L-tryptophan-(S)-methyl-benzylamide.*

SM-induced pulmonary and ocular injury (Guignabert et al., 2005; Horwitz et al., 2014). However, doxycycline does not protect HaCaT viability; cells lose adherence and undergo apoptosis (Lindsay et al., 2007).

Microvesication of human skin *in vitro* was inhibited by treatment with MMP inhibitors such as Ilomastat, but HGP skin had no decrease in microvesication or necrosis after repeated treatments with Ilomastat (Mol and van den Berg, 2006). Mol and van den Berg suggest that this may be due to lack of delivery of the MMP inhibitor to the site of action. Although MMP inhibitors may reduce the ongoing injury caused by upregulation of MMP-9, most inhibitors have difficulty penetrating the skin. Another study demonstrated that treatment of an ex vivo human skin model with the metalloprotease inhibitor GM6001 blocked microvesication even when given 8 h after HD exposure (Mol et al., 2009). The application of pharmaceutical concepts may aid in the development of better delivery systems that would enhance the efficacy of this class of drugs.

More recent studies have suggested that MMP-9 is released by fibroblasts in response to keratinocyte stimulation by SM (Ries et al., 2008). Mustard-stimulated release of MMP-9 only occurred in coculture of human dermal fibroblasts with HaCaT keratinocytes or with culture of human dermal fibroblasts with conditioned media from HD-treated HaCaT cells, suggesting that paracrine signaling is responsible (Ries et al., 2008). Our work showed a dramatic increase in cytokine expression and activation of mediators of inflammation within 24 h after HD exposure, which may contribute to MMP-9 induction (Figure 39.6) (Gerecke et al., 2009). Together these data suggest that inhibition of fibroblast activation after vesicant exposure may also reduce MMP-9 activation and release.

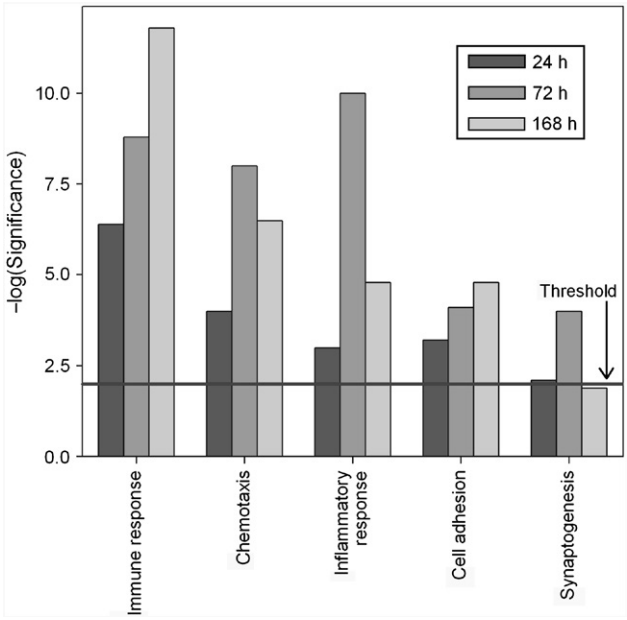

FIGURE 39.6 Results of biological processes sorting for microarray data from three different time points after SM exposure using the MEVM. The time points were 24, 72, and 168 h after exposure. The bars represent positive fold change increases over the carrier solvent alone samples. The immune response genes were within the top three biological processes activated for all the time points studied.

Steroids, Corticosteroids, and Glucocorticoids

Steroids have shown some efficacy in reducing inflammation and blister formation in response to SM or genetic blistering diseases such as bullous pemphigoid (Di Zenzo et al., 2007). Early studies showed that several types of corticosteroids were effective in reducing edema induced by SM (Dannenberg and Vogt, 1982). Glucocorticoids were shown to be effective in reducing edema in the initial phase of injury but did not affect the overall rate of healing (Vogt et al., 1984). Hydrocortisone given systemically or topically 2 h before HD administration resulted in a reduction in ear inflammation in the MEVM (Babin et al., 2000; Casillas et al., 2000a,b). Steroids given after SM exposure also enhance wound healing in a Yorkshire pig model of SM injury (Reid et al., 2008). Dexamethasone in combination with the nonsteroidal anti-inflammatory diclofenac produced more than a 60% reduction in edema in mouse ears treated with SM (Dachir, et al., 2004a,b). However, reduction of edema, although important, did not necessarily correlate with a reduction in the progression of injury, as seen later in this work. Studies using newer steroids such as clobetasol showed improved healing, lower severity of basal cell necrosis, and less inflammation (Reid, et al., 2008). Co-treatment of steroids with nonsteroidal anti-inflammatory drugs (NSAIDs) shows greater promise: co-treatment with diclofenac and tacrolimus in the HGP exposed to SM vapor showed less erythema, reduced lesion area, and fewer lesions (Dachir et al., 2008). A similar combination of steroid (Adexone) and NSAID (Voltaren) applied to mouse ears treated with SM led to reduced inflammation, less edema, reduced area of clinical damage, and reduced damage to epithelial cells (Dachir et al., 2004a,b).

Nonsteroidal Anti-Inflammatory Drugs

Several studies have demonstrated that NSAIDs administered systemically or topically offer protection against continued SM-mediated toxicity. This suggests that inflammation is a key component of SM-induced injury, as it is with other cutaneous injuries, such as those induced by UVA (Yourick et al., 1995). These results suggest that inflammation is involved in the ongoing pathology of SM-induced injury, and that anti-inflammatory drugs should be considered as part of a

drug cocktail for treatment of SM injury. Early studies with indomethacin in the HGP model showed that oral pretreatment could attenuate erythema and cutaneous injury (Yourick et al., 1995). Partial protection by indomethacin against microvesicle formation was found in the IPPSF model (Zhang et al., 1995b). Significant reductions in SM-induced early edema were found when indomethacin was administered from 24 h before exposure to 24 h after exposure (Babin et al., 2000) or when given 20 min after SM challenge (Kiser et al., 2001). Topical indomethacin given 2 h before SM exposure in the MEVM protected against early (24 h) but not late (72 h) edema (Casillas et al., 2000a,b).

Early studies using the MEVM showed that posttreatment with NSAIDs, particularly in combination with steroids, could diminish SM-induced inflammation at early time points, although later effects were not measured (Dachir et al., 2002). As mentioned, Voltaren, an NSAID, administered in combination with the steroid Adexone, reduced skin injury (Dachir et al., 2004a,b). More recently, bifunctional compounds containing NSAIDs (ibuprofen or diclofenac) tethered to pyridostigmine, an acetylcholinesterase inhibitor, were somewhat effective against SM dermal toxicity (Amitai et al., 2005). This combination was effective in the MEVM in reducing subepidermal blistering (Amitai et al., 2006). In fact, subepidermal blistering in the MEVM was reduced by these tethered pharmaceuticals (Amitai et al., 2006).

TRPV1 Ligands

An emerging hypothesis suggests a neurological component to SM toxicity in the skin. Nonmyelinated sensory C-fibers arising from the dorsal route ganglion transmit sensory information from the skin to the central nervous system in response to noxious stimuli, such as pain and heat. These fibers function as dual sensory efferents and release nociceptive and inflammatory neuropeptides such as substance P, peripherally (Szallasi and Blumberg, 1999; Szolcsanyi, 2004). Agents such as capsaicin, the active ingredient in hot peppers, produce analgesia by binding as an agonist to the transient receptor potential V1 channel (TRPV1) (Szolcsanyi, 2004). Capsaicin rapidly produces desensitization and interferes with the release of neuropeptides from sensory fibers (Campbell et al., 1993). Moreover, TRPV1 are expressed on cells in a number of nonneuronal cutaneous tissues, including keratinocytes and mast cells (Li, et al., 2007). Capsaicin and its structural analogs, known collectively as vanilloids, have been shown to have anti-inflammatory activity, as demonstrated by inhibition of edema, mast cell degranulation, and leukocyte migration (Brand et al., 1990; Bunker et al., 1991). Pretreatment of skin with vanilloids before SM exposure was shown to significantly reduce edema formation (Babin et al., 2000, 2003; Casillas et al., 2000a,b; Sabourin et al., 2003). One such vanilloid, olvanil, is a highly lipophilic analog of capsaicin and has been shown to reduce SM-induced histological damage and edema as well as cytokine and chemokine mRNA induction (Casillas et al., 2000a,b; Sabourin et al., 2003). Analogs octyl homovanillamide and heptyl isovanillamide were shown to display similar protective activities against SM (Casbohm et al., 2004). There appears to be much promise in following these leads and more closely examining the role of neurogenic inflammation and neuropeptides in the cutaneous damage induced by SM.

Cooling

Because of its high vapor pressure, exposure to SM in hot environments can exacerbate exposure. The toxicity of SM also relies on its ability to chemically react with biological molecules within the skin in a temperature-dependent manner. *In vitro* tests showed that human skin keratinocytes treated with SM and cultured at 25°C had less injury after 24 h than keratinocytes grown at 37°C (Sawyer and Risk, 1999). Similarly, HGP with skin exposed to SM had less injury after 72 h if treated for 4.5 h after exposure with cold (Sawyer and Risk, 1999). Other studies showed that anesthetized swine skin exposed to mild cooling (15°C) for 2–4 h after SM exposure had significantly less injury progression after 7 days (Sawyer et al., 2002). However, later studies demonstrated that this effect was temporary, slowing the rate of injury progression rather than reducing overall injury. Tissue and animal studies showed that temperature-mediated inhibition of injury was reversible on return of the tissue to normal body temperature (37°C). Sawyer et al. (2002) have suggested that cooling might be used as a temporary measure that "increases the therapeutic window in which other medical countermeasures are useful (Sawyer and Nelson, 2008)." Later tests with VX poisoning showed similar results, lengthening the time for entry of VX into the bloodstream (Sawyer et al., 2011). Although this work has been largely ignored by literature, cooling would be inexpensive, effective, and easily applied by first responders.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Countermeasure research to date has focused primarily on the development of therapeutics, but little attention has been given to the use of advances in drug delivery or pharmaceutics. Weak efficacy can sometimes be enhanced by the use of better delivery mechanisms. Drug delivery by encapsulation of active moieties in nanoparticles has been increasingly used for chemotherapeutic agents such as doxorubicin (Haley and Frenkel,

2008). However, with the exception of barrier creams, nanoparticle-mediated cutaneous SM detoxification has largely been limited to decontamination (Hobson, et al., 2002a,b,c; Braue and Hobson, 2005; Stengl et al., 2005; Prasad et al., 2007a,b,c; Singh et al., 2009). Most studies with creams focus on the use of nanoparticles that increase the rate of deactivation of the chemical agent (Koper et al., 1999). Liposomes used as delivery devices for hydrophilic or hydrophobic antioxidants have shown enhanced drug delivery efficacy using *in vitro* models (Paromov et al., 2011). NAC-containing liposomes have been used to treat CEES-induced lung injury, suggesting that encapsulated scavengers may be effective at restoring intracellular reducing agents after SM exposure (Hoesel et al., 2008). Formulations that allow effective dermal delivery may enable the development of scavengers, antioxidants, or protease inhibitors for SM treatment. NAC-containing liposomes tested on an *in vitro* model of the skin (Epiderm) maintained cell viability if given concomitantly with SM; whether posttreatment was efficacious is unknown (Paromov et al., 2011). These studies demonstrate that advances in pharmaceutics may dramatically improve the efficacy of previously discarded therapies.

Effective management of SM-exposed patients is likely to include combination therapies that act at the same stage or at different stages of the SM injury and recovery process to enhance overall efficacy. Dermabrasion has been shown to be effective in conjunction with split-thickness wound grafting (Graham et al., 2002b). Other studies have shown increased efficacy when NSAIDs are administered in conjunction with steroids (Dachir et al., 2002, 2004a,b, 2008). It is therefore likely that several therapies with limited efficacy could have an additive or synergistic effect for enhancement of SM-mediated injury healing.

Perhaps the largest problem inhibiting the development of effective preventive and therapy-based countermeasures is our continued lack of understanding of which of the many actions of SM are critical events leading to SM injury. The major difficulty of SM is that, although a large number of biochemical pathways are altered, the identification of pathways with pharmacological significance has yet to be clearly established. Previous studies suggested that the PARP pathway, calcium mobilization, and intracellular pH are affected in SM-mediated injury, but therapies designed against these pathways have not demonstrated effectiveness beyond *in vitro* approaches or have not shown feasibility for development as drugs. It is likely that these pathways, although important, are not the initiating events of toxicity, and therefore targeting these pathways might have limited effectiveness. Only anti-inflammatory drugs seem to be moderately effective in the treatment of SM injury. If SM is to be effectively treated, then more research into the basic mechanisms of SM injury needs to be performed. As Brimfield suggests, "the search for the primary biochemical lesion is still underway (Brimfield, 2004)." Given the complex progression of SM injury, multiple interventions used in combination against various components of the progression of injury are likely to be required to deliver the best result.

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CHAPTER

40

Skeletal Muscle

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INTRODUCTION

The skeletal muscles account for about half of a mammal's body weight, and as a result, they receive proportionately large amounts of administered doses of chemicals. Skeletal muscle is a target organ for a variety of chemicals and adverse or toxic effects can range from minor muscle weakness or slight pain to complete paralysis. Skeletal muscles present major targets for the toxicity of organophosphate (OP) nerve agents, exceeded only by the brain. Morbidity and mortality associated with OP intoxication occurs due to the effects of these compounds on skeletal muscles in general and the muscles of respiration in particular. Deaths from overdose of OPs are due in part to respiratory paralysis following depolarization of the neuromuscular blockade. Understanding the skeletal muscle system in the context of OP poisoning is essential, but it is also very complex because different fiber types of muscles often respond differently, even to the same OP compound. The distinct features of slow and fast muscles are the most fascinating research aspects of skeletal muscles.

Skeletal muscles are enriched with both cholinergic and noncholinergic elements that are directly or indirectly modulated by OP nerve agents. Motor innervation plays an important role in the regulation of many properties of skeletal muscles, including neuromuscular activity. Changes in the activities of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) appear to greatly modulate neuromuscular activity and can modify neuromuscular transmission. At the cholinergic synapse, AChE plays an important role in the removal of acetylcholine (ACh) from the synaptic cleft. Inhibition of this enzyme by compounds such as OP nerve agents profoundly modifies neuromuscular transmission, as seen in twitch potentiation, fasciculation, muscular weakness, and muscle cell death by necrosis or apoptosis. Because of their high metabolism, skeletal muscles are very vulnerable to OP-induced oxidative/nitrosative stress due to excess free radical generation. In the past two decades, interest in skeletal muscles has been rising because of their involvement in intermediate syndrome (IMS) and tolerance development related to the toxicity of OP pesticides. OP-induced effects on skeletal muscles can occur at one or multiple sites (the nerve fiber, the nerve terminal, the junctional cleft, the motor endplate, and the myofibrils). This chapter describes structural and functional aspects of skeletal muscles in the context of OP nerve agents' toxicity.

BEHAVIORAL EFFECTS

In general, exposure to sublethal sign-producing doses of an OP nerve agent exerts prominent motor, behavioral, and autonomic symptoms. The motor symptoms are fasciculations, fibrillations, and body tremors. Fasciculations and fibrillations occur due to antidromic neural discharge from excess junctional ACh, while tremors are of a central origin (Gupta et al., 1986; Misulis et al., 1987). OP-induced behavioral studies indicate that different nerve agents [e.g., soman, sarin, tabun, and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX)] require different concentrations to produce equitoxic effects, degree of AChE inhibition, and myonecrosis. Acute symptoms of equal severity in male Sprague-Dawley rats by soman, sarin, tabun, and VX can be achieved at doses of 100, 110, 200, and 12 μg/kg, administered subcutaneously (s.c.), respectively (Gupta et al., 1987a,b, 1991). Based on the equitoxic doses, VX is the most toxic and tabun is the least toxic OP nerve agent.

Rats receiving an acute dose of soman (100 μg/kg, s.c.) exhibit symptoms of toxicity, such as salivation, muscle fasciculations, and severe tremors, within 5–15 min. Within 20–30 min, signs of toxicity are maximal and can

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persist for 4–6 h. Thereafter, the intensity is reduced to a mild form, but the signs of toxicity can still be observed after 24 h. Rats usually become free of overt signs after 72 h. With VX, onset of symptoms and appearance of maximal severity are delayed by approximately 20 min compared to other nerve agents (Gupta et al., 1987a,b, 1991). During peak toxicity of soman, signs such as complex posturing movements and tremors are indicative of pronounced CNS effects compared to moderate peripheral muscle fasciculations. Muscle fasciculations superimposed upon this activity are less prominent than the signs of gross motor unit activity. This is in contrast to the OP compound diisopropylphosphorofluoridate (DFP), which exerts greater peripheral activity with fasciculations and fewer central toxicity signs (Gupta et al., 1985, 1986, 1987a,b). This suggests that AChE inhibitors differ in their propensity to produce central or peripheral effects and that peripheral effects are required for muscle necrosis (Gupta et al., 1985, 1986, 1987a,b; Misulis et al., 1987).

CHOLINERGIC SYSTEM

Key elements of the cholinergic system include a neurotransmitter, ACh; an enzyme, AChE, which hydrolyzes ACh; and an enzyme, ChAT, which synthesizes ACh. All skeletal muscles contain these cholinergic components, but their quantities can significantly vary from muscle to muscle; i.e., fast fiber-containing muscle has greater values than slow fiber-or mixed fiber-containing muscle.

Normal Activity of AChE and its Molecular Forms

Muscle AChE (E.C. 3.1.1.7) is partly concentrated in the endplate region; i.e., 20–40% of the total amount found in the whole muscle (Hall, 1973; Younkin et al., 1982). Using a histochemical technique, Müntener and Zenker (1986) demonstrated the presence of AChE in the sarcoplasm of limited areas in sections of normal rat muscles. Normal activity of AChE varies from muscle to muscle; i.e., higher AChE activity is found in fast fibers (type II fibers in general, and type IIB fibers in particular) than in slow fibers (type I fiber) containing muscle. Enzyme activity in mixed fiber muscle is found in between the values of slow and fast muscles (Table 40.1). AChE plays an essential role in the removal of ACh at the neuromuscular junction (NMJ). Inhibition of AChE activity, which results in accumulation of ACh, profoundly modifies neuromuscular transmission by producing fasciculations and twitch potentiation. At high rates of stimulation, the muscle is unable to maintain a normal contraction, and muscle hyperactivity often ensues in muscle fiber necrosis.

TABLE 40.1 Normal Values of AChE and BuChE Activity in Rat Skeletal Muscles

Muscle	AChE Activity (μmol Substrate/g/h)	BuChE Activity (μmol Substrate/g/h)
EDL (fast fiber muscle)	105.8 ± 1.0	9.7 ± 0.8
Soleus (slow fiber muscle)	60.1 ± 1.2	10.3 ± 0.3
Diaphragm (mixed fiber muscle)	78.3 ± 1.6	10.2 ± 0.2

The substrates used were acetylthiocholine iodide and butyrylthiocholine iodide for AChE and BuChE activity, respectively. Each value represents mean ± SEM (n=5–6).

It is well established that AChE exists in nerves and muscles in a range of globular and asymmetric molecular forms. A wide variety of sedimentation profiles have been established for AChE molecular forms in different mammalian muscles (Massoulie and Bon, 1982). The variations seen in the ratios of these molecular forms between different muscles are wide and complex. Qualitative and quantitative variations exist among different species, as well as the young versus adults (Barnard et al., 1984). In the rat extensor digitorum longus (EDL, a fast-twitch muscle), the G_1 (4S), G_4 (10S), and A_{12} (16S) molecular forms are predominant, while in the soleus (SOL, a slow-twitch muscle) and diaphragm (a mixed muscle), a fourth major molecular form is also present, called the A_8 (12S). In rat SOL, the majority of the total AChE activity is contributed by the 12S and 16S forms, whereas in the diaphragm, it is 4S and 10S, and in the EDL, it is 4S (Grosswald and Dettbarn, 1983a,b; Patterson et al., 1987) (Table 40.2).

In the rat, the 16S form is found in high concentrations at the endplates, and it is thought to be involved in neuromuscular transmission. The different molecular forms of AChE in SOL and EDL have apparent K_m values similar to that found in the diaphragm muscle (Grosswald and Dettbarn, 1983a,b). There appears to be no difference between catalytic sites of the molecular forms of AChE in fast-EDL and slow-SOL muscles, despite the different molecular form patterns and activity in these muscles.

Inhibition of AChE and its Molecular Forms by Nerve Agents

In general, following an acute exposure to OP nerve agents, signs of cholinergic toxicity appear within a few minutes and are caused by irreversible inhibition of AChE activity in neuronal tissues. Inhibition of AChE causes excess accumulation of ACh at central and peripheral synaptic sites, leading to failure of neuromuscular,

TABLE 40.2 Total AChE Activity and Percent Contribution of Molecular Forms to Total AChE Activity in Rat Skeletal Muscles

			Molecular Forms			
		Total Activity	A_{12} (16S)	A_8 (12S)	G_4 (10S)	G_1 (4S)
EDL	w/ PI	93.8 ± 7.5	18.7 ± 1.8	–	26.4 ± 3.5	54.9 ± 4.1
	w/o PI	102.9 ± 4.7	18.7 ± 1.6	–	30.2 ± 2.8	51.1 ± 3.1
Soleus	w/ PI	53.6 ± 4.7	26.8 ± 1.9	33.7 ± 2.3	15.2 ± 1.0	24.3 ± 0.9
	w/o PI	61.4 ± 5.3	25.9 ± 2.2	33.0 ± 2.2	16.3 ± 1.3	24.8 ± 1.4
Diaphragm	w/ PI	81.4 ± 6.1	26.9 ± 1.4	14.2 ± 0.9	28.8 ± 0.3	30.1 ± 1.7
	w/o PI	89.5 ± 4.9	24.6 ± 1.8	14.8 ± 0.9	29.7 ± 1.7	30.9 ± 1.5

Activity values are expressed as μmol ACh hydrolyzed/g/h tissue for total activity and as a percentage of the total distribution for molecular forms. Values are the mean ± SD (n=6–8 muscles); w/ PI, protease inhibitors present; w/o PI, protease inhibitors absent. No statistical significance was found comparing the presence versus absence of protease inhibitors for values of the individual molecular forms or total activities.

respiratory, and cardiovascular functions. For some OP compounds, a close relationship exists between the severity of toxic signs and the inhibition of AChE during the acute phase of intoxication. The observable toxic effects usually do not persist for more than 4–6 h, while recovery of AChE activity occurs at a much slower rate, such as 7–14 days or even 3–4 weeks, depending on the tissue and the inhibitor.

Gupta et al. (1987a) demonstrated that selective inhibition of AChE activity in skeletal muscles was apparent within 1 h of soman administration (100 μg/kg, s.c.) in rats when SOL showed the maximum inhibition (87%), whereas EDL showed the least inhibition (47%). AChE activity in skeletal muscles was maximally depressed during the following 3–6 h, the time when animals showed severe signs of toxicity. Recovery was clearly apparent during the next 3–7 days in all three skeletal muscles (SOL, EDL, and diaphragm); 7 days after soman treatment, enzyme recovery was greater than 90% in EDL and diaphragm compared to 75% in SOL. Within 1 h, all molecular forms of AChE were reduced to less than 10% of control in SOL and diaphragm (Figure 40.1). In EDL, the 16S form, mainly localized at the NMJ, was not affected at this time, while the 10S form was completely inhibited and the 4S form was reduced to 50%. Further inhibition was seen after 24 h but even then the 16S form was the least inhibited in EDL. After 3 days of soman treatment, the AChE molecular forms in all three muscles showed signs of recovery. This was particularly evident in the 16S form in EDL and diaphragm where it appeared to be approximately 75% of controls. In the SOL, the 16S form had recovered to only about one-third of the control when measured after 3 days; 7 days after soman, the 16 and 4S forms in the EDL and diaphragm had fully recovered. The activities of the 10S EDL form and the 10 and 12S diaphragm forms were still reduced, while in the SOL the activity of all forms remained below the control.

In similar experiments, tabun (200 μg/kg, s.c.) produced differential AChE inhibition in various skeletal muscles (SOL = diaphragm > EDL), which was similar to that seen with soman (Gupta et al., 1987a,b). The varying degrees of AChE inhibition in the skeletal muscles treated with tabun, however, correlated well with the observed difference in the number of myonecrotic lesions; i.e., the greater the AChE inhibition (during the initial 24 h period), the higher the number of lesions found in the diaphragm and SOL. EDL, with a low level of AChE inactivation, had the smallest number of lesions. This observation was in agreement with a similar pattern of histochemical observations following soman and sarin administration (Meshul et al., 1985; Gupta et al., 1987a, 1991). The reason for this difference in susceptibility to a particular nerve agent may be due to (i) variations in location of AChE in different muscles (Grosswald and Dettbarn, 1983a), (ii) changes in ACh release due to a different firing pattern (Misulis et al., 1987), and (iii) pharmacokinetic variables, which influence the delivery of a particular OP compound. With tabun toxicity, inhibition and recovery of AChE molecular forms, especially the 16S form associated with the NMJ (endplate region), corresponded well with the appearance and disappearance of necrotic lesions. The delay in the molecular form's return to normal is due to the fact that the heavier molecular forms (16, 12, and 10S) are based on an assembly of the monomeric 4S form. Synthesis of the 4S form increases when the assembly of the heavier forms lags behind.

Following an acute exposure to sarin (110 μg/kg, s.c.), AChE activity in skeletal muscles was reduced to 23% in SOL and 48% in diaphragm within 1 h, while EDL AChE was significantly unaffected. By 24 h, however, a still-greater inhibition was seen for these muscles, and in EDL, AChE activity was reduced to 43%. In an early phase, recovery of AChE was rapid, but still not complete

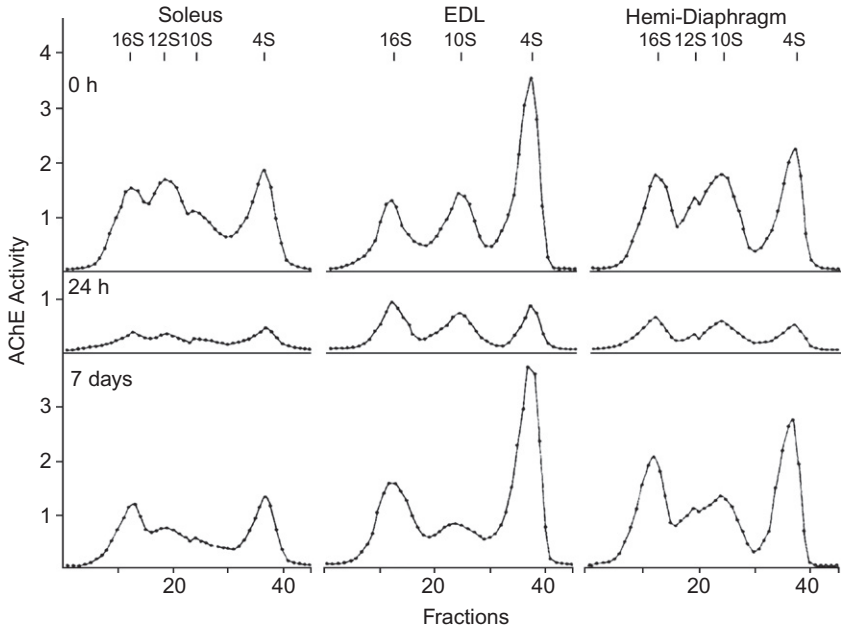

FIGURE 40.1 Representative profiles of the activity of the AChE molecular forms in the SOL, EDL, and hemidiaphragm muscles. Profiles at the top of each column are from untreated muscles, followed by profiles of activity of AChE molecular forms of muscles 24 h and 7 days after receiving an acute dose of soman (100 μg/kg, s.c.). The AChE activity scale is in arbitrary units based on the μmole substrate hydrolyzed/min by the enzyme activity in each fraction. The sedimentation values of the AChE molecular forms are given in the profiles of untreated muscles above the associated peaks. Sedimentation values were determined by the location of the added sedimentation standards, β-galactosidase (16.0 S), catalase (11.1 S), and alkaline phosphatase (6.1 S), following velocity sedimentation of the gradients.

when measured after 7 days of treatment. Activities of the AChE molecular forms, after 1 h of sarin injection, were significantly depressed in the SOL and diaphragm, while those in the EDL showed significant inhibition only after 24 h (Figure 40.2). By day 7 in SOL, activities of the 4 and 10 S molecular forms of AChE had recovered to higher than control levels, while in the diaphragm, a significant shift toward the 4 S molecular form had taken place so that the AChE profile resembled that of a control EDL. In EDL, the activity of 4 and 16 S molecular forms recovered at a faster rate than the 10 S molecular form.

Following VX administration (12 μg/kg, s.c.), within 1 h, AChE activity of all three skeletal muscles was reduced to between 8% and 17% of control with incomplete recovery by the end of 7 days. At the same time, VX caused significant inhibition in the activity of all molecular forms in all three muscles (Figure 40.3). In EDL, the activity of 4 and 10 S showed significant recovery 24 h later, while those of SOL and diaphragm remained inhibited. By day 7, the activity of all forms had recovered and an excess of activity was seen in the 4 S form of EDL and diaphragm, shifting the latter profile toward that of the EDL and contributing a higher protection to the total AChE activity.

Butyrylcholinesterase

Butyrylcholinesterase (BuChE, E.C. 3.1.1.8) activity in skeletal muscles is significantly lower than AChE activity (Table 40.1). Also, unlike variable AChE activity, all three muscles (SOL, EDL, and diaphragm) contain equal levels of BuChE activity. Following soman administration (100 μg/kg, s.c.) in rats, maximal inhibition of BuChE activity in skeletal muscles was observed after 24 h. At this time, SOL was greatly affected (98%), followed by the diaphragm (87%) and EDL (60%). A rapid recovery of BuChE was noticed during 48–72 h after soman treatment and enzyme activity returned to baseline values when measured after day 7 (Gupta et al., 1987a).

Tabun (200 μg/kg, s.c.) caused significant inhibition of BuChE activity within 1 h in skeletal muscles (in the SOL, EDL, and diaphragm, 14%, 50%, and 35% remaining activity, respectively), but maximal inhibition appeared after 3 h in SOL (6% remaining activity), and after 24 h in diaphragm and EDL (7% and 11% remaining activity, respectively) (Gupta et al., 1987b). Unlike AChE, the recovery rate of BuChE appeared to be rapid, as the enzyme activity in the diaphragm and EDL recovered to baseline values by day 7. At this time, SOL BuChE activity still remained significantly inhibited. After 1 h of sarin administration (110 μg/kg, s.c.), BuChE activity was significantly inhibited in the SOL and EDL (78% and 79% remaining activity, respectively), but not in diaphragm. With sarin, maximal BuChE inhibition was noted after 24 h in SOL, EDL, and diaphragm (39%, 71%, and 43% remaining activity, respectively). In similar studies, within 1 h VX (12 μg/kg, s.c.) caused significant

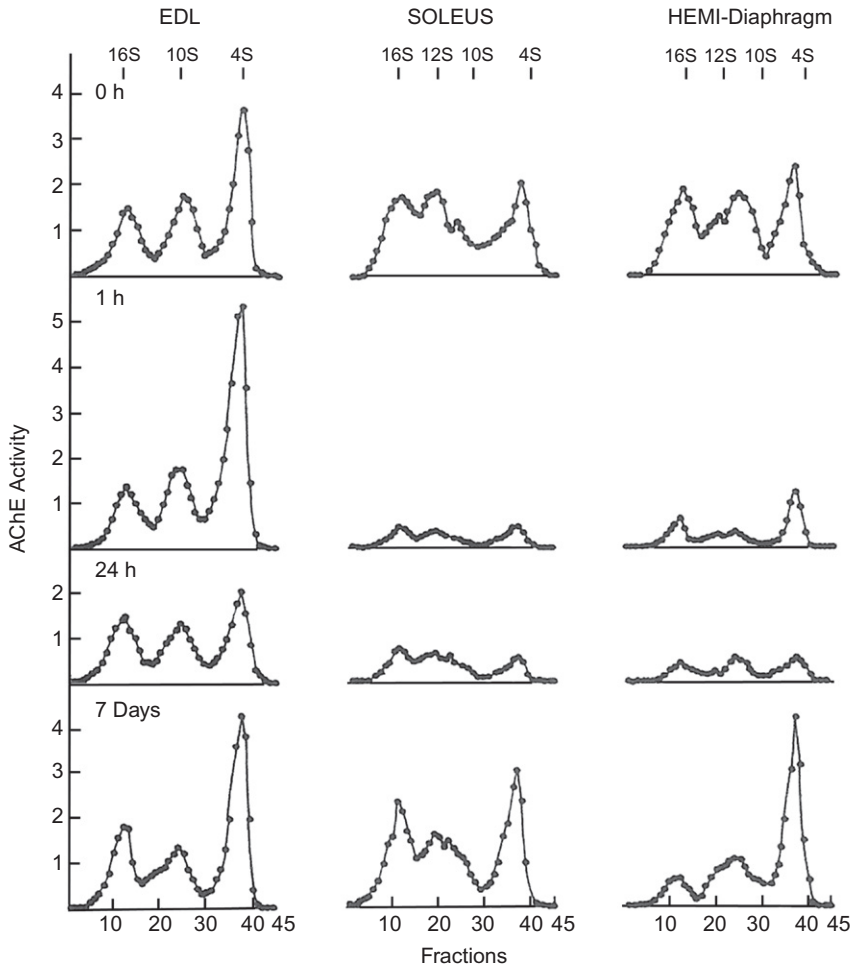

FIGURE 40.2 Representative profiles of the AChE molecular forms in the EDL, SOL, and hemidiaphragm muscles from rats following an acute sublethal injection of sarin (110 µg/kg, s.c.). Profiles at the top of each column are from untreated muscles, and subsequent profiles show the activity of the AChE molecular forms 1 h, 24 h, and 7 days, respectively, after sarin treatment. For further details, see Figure 40.1.

and maximal inhibition of BuChE in all three muscles (29%, 60%, and 60% remaining activity, respectively). When measured after 24 h, EDL and diaphragm showed marked recovery, while SOL still had 41% inhibition. All three muscles showed complete recovery of BuChE when measured after 7 days.

Choline Acetyltransferase

Existence of ChAT (acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6) in skeletal muscles is probably of neural origin, and its activity varies among skeletal muscles. ChAT activity can be altered by increased or decreased neuromuscular activity. It appears that neuromuscular activity exerts a regulatory influence on neuronal production of ChAT. Alterations in ChAT activity in response to variations in muscular activity represent changes in enzyme synthesis, although effects on catabolism of the enzyme or on exoplasmic transport of enzyme to the nerve terminal cannot be ruled out. Acute exposure to an OP compound DFP (1.5 mg/kg, s.c.) caused insignificant increase, while repeated administration at a low dosage (0.5 mg/kg/day for 5 days) resulted in significant increase in ChAT activity (diaphragm 140%, EDL 150%, and SOL 156%). Significant increase in ChAT activity was noted even after 2 weeks of repeated administration.

Acetylcholine Receptors

Acetylcholine receptors (AChRs) are of two types: muscarinic (mAChR) and nicotinic (nAChR) based on the agonist activities of the natural alkaloids, muscarine and nicotine, respectively. These receptors are functionally different. The muscarinic type being G protein-coupled receptors mediate a slow metabolic response via second messenger cascades, while the nicotinic type are ligand-gated ion channels, which mediate a fast

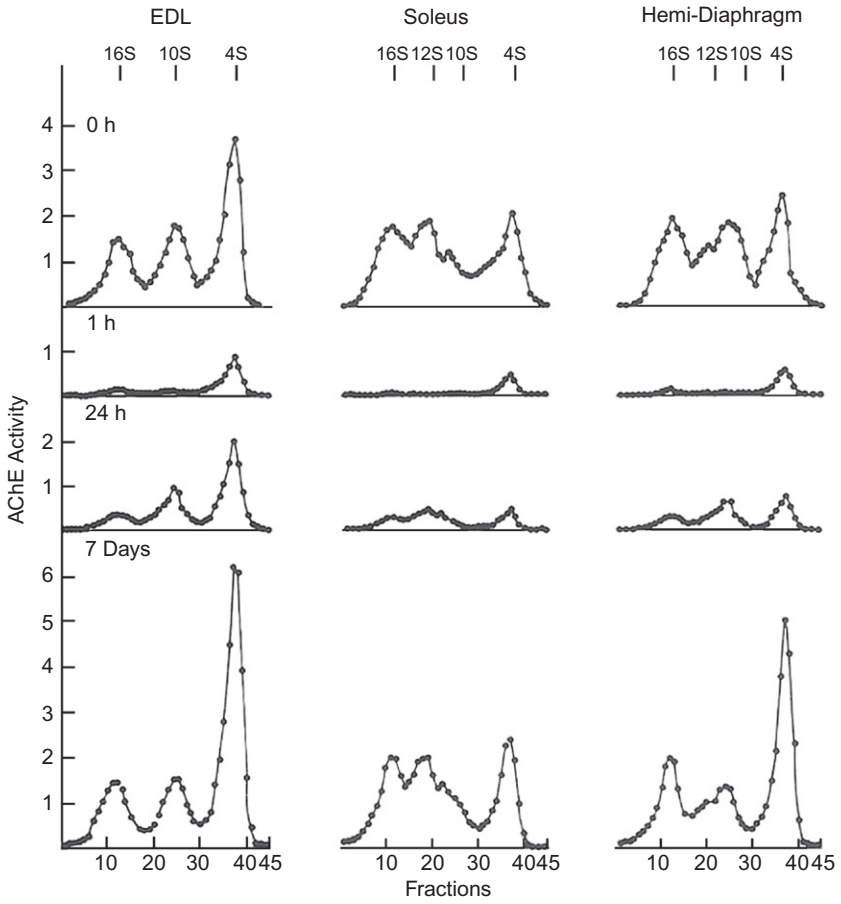

FIGURE 40.3 Representative profiles of the activity of the AChE molecular forms in the EDL, SOL, and hemidiaphragm muscles from rats following an acute, sublethal injection of VX (12 μg/kg, s.c.). For further details, see Figure 40.1.

synaptic transmission of the neurotransmitter. Skeletal muscles are enriched with nAChRs and are devoid of mAChRs.

The nAChRs, with a molecular mass of 290 kDa, are composed of five receptor subunits (α, β, δ, ε, and γ) arranged symmetrically around the central pore. Adult muscle is composed of α2βαδγ, whereas fetal muscle has α2βδε (Mishina et al., 1986). Experimental studies in the late 1970s revealed that in relatively immature muscle cells, the AChRs are distributed uniformly over the entire sarcolemma (Bevan and Steinbach, 1977). At about the time of the initial innervation, aggregations/clusters of AChRs appear, in addition to the relatively low-density receptors (Braithwaite and Harris, 1979). During the course of embryonic development, the low-density receptors disappear, and almost all AChRs in the adult muscle fibers are localized to the region of the NMJ (Fambrough, 1979). Sohal and Boydston (1982) suggested that neither the total receptor content nor the ability of the receptors to cluster seems an essential element for the formation and maintenance of the morphological aspects of the NMJs or for the growth and maturation of the muscle. It is yet to be established what exactly, if any, role nAChRs have in the formation of the NMJ.

At the mammalian NMJ, where nAChRs are localized, stimulation of the presynaptic nerve causes release of the neurotransmitter ACh from the nerve terminals. Released ACh diffuses across the gap, separating nerve and muscle cells and interacts with specific receptors associated with the postsynaptic muscle membrane to produce increased membrane permeability to some cations. When two molecules of ACh bind to an nAChR, a conformational change occurs in the receptor, resulting in the formation of an ion pore. The nAChRs are a family of cationic channels whose opening is controlled by ACh and nAChR agonists and they are the key molecules in cholinergic nicotinic transmission at the NMJ. At this site, the opening of a pore produces a rapid increase in the cellular permeability of Na^+ and K^+ ions, resulting in the depolarization and excitation of the muscle cell, thereby producing a muscular contraction. Adult vertebrate muscle fibers are highly sensitive to ACh only in the region of the NMJ. Muscle fibers of fetal and neonatal rats are also sensitive to ACh in regions outside the NMJ.

Excessive ACh or long-acting cholinomimetic agents can produce muscle paralysis due to prolonged depolarization of the endplate, a phenomenon referred to as depolarization block. Prolonged transmitter (ACh) receptor (nAChR) interactions, as a result of the high concentrations of accumulated ACh, produce a depolarization block, similar to that seen with agents such as decamethonium. Furthermore, there is a possibility of the same postjunctional area being activated repeatedly, resulting in desensitization of receptors (desensitization block). These forms of neuromuscular block usually produce muscle weakness and paralysis.

The binding site for cholinergic effectors is believed to reside primarily in the α-subunits. Evidence for this fact is based on affinity-labeling experiments in which analogs of ACh (bromoacetylcholine and 4-(-*N*-maleimidophenyl) trimethylammonium) have been shown to label only the α-subunits. Receptors also demonstrate high-affinity binding of the polypeptide α-neurotoxins, such as α-bungarotoxin (αBT), which interacts specifically and in an essentially irreversible manner with nAChRs. At the nAChRs, αBT competes for binding with both cholinergic agonists (e.g., carbamylcholine chloride and decamethonium bromide) and antagonists (e.g., curare) (Karlin, 1980). Berg et al. (1972) reported binding of αBT to AChRs in rat diaphragm muscle and found that 90% of the binding that occurred was "endplate-specific."

Some of the toxic effects of OP nerve agents and pesticides are unrelated to inhibition of AChE. However, the exact mechanism by which OP nerve agents and other anti-AChE compounds affect nAChRs is yet to be elucidated. Some of the biochemical and morphological alterations have been attributed to an excess amount of ACh in the synaptic cleft resulting from AChE inactivation. Many anti-AChE agents have been shown to produce postsynaptic morphological, biochemical, and electrophysiological alterations in adult mammalian skeletal muscle after acute and long- term treatment (Fenichel et al., 1972; Laskowski et al., 1977; Hudson et al., 1978). Normally, at the motor endplate, the small electrical nerve impulse releases the chemical transmitter ACh which diffuses across the synaptic membrane and attaches to the postsynaptic receptors on the muscle membrane. However, during AChE inhibition by OPs, unhydrolyzed ACh does not diffuse from the cleft, but repeatedly combines with postsynaptic receptors. The prolonged presence of ACh in the synaptic area appears to cause some of the myopathic changes. This was supported by observations of paraoxon, causing antidromic firing and increasing spontaneous miniature endplate potential (MEPP) frequency to 38 times the control rates (Laskowski and Dettbarn, 1971).

In an early study, Gupta et al. (1986) demonstrated that an acute exposure to DFP (1.5 mg/kg, s.c.) changed neither nAChR density (B_{max}) nor the affinity constant (K_D) in the diaphragm muscle. Subchronic DFP treatment (0.5 mg/kg/day, s.c., for 5 days) caused a marked decrease in B_{max} (56%), without significant change in K_D. Chronic treatment with anti-AChEs reduced the total number of nAChRs by 42–45% in the endplate region (Chang et al., 1973). Later studies revealed that in sublethal doses, OPs induce symptoms that cannot be solely attributed to AChE inhibition, indicating a direct interaction with postsynaptic nAChRs (Menking et al., 1990). Tattersall (1990) investigated the effects of DFP, sarin, soman, VX, and ecothiophate on the nAChR ion channel at the adult mouse muscle endplate by using single-channel recording techniques. DFP, sarin, and soman had no effect on open times at concentrations up to 100 μM, but ecothiophate and VX were found to have voltage- and concentration-dependent, open-channel blocking at concentrations of 1–50 μM. In similar experiments, Bakry et al. (1988) demonstrated that OP nerve agents and echothiophate bind to AChRs, inhibit or modulate binding of radioactive ligands to these receptors, and modify events regulated by them. The OPs also bound to allosteric sites on the nAChR (identified by inhibition of ^{3}H-phencyclidine bonding), but some bound also to the receptor's recognition site (identified by inhibition of ^{125}I-αBT binding). Soman and echothiophate in micromolar concentrations acted as partial agonists of the nAChR and induced receptor desensitization. On the other hand, VX acted as an open-channel blocker of the activated receptor and also enhanced receptor desensitization. Membrane fragments from Torpedo electric organs were used to determine these interactions using ^{3}H-phencyclidine as a probe. The results were consistent with the hypothesis that OPs bind to, and irreversibly phosphorylate, an allosteric site on the ion channel associated with the nAChR.

In the context of OP toxicities, it needs to be emphasized that for a normal muscle contraction, the ratio of AChE to nAChRs is crucial in determining the minimum AChR density. Since the AChE recovers at almost the same rate as do the AChRs, a balance of AChE to AChR is maintained over the postsynaptic surface during recovery. A relatively constant ratio of AChE to AChR is very important for maintaining normal neuromuscular function.

NONCHOLINERGIC SYSTEM

Muscle Excitotoxicity

Involvement of the cholinergic neurotransmitter ACh in muscle excitotoxicity has been known for a long time. There is also mounting evidence showing the presence of glutamatergic machinery (receptors, transporters,

and glutamate itself) at the NMJ (Pinard and Robitaille, 2008). It has been suggested that glutamate and ACh are co-released at the NMJ. Glutamate might be a mediator or modulator of neuromuscular transmission. Glutamate receptors present at the NMJ are predominantly an *N*-methyl-D-aspartate (NMDA) subtype. Furthermore, it was demonstrated in *in vivo* studies that a noncompetitive NMDA receptor (NMDAR) antagonist, known as memantine, blocks muscle fasciculations induced by AChE inhibitors (DFP, soman, sarin, tabun, and VX), suggesting an involvement of NMDARs (Gupta and Dettbarn, 1992; McLean et al., 1992). Of course, memantine exerts several additional pharmacological actions, including nAChR blockage. Unlike the well-understood role of cholinergic excitotoxicity, the role of glutamate excitotoxicity is yet to be established in AChE inhibitor-induced muscle toxicity.

Oxidative/Nitrosative Stress

Although the exact mechanism underlying skeletal muscle damage by OP nerve agent-induced hyperactivity still remains unclear, mounting evidence indicates that free radicals play an important role. During normal conditions, free radicals are generated at a low rate and subsequently taken care of by the well-developed scavenger and antioxidant systems. However, during exhaustive hyperactivity of the skeletal muscles caused by anti-ChE, excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (hereafter referred to collectively as *ROS*) are generated and exceed the capacity of the muscle defense system, thus producing oxidative stress. Excessively generated ROS can cause muscle injury by reacting with cellular components, such as membrane phospholipids, mitochondrial enzymes/proteins and nucleic acids. Consequently, this leads to skeletal muscle cell apoptosis/necrosis, inflammation, and loss of cell viability.

A causal relationship appears to exist between excitotoxicity, ROS, lipid peroxidation, and muscle cell injury/death. One of the well-recognized targets of ROS-induced injury is peroxidation of lipids and the formation of prostaglandin F_2-like compounds, such as F_2-isoprostanes (F_2-IsoPs). Studies have shown that the assay of these compounds provides an accurate measure of lipid peroxidation (Roberts and Morrow, 2000; Dettbarn et al., 2001). Quantification of F_2-IsoPs and nitric oxide (NO) in skeletal muscles (SOL, EDL, and diaphragm) has provided strong evidence that AChE inhibitor-induced toxicity initiates lipid peroxidation and muscle cell injury (Dettbarn et al., 2001; Gupta et al., 2001a,b; Milatovic et al., 2001). Yang and Dettbarn (1998) found a significant increase of F_2-IsoPs in diaphragm muscle (156%) 1 h after DFP injection (1.7 mg/kg, s.c.), when muscle hyperactivity was maximal.

The primary reason for the increased generation of ROS appears to be a decreased rate of ATP synthesis in the mitochondria, which is related to a loss of cytochrome oxidase (COX) activity. COX is the terminal complex in the mitochondrial respiratory chain, which generates ATP by oxidative phosphorylation. During intense muscle hyperactivity, the activity of COX is reduced, leading to an increase in the electron pressure within the electron transport chain and to increased ROS production (Yang and Dettbarn, 1998). More than 90% of O_2 consumption in the cells is catalyzed by COX. The chance of intermediary products, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical, escaping is small under conditions where COX remains active. A reduced capacity of this enzyme, however, increases the risk for an incomplete reduction of O_2 and further O_2 radical formation.

Xanthine oxidase (XO) is another enzyme that contributes to increased ROS generation. During normal conditions, 80–90% of native XO exists as xanthine dehydrogenase (XD), but during metabolic stress and increased Ca^{2+}, XD is converted to a reversible oxidase form. XO uses molecular O_2 instead of NAD^+ as an electron acceptor. Molecular O_2 is thereby reduced and the superoxide radical (O_2^-) is formed. During hyperactivity of the muscle by AChE inhibitors, regeneration of ATP is insufficient, not due to the lack of O_2, but due to greater utilization and impaired synthesis of ATP (Gupta et al., 1994, 2000a,b, 2001a, 2002). Unlike ischemia, O_2 is present during oxidative stress caused by prolonged contractile activity. This suggests that subsequent to the conversion of XD to XO, O_2 is continuously univalently reduced to superoxide anions. This occurs during oxidative stress when ATP utilization exceeds the rate of ATP synthesis during increased muscle activity. Yang and Dettbarn (1998) provided direct evidence for the role of COX and XO in muscle injury by its hyperactivity, showing that during DFP-induced muscle hyperactivity, a decrease in COX activity and an increase in XO activity occurred. The NAD(P)H oxidases can also be the major O_2^- generating sources in contracting skeletal muscle (Sakellariou et al., 2014). Blockage of muscle fasciculations provided prevention of these enzyme changes.

Another ROS contributing to oxidative stress is peroxynitrite ($ONOO^-$), which is formed by the reaction of NO with superoxide (O_2^-). $ONOO^-$ has the potential to modify biomolecules through several different mechanisms and is a good candidate for mediation of the NO-dependent pathophysiological process. Under normal conditions, NO is widely regarded as a multifunctional messenger/signaling molecule and is thought to have two physiological functions in skeletal muscle. The first is to promote relaxation through the cGMP pathway, and the second is to modulate muscle contractility that depends on reactive oxygen intermediates. At the NMJ,

NO appears to be a mediator of (i) early synaptic protein clustering, (ii) synaptic receptor activity and transmitter release, and (iii) downstream signaling for transcriptional control (Blottner and Luck, 2001). NO has also been demonstrated to modulate excitation–contraction coupling in the diaphragm muscle (Reid et al., 1998). Within skeletal muscle cells, all three known nitric oxide synthase (NOS) isoforms (neuronal, nNOS; endothelial, e-NOS; and inducible, iNOS) are present, but nNOS, which is Ca^{2+} dependent, seems to predominate, and is concentrated at the sarcolemma and postsynaptic surface of the NMJ (Stamler and Meissner, 2001). In a recent study, NOS has been demonstrated to play the role of mechanosensor in skeletal muscle fibers (Smith et al., 2002). Neuronal NOS in skeletal muscles is involved in the regulation of metabolism and muscle contractility. Kobzik et al. (1994) reported for the first time that the NOS activity of individual muscles could be correlated primarily with type II fibers (being highest in the EDL), while Frandsen et al. (1996) found the distribution of nNOS homogeneous.

Data presented in Table 40.3 show the levels of citrulline (determinant of NO/NOS) in skeletal muscles of control rats and those treated with an acute dose of DFP (1.5 mg/kg, s.c.). In control muscles, markedly higher citrulline levels were found in the SOL, followed by the EDL and diaphragm. The observed higher level of NO in the SOL could be due to a greater activity of NOS. Within 1 h of DFP exposure, when rats exhibited signs of peak severity and maximal reduction of AChE activity (90–96%), the levels of citrulline were maximally increased (272–288%), and remained significantly elevated in all three muscles (> twofold) when measured after 2 h. The finding of elevated NO by DFP-induced muscle hyperactivity (Gupta et al., 2002) was supported by previous studies showing that increased muscle contractility generates significantly greater quantities of ROS/RNS (Yang and Dettbarn, 1996; Clanton et al., 1999). A significant increase in NO is known to cause the inhibition of mitochondrial function and thereby appears to be the cause of the impaired synthesis of ATP.

TABLE 40.3 Citrulline Levels (nmol/g) in Skeletal Muscles of Rats after 1 h of DFP (1.5 mg/kg, s.c.) Injection

Treatment	Soleus	EDL	Diaphragm
Control	451.2 ± 5.3	381.3 ± 8.2	331.2 ± 9.9
	(100%)	(100%)	(100%)
DFP	1227.1 ± 47.2[a]	1061.0 ± 38.9[a]	952.3 ± 49.8[a]
	(272%)	(278%)	(288%)

Values are means ± SEM (n=4–5).
[a]*Significant difference between control and DFP-treated rats ($p<0.05$).*

High-Energy Phosphates Depletion and Myonecrosis

Recently, interest in the role of energy metabolites (especially ATP) in chemical-induced mitochondrial/cytotoxicity has been reinvigorated, since ATP appears to be a switch to decide whether cells die from apoptosis or necrosis. Muscle necrosis is probably caused by increased contractile activity in individual muscle fibers. Calcium is believed to regulate mitochondrial oxidative phosphorylation, thereby contributing to the maintenance of cellular energy homeostasis (Glancy et al., 2013). In normal cells, the mitochondrial Ca^{2+} content is relatively low compared to that in the cytoplasm. However, abnormally high cytosolic Ca^{2+} levels (due to NMDAR activation and impaired Ca^{2+} extrusion, which is an energy-dependent process) perturb many cellular processes. The major changes include (i) reduced cytochrome-c oxidase activity, (ii) increased XO activity, (iii) mitochondrial damage due to excessive mitochondrial Ca^{2+} accumulation, (iv) reduced ATP synthesis, and (v) increased production of O_2^- and NO^-, resulting in increased $OONO^-$ formation. The extreme vesiculation and disruption of the sarcoplasmic reticulum (SR) under the endplate, as well as the swollen mitochondria may reflect Ca^{2+} overloading of the muscle Ca^{2+}-binding capacity, which could result in high sarcoplasmic Ca^{2+} levels, which in turn leads to necrosis (Salpeter et al.,1982). The control of intracellular Ca^{2+} concentration is of great importance to muscle fibers, and a transient rise leads to contraction. An increased net influence of Ca^{2+} forces the mitochondria and SR to maintain Ca^{2+} homeostasis by sequestering the excessive amounts of this ion. This is an energy-consuming process and occurs in preference to ATP formation. The ensuing lack of energy ultimately causes the free sarcoplasmic Ca^{2+} to rise and an excessive ROS formation, leading to necrosis. The energy required for muscle contraction is derived from the breakdown of ATP. As soon as ATP is broken down, it is promptly restored in the so-called Lohman reaction at the expense of phosphocreatine (PCr).

Table 40.4 presents the data of high-energy phosphates (i.e., ATP and PCr) and their metabolites in the skeletal muscle of control rats and those treated with an acute dose of DFP (1.5 mg/kg, s.c.). Analyses of control muscles revealed the levels of ATP and PCr to be higher in the EDL, followed by the diaphragm and the SOL. The values of energy charge potential (ECP = ATP + 0.5ADP/TAN) in SOL, EDL, and diaphragm were 0.86± < 0.01, 0.91± < 0.01, and 0.86± < 0.01, respectively. At the time of maximal severity (i.e., 1 h after DFP exposure), the levels of ATP, total adenine nucleotides (TAN = ATP + ADP + AMP), PCr, and total creatine compounds (TCC = PCr + Cr) were maximally reduced in all three

TABLE 40.4 Energy Phosphates and Their Metabolites in Muscles of Rats Intoxicated with DFP (1.5 mg/kg, s.c.)

		μmol/g (Means ± SEM; n=4–5)			
	Treatment	**ATP**	**TAN**	**PCr**	**TCC**
Soleus	Control	3.42 ± 0.05	4.42 ± 0.04	6.38 ± 0.19	20.41 ± 0.37
		(100)	(100)	(100)	(100)
	DFP	2.06 ± 0.06[a]	2.78 ± 0.08[a]	3.67 ± 0.19[a]	12.87 ± 1.01[a]
		(60)	(63)	(57)	(63)
EDL	Control	4.91 ± 0.37[b]	5.80 ± 0.38[b]	9.63 ± 0.59[b]	26.30 ± 1.64[b]
		(100)	(100)	(100)	(100)
	DFP	3.42 ± 0.02[a]	4.15 ± 0.02[a]	6.82 ± 0.23[a]	21.74 ± 0.33[a]
		(70)	(72)	(71)	(83)
Diaphragm	Control	4.47 ± 0.23	5.76 ± 0.26	6.18 ± 0.03	23.34 ± 0.17
		(100)	(100)	(100)	(100)
	DFP	2.74 ± 0.43[a]	3.66 ± 0.05[a]	3.96 ± 0.12[a]	15.86 ± 0.45[a]
		(61)	(64)	(64)	(68)

[a]*Significant difference between control rats and DFP-treated rats ($p<0.05$).*
[b]*Significant difference between soleus and EDL values of control rats ($p<0.05$); numbers in parentheses are percent remaining values compared to controls (100%).*

muscles, and they remained reduced to the same degree after 2 h. Similar results were found with soman toxicity.

AChE inhibitors cause depletion of energy-rich phosphates (ATP and PCr) due to a combination of impaired synthesis and greater utilization of ATP during muscle hyperactivity. In fact, the time course of necrosis correlates with the reduced levels of PCr, the reduction of which may have been the result of an increased demand for energy and a low rate of ADP phosphorylation, caused by an increased level of sarcoplasmic Ca^{2+}. AChE inhibitor-induced increases in NO (Table 40.3) can exert cellular toxicity primarily by depleting energy stores through multiple mechanisms: (i) by prolonging poly-(ADP-ribose) polymerase activation; (ii) by inhibiting mitochondrial enzymes, such as COX, aconitase, and creatine kinase (CK); and (iii) by inhibiting the glycolytic enzyme phosphofructokinase. NO, at nanomolar concentrations, can directly and specifically inhibit mitochondrial respiration by competing with molecular O_2 for binding to COX, thereby causing an inhibition of ATP synthesis (Brown and Cooper, 1994). Other factors that contribute to the decline of energy metabolites may include damage to mitochondria, a higher rate of ATP utilization needed to generate NAD^+ in the ADP-ribosylation of nuclear proteins, enhanced influx of sarcoplasmic Ca^{2+}, an increased number of contractile protein cross-bridges, and the release of ATP in concert with ACh from the nerve terminals. The net effect of AChE inhibitor-induced muscle hyperactivity is a reduced cellular energy level.

MUSCLE ACTIVITY—EMG

AChE inhibitors (DFP, 1.5 mg/kg, s.c.; or soman, 100 μg/kg, s.c.) at a toxic sign-producing sublethal dose elicit prominent motor, behavioral, and autonomic symptoms. As mentioned earlier, the motor symptoms are fasciculations, fibrillations, and body tremors. Fasciculations and fibrillations are due to antidromic neural discharge from excess junctional ACh, while tremors are of a central origin. Misulis et al. (1987) demonstrated a difference in the pattern of motor symptoms in rats treated with soman or DFP. Soman produces complex posturing movements and tremors affecting virtually the entire body, while DFP produces movements that are similar to fasciculations or myokymia. Muscle fiber necrosis is also more frequent at symptom-producing doses of DFP than equivalent doses of soman. This suggests that the AChE inhibitors differ in their propensity to produce central or peripheral effects and that peripheral effects are required for muscle necrosis.

Electromyographic (EMG) findings indicate that soman and DFP produce different responses (Figure 40.4). The majority of the motor symptoms induced by soman are due to impulses descending from the CNS, and a proportion of these symptoms are epileptiform activity. This activity is not generated at spinal levels. In contrast, DFP produces motor symptoms mainly by peripheral action that depends on a functioning nerve terminal (Misulis et al., 1987). Anderson (1987) also demonstrated that DFP and soman have opposite effects on skeletal muscle

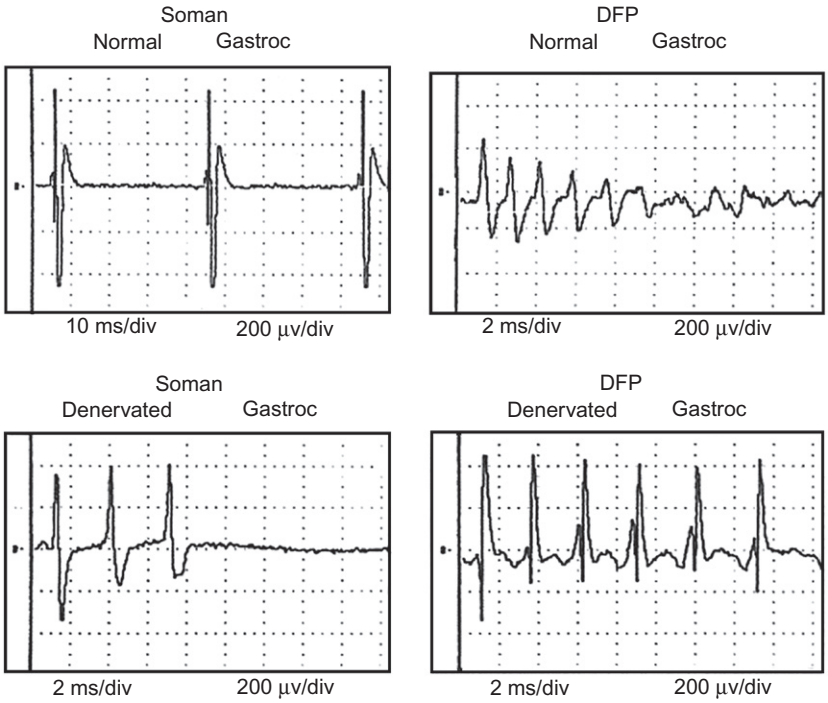

FIGURE 40.4 EMG recordings from normal and acutely denervated lateral gastrocnemius in response to soman (100 μg/kg, s.c.) or DFP (1.5 mg/kg, s.c.). Left, soman; right, DFP; top, nondenervated muscle; bottom, denervated muscle. Note the difference in time base and voltage scale. The differing morphology of the normal DFP waveform occurs due to electrode orientation and distance from the muscle fiber and does not represent a systematic difference between the effects of individual agents.

contracture during tetanic stimulation. DFP significantly decreased the ability of rat skeletal muscle to maintain a contracture. Soman, on the other hand, increased muscle force in a frequency-dependent manner. The opposite actions of DFP and soman on muscle contractility might be the consequence of a difference in effects of these two agents on postsynaptic muscle ACh receptors. The finding of Anderson (1987), which saw an increase in contracture following soman, was somewhat surprising in view of the work of Dettbarn (1984) and Gupta et al. (1987a,b), who reported that soman is as effective as other AChE inhibitors in producing muscle necrosis (Table 40.6). However, Meshul et al. (1985) reported that soman appears to spare motor endplates from structural damage and thereby may account for maintaining muscle function (Anderson, 1987). Thus, different AChEIs can produce opposite effects on muscle contracture.

MUSCLE FIBER HISTOPATHOLOGY

By using actomyosin ATPase reaction, analysis of untreated rat SOL revealed predominantly type 1 fibers with a few type 2A and 2B fibers. In contrast, EDL is composed predominantly of type 2 fibers with few type 1 fibers. The total fiber numbers are approximately 1800 in SOL and 2500 in EDL (Gupta et al., 1989).

OP pesticides and nerve agents and some carbamates in a single sublethal dose are known to cause fasciculations and induce myopathy (histopathological changes) in the diaphragm, SOL, EDL, sternomastoid, gastrocnemius, triceps, and tibialis of experimental animals. Pathological changes in skeletal muscles by AChE inhibitors are observed in the region adjacent to the motor endplate. Patterson et al. (1987) determined the necrotic lesions by assessing endplates in skeletal muscles of rats acutely intoxicated with DFP (1.5 mg/kg, s.c.). In SOL, these are found only in the midsection of the muscle, while in EDL, the endplates are found throughout the muscle length. In EDL, the fibers are innervated at different levels throughout the length of the muscle, while in SOL, the nerve makes functional contact just in the midsection. No endplates or lesions are found outside this area. Gupta et al. (1985, 1986) found the highest number of lesions in all three muscles (diaphragm, SOL, and EDL) of rats within 24–48 h after a single injection of DFP (1.5 mg/kg, s.c.), when the inhibitory effect on AChE activity was also maximal (Table 40.5). The diaphragm muscle had the highest number of lesions, followed by the EDL and SOL.

Data on soman (100 μg/kg)-, sarin (110 μg/kg)-, tabun (200 μg/kg)-, and VX (12 μg/kg)-induced pathological changes in skeletal muscles of rats are presented in Table 40.6. Following soman treatment, the number of

TABLE 40.5 Number of Necrotic Fibers Following a Single Sublethal Injection of DFP (1.5 mg/kg, s.c.)

Time After DFP Administration	Soleus		EDL		Diaphragm	
	AChE	Lesions	AChE	Lesions	AChE	Lesions
Control	100%	0	100%	0	100%	0
1 day	24%	27 ± 6[a]	17%	64 ± 11[a]	13%	308 ± 58[a]
2 day	24%	39 ± 9[a]	14%	71 ± 12[a]	13%	333 ± 63[a]
3 day	39%	5 ± 1[a]	31%	49 ± 5[a]	25%	174 ± 35[a]
7 day	65%	0	53%	0	69%	0

Values of AChE activity are expressed as % remaining activity of control (100%); numbers of lesions are presented per cross-section from the mid-belly region of muscle; data are presented as the means ± SEM (n=5–10).
[a]*Significant difference between control and DFP-treated rats ($p<0.05$).*

TABLE 40.6 Necrotic Fibers in Skeletal Muscles of Rats Following a Sublethal dose of Soman, Sarin, Tabun, or VX

Time (Posttreatment)	Muscle	Number of Lesions/1,000 Fibers			
		Soman (100 μg/kg, s.c.)	Sarin (110 μg/kg, s.c.)	Tabun (200 μg/kg, s.c.)	VX (12 μg/kg, s.c.)
1 h	DIA	0	9 ± 1	6 ± 2	26 ± 5
	SOL	0	12 ± 4	9 ± 2	16 ± 3
	EDL	0	0	0	0
6 h	DIA	3 ± 2	100 ± 30	48 ± 12	233 ± 45
	SOL	13 ± 3	111 ± 38	21 ± 4	97 ± 17
	EDL	0	0	0	23 ± 10
24 h	DIA	260 ± 48	435 ± 154	302 ± 32	322 ± 49
	SOL	48 ± 9	101 ± 24	66 ± 14	99 ± 20
	EDL	1 ± 0	103 ± 45	33 ± 5	28 ± 14
3 days	DIA	74 ± 24	490 ± 66	541 ± 31	305 ± 78
	SOL	9 ± 3	135 ± 42	192 ± 29	186 ± 37
	EDL	0	18 ± 17	66 ± 12	79 ± 16
7 days	DIA	0	216 ± 69	40 ± 10	75 ± 12
	SOL	0	237 ± 61	28 ± 4	62 ± 7
	EDL	0	30 ± 11	3 ± 1	27 ± 11

Value are means ± SEM of necrotic lesions (n=5).

necrotic fibers in the SOL and the diaphragm was found to increase for up to 24 h, and no new lesions appeared thereafter. By day 7, these two muscles appeared to have fully recovered from the soman effect. In EDL, no morphological changes were seen at any time. While the acute toxic effects of tabun are more or less similar to soman, there are also some significant differences between these two agents. Unlike soman, tabun caused prolonged AChE inhibition and progressive development of muscle necrosis over a period of 3 days. While the primary toxic effect of tabun occurs due to inhibition of AChE activity, some of the differences seen in regard to other nerve agents may be due to additional actions of the cyanide group that is released during AChE inhibition. The inhibitory effect of CN on oxidative metabolism could result in reduced *de novo* synthesis, delaying synthesis of AChE and prolonged inhibition of Ca^{2+} sequestration into the SR of skeletal muscles. Therefore, in the context of OP nerve agent-induced myopathy, soman, sarin, tabun, or VX caused the greatest number of lesions in the diaphragm,

followed by the SOL, and then in the EDL during the first 24–48 h (Gupta et al., 1987a,b, 1991). With all four OP nerve agents, the greatest number of lesions occurred in animals exhibiting severe muscle fasciculations (Gupta et al., 1986, 1987a,b, 1991; Inns et al., 1990; Bright et al., 1991). By day 7, muscles recovered from soman-induced myopathy, but not from any other nerve agents since the necrotic lesions were still evident.

The earliest lesions are focal areas of abnormality in the subjunctional section of the muscle fiber adjacent to the motor endplate, including eosinophilia and sarcoplasmic swelling (Laskowski et al., 1977; Gupta et al., 1985, 1986; Patterson et al., 1987). Mitochondria are disrupted as evidenced by clumping of highly reactive material of lactate dehydrogenase (LDH) and reduced nicotinamide adenine dinucleotide (NADH) reactions. These focal changes progress to a breakdown of subjunctional fiber architecture, loss of striations, and then phagocytosis. Longitudinal sections reveal that necrosis affects only a small segment of fiber lengths. During the latter stages, progressively greater lengths of muscle fibers are affected (Gupta et al., 1986; Patterson et al., 1987). Serial cross sections of 10 μm thickness indicate that the number of lesions correlates with the greatest density of endplates (Patterson et al., 1987). The longer the lag between injection and sacrifice, the greater the extent of the lesions. A significant increase in blood CK activity coincides with the appearance of myonecrosis, indicating destruction of the muscle membrane (Sket et al., 1989; Gupta et al., 1991, 1994).

Subjunctional changes in the muscle fibers, such as supercontraction of subjunctional sarcomeres, as well as disruption of cytoarchitectural organization, are always present (Gupta et al., 1986, 1987a,b, 1991; Inns et al., 1990; Bright et al., 1991). The initial changes are in the mitochondria which first swell and then show lysis of the central cristae. Myelin figures beneath the endplate are frequently observed, while the region more distal to the endplate is less affected. The nucleoli of the muscle cell nuclei are enlarged and move to the periphery of the nucleus. This myopathy can be induced with OP prototype DFP (Gupta et al., 1986; Misulis et al., 1987; Patterson et al., 1987, 1988; Sket et al., 1991a,b), or the OP nerve agents soman, sarin, tabun, and VX (Gupta et al., 1987a,b, 1991; Inns et al., 1990; Gupta and Dettbarn, 1992).

Laskowski et al. (1975) reported ultrastructural changes in the subsynaptic folds that were quite varied, even between muscle fibers from the same diaphragms of rats acutely treated with paraoxon. The fact that some endplates were totally degenerated after 2 days of paraoxon treatment, while others appeared almost normal even after 5 days, indicates that some endplates are more resistant than others. The most consistent change at the endplates was the presence of vesicular structures in the synaptic clefts. Some regions of the subsynaptic folds contained varying sizes of vacuoles and vesicles. There appears to be a wide variation in the severity of the lesions in the subsynaptic folds even in the same muscle. After 2 days of paraoxon treatment, the muscle surrounding the motor endplate often showed less cytoarchitectural organization than control muscles. Myelin figures beneath the endplate were frequently observed, while the region of muscle distal to the endplate was less severely affected.

Regardless of the diversity in OP structures, it is evident that induced myopathy is dose-dependent and rests on both a critical duration and degree of AChE inhibition. The higher number of necrotic lesions found with faster rates of AChE inactivation indicate the involvement of ACh in the generation of myopathy (Wecker et al., 1978a,b; Gupta et al., 1986, 1987a,b, 1991). Regardless of the OP involved, AChE inhibition of more than 80% for about 2 h is necessary to initiate severe muscle fiber necrosis. AChE inhibition resulting in an excess of ACh and its prolonged functional interactions with the nAChRs is responsible for producing lesions. However, some anticholinesterase (anti-ChEs) agents have been shown to interact directly with the nicotinic receptors (Tattersall, 1990, 1992). The OP compounds acting directly on postsynaptic membranes or on components of the muscle cell were also considered in the etiology of myopathy (Laskowski and Dettbarn, 1971). However, the prior injection of either tubocurarine or α-bungarotoxin can prevent development of the myopathy and indicates that neither the OP *per se* nor ACh by itself causes the damage; rather, the damage is due to changes from transmitter–receptor interaction. Tubocurarine and α-bungarotoxin, by occupying the postjunctional receptors, prevent the ACh from interacting with the receptor. The primary defect is at both presynaptic and postsynaptic sites. This not only supports the concept of an abnormal neurotrophism as a mechanism of myopathy, but it also allows the possibility that the primary defect is in the cholinergic system of nerve and muscle.

Evidence for the involvement of locally elevated levels of ACh was confirmed since denervation prevented myopathy or nicotinic ACh receptor blockade with α-bungarotoxin (Wecker and Dettbarn, 1976; Dettbarn, 1984). It was clearly demonstrated that transection of the phrenic nerve to the rat hemidiaphragm prevented myopathic development, while in the contralateral innervated hemidiaphragm, the number of lesions increased. Thus, the common denominator is muscle hyperactivity, such as fasciculations (Gupta et al., 1986, 1987a,b, 1991; Adler et al., 1992). The longer the muscle hyperactivity lasts, the greater the number of necrotic muscle fibers found.

There is ample evidence that ACh accumulation is involved in causing Ca^{2+} influx into skeletal muscle fibers during anti-ChE poisoning. Experiments with sarin

(25–150 µg/kg, s.c.) in mice revealed a similar role of Ca^{2+} in muscle fiber damage (Inns et al., 1990). Ca^{2+} increase was found in the diaphragm of those mice to which sarin had been administered at doses of 50 µg/kg or above. Calcium accumulation, which was confined to the region of the motor endplates, occurred earliest and remained the longest in the diaphragm from those animals receiving the highest doses of sarin. Generally, Ca^{2+} accumulation can be detected on day 1 after injection and none after day 7. This observation coincides with the duration of appearance and disappearance of muscle lesions. It was suggested that Ca^{2+} is among a series of events that ultimately lead to myonecrosis. Mitochondrial damage by excessive Ca^{2+} is expected to cause a partial or complete depletion of ATP synthesis and consequently excessive generation of oxygen- and nitrogen-free radicals and eventually myotoxicity (Gupta and Dettbarn, 1987, 1992; Gupta et al., 2001a,b, 2002). Together, following an acute exposure to an OP compound at a higher dose, a causal relationship exists between a critical level of AChE inhibition, Ca^{2+} accumulation, depletion of ATP, and appearance of muscle fiber necrosis.

Chronic exposure (30–60 days) of rats to paraoxon, at doses (0.05–0.1 mg/kg, s.c./day) that do not produce parasympathomimetic effects, led to necrosis of muscle fibers in diaphragm muscle, which was qualitatively similar to that following the administration of a single high dose of paraoxon (0.23 mg/kg, s.c.). The lesion was characterized by the presence of central nuclei, fiber splitting, and breakdown of fiber architecture followed by phagocytosis and necrosis. With either dose, AChE inhibition occurred in the endplate regions, not in the nonendplate regions (Wecker and Stouse, 1985).

MUSCLE CYTOTOXICITY BIOMARKERS

CK and CK isoenzymes

CK catalyzes the synthesis of ATP and PCr in a reversible Lohmann reaction. Table 40.7 shows the normal distribution of CK and its isoenzymes in the skeletal muscle of untreated control male Sprague-Dawley rats. The findings revealed that the fast-muscle EDL had the maximal CK activity, followed by diaphragm, with the lowest in the SOL. Electrophoretic separation of CK isoenzymes in all three muscles revealed the existence of only CK-MM isoenzyme. Further separation of CK-MM isoenzyme for subforms showed only CK-MM3 in all three muscles. Compared to muscles, serum had very little CK activity; however, the CK consisted of three distinct isoenzymes: CK-BB (15.3%), CK-MB (3.9%), and CK-MM (80.8%). Further electrophoresis of the serum CK-MM isoenzyme revealed the presence of three subforms: CK-MM1 (6.3%), CK-MM2 (24%), and CK-MM3 (69.7%) (Gupta et al., 1994). Literature abounds showing that the CK-MM3 subform secretes from muscles into the plasma, where it converts into the MM2 and MM1 subforms by carboxypeptidase-N2.

Within 1 h of exposure to carbofuran (1.5 mg/kg, s.c.), CK activity was significantly reduced in the SOL, while it increased in the diaphragm. At the same time, activities of CK and all three CK isoenzymes were significantly elevated in serum. An important finding was that carbofuran or methyl parathion caused a shift in the serum CK-MM subform; i.e., higher sequential conversions of CK-MM3 subform to CK-MM2 and CK-MM2 to CK-MM1, possibly due to enhanced carboxypeptidase-N2 activity (Gupta et al., 1994).

LDH and LDH Isoenzymes

LDH catalyzes the synthesis of lactate and pyruvate in a reversible reaction and is commonly used as a biomarker of cell damage or death. Normal distribution of LDH and its isoenzymes in skeletal muscles of rats revealed that in controls, LDH activity was found to be highest in the EDL, followed by the diaphragm, and lowest in the SOL (Table 40.8). Compared to muscles, the enzyme activity was meager in serum. Interestingly, all three muscles and serum contained all five electrophoretically

TABLE 40.7 Normal Distribution of CK and CK Isoenzymes in Skeletal Muscles and Serum of Rats

		CK Isoenzymes		
	Total CK	**CK-BB (CK-1)**	**CK-MB (CK-2)**	**CK-MM (CK-3)**
Soleus	2,062,800 ± 71,065 (100)	ND	ND	2,062,800 ± 71,065 (100)
EDL	4,659,125 ± 185,583 (100)[a,b]	ND	ND	4,659,125 ± 185,583 (100)[a,b]
Diaphragm	3,018,240 ± 110,777 (100)	ND	ND	3,018,240 ± 110,777 (100)
Serum	3,769 ± 240 (100)	576 ± 45 (15.3)	148 ± 17 (3.9)	3,086 ± 209(80.8)

Values expressed in terms of IU/L are presented as means ± SEM (n=4–6); numbers in parentheses are percentages of isoenzymes to total CK activity (100%); ND, none detected.
[a]*Significant difference between EDL and soleus ($p<0.001$).*
[b]*Significant difference between EDL and diaphragm ($p<0.001$).*

TABLE 40.8 Normal Distribution of LDH and LDH Isoenzymes in Skeletal Muscles and Serum of Rats

		LDH Isoenzymes				
	Total LDH	**LDH-1 HHHH**	**LDH-2 HHHM**	**LDH-3 HHMM**	**LDH-4 HMMM**	**LDH-5 MMMM**
Soleus	72,720 ± 2,484 (100)	14,962 ± 476 (20.7)	19,054 ± 617 (26.3)	16,118 ± 692 (22.2)	14,254 ± 1,332 (19.5)	8,316 ± 890 (11.3)
EDL	207,300 ± 22,945 (100)[a,b]	7,290 ± 1,890 (3.5)[a,b]	12,300 ± 2,033 (5.9)[a,b]	18,105 ± 1,498 (8.7)	40,420 ± 4,100 (19.5)[a,b]	129,870 ± 15,298 (62.6)[a,b]
Diaphragm	129,120 ± 4,828 (100)	17,020 ± 504 (13.2)	17,498 ± 571 (13.5)	16,190 ± 780 (12.6)	26,738 ± 1,080 (27.7)	51,680 ± 3,081 (40.0)
Serum	740 ± 28 (100)	9.8 ± 1.4 (1.3)	17.4 ± 2.0 (2.3)	10.2 ± 1.2 (1.4)	38.0 ± 3.8 (5.2)	643 ± 65 (87.0)

Values expressed in terms of IU/L are presented as means ± SEM (n=4–6); numbers in parentheses are percentages of isoenzymes to total LDH activity (100%).
[a]*Significant difference between EDL and soleus ($p<0.01$).*
[b]*Significant difference between EDL and diaphragm ($p<0.01$).*

distinct LDH isoenzymes, although with varying quantities. Each muscle presented a characteristic LDH isoenzyme pattern. For example, EDL contained a large proportion of LDH-5 (62.6% of total activity) and very little LDH-1 (3.5%). SOL, on the other hand, had a very small amount of LDH-5 (11.3%) and about 20% each of the other four isoenzymes (LDH-1–LDH-4). In general, values of LDH isoenzymes in diaphragm were intermediate to the EDL and soleus. Diaphragm had predominantly LDH-5 and LDH-4 (40% and 27.7%, respectively). In control serum, isoenzyme LDH-5 was 87% of the total LDH activity (100%).

Total LDH activity was significantly enhanced in EDL, diaphragm, and serum by carbofuran (1.5 mg/kg, s.c.) or methyl parathion [5 mg/kg, administered intraperitoneally (i.p.)] within 1 h of injection. Each AChE inhibitor caused marked elevation of all five isoenzymes in serum, with maximum increases in LDH-1 and LDH-4 (threefold). Unlike serum, muscle LDH isoenzymes depicted variable patterns by carbofuran or methyl parathion intoxication. A significant decline in ATP appears to be the mechanism involved in leakage of cytoplasmic/mitochondrial enzymes into circulation (Gupta et al., 1994). For further details on muscle toxicity biomarkers, see Gupta et al. (2014).

SKELETAL MUSCLE AND TOLERANCE DEVELOPMENT

Repeated application of OP nerve agents or pesticides for a prolonged period in concentrations that initially do not produce obvious symptoms of toxicity can lead to a limited degree of adaptation, as seen in a reduction of the duration and intensity of muscle fasciculations, muscle fiber necrosis, and behavioral tolerance (Wecker and Dettbarn, 1976; Gupta et al., 1985, 1986). Various cholinergic mechanisms in skeletal muscles underlying this phenomenon have been well-documented, including (i) reduced cholinergic-binding sites of muscarinic and nicotinic receptors, (ii) reduced uptake of choline, and (iii) stimulation of AChE synthesis (Gupta et al., 1985, 1986). In an *in vivo* subchronic study (DFP, 0.5 mg/kg, s.c./day for 14 days) in male rats, Gupta and Dettbarn (1986) demonstrated complete recovery of protein synthesis in skeletal muscles (the SOL, EDL, and diaphragm) during the tolerance phase (day 14) from inhibition of protein synthesis observed during the toxicity phase (day 5). Further, during the toxicity phase (day 5), inhibition of *in vivo* protein synthesis was comparable to that seen from an acute dose of DFP (1.5 mg/kg, s.c.). Additional mechanisms that may contribute to general tolerance are the availability of other serine active site enzymes, such as carboxylesterase (CarbE) and BuChE. Although the functional role of these enzymes is unknown, binding to and inhibition of these enzymes reduce the free concentration of inhibitors that otherwise would have been available to interact with AChE (Gupta et al., 1985). This is supported by studies showing that the toxicity of OPs can be potentiated by inhibition of CarbE, and tolerance to soman develops when plasma CarbEs recover during chronic exposure (Sterri et al., 1981).

A major mechanism causing adaptation to the necrotic action of DFP at the NMJ is the reduction of nAChRs (B_{max}, 56%) without significant change in affinity constant (K_D). This loss of receptors can also explain the disappearance of fasciculations that was observed between days 3 and 5 of treatment (Gupta et al., 1986). Whether presynaptic receptors regulating fasciculations are involved in this process remains to be determined. Changes in the postsynaptic receptor density could occur as an adaptation mechanism of the cell to the excessive cholinergic stimulation caused by AChE inhibition. The loss of ACh receptors may be caused by a reduction of junctional folds (Laskowski et al., 1975) on which most of the nicotinic ACh receptors are located. Changes in the ionic milieu, especially increases in Ca^{2+} caused by the increased neuromuscular activity during DFP-induced fasciculations, may be involved in the loss of secondary junctional folds. In essence, at the NMJ, the two major mechanisms leading to adaptation are (i) an increased recovery of AChE activity as a result of *de*

novo synthesis (Gupta et al., 1986) and (ii) a reduction in nAChR binding sites (Gupta et al., 1985, 1986, 1987a,b). Finally, mechanisms such as modification of ACh release from presynaptic sites may be an additional contributing factor (Carlson and Dettbarn, 1988).

SKELETAL MUSCLE INVOLVEMENT IN IMS

OP insecticide-induced IMS was reported for the first time in human patients in Sri Lanka in 1987 (Senanayakeand Karalliedde, 1987; Karalliedde and Henry, 1993). Thereafter, this syndrome has been reported in South Africa (1989), Turkey (1990), Belgium (1992), India (2003), and many other countries. IMS is clearly a separate entity from acute cholinergic crisis and delayed neuropathy. IMS is a life-threatening complication of OP poisoning, which most commonly occurs 48–72 h after exposure. Thus far, IMS has not been documented with OP nerve agents. Clinically, IMS is characterized by acute respiratory paralysis and weakness in the territories of several cranial motor nerves, neck flexors, and facial, extraocular, palatal, nuchal, and proximal limb muscles. Despite severe AChE inhibition, muscle fasciculations and muscarinic receptor-associated hypersecretory activities are absent. Based on EMG findings from OP-poisoned patients and experimental studies on laboratory animals, it has been found that the defect in IMS is at the neuromuscular endplate and postsynaptic levels, but the effects of neural and central components in muscular weakness have not been ruled out. EMG in the early stages reveals marked decrements at low rates of repetitive nerve stimulation and increments at a high rate, suggesting diverse types of impaired neuromuscular transmission. IMS seems to be due to persistent AChE inhibition at the endplate, presumably leading to combined presynaptic and postsynaptic impairment of neuromuscular transmission. Currently, very little is known about the type of damage at the motor endplate or about risk factors associated with IMS. For details on the involvement of muscles in IMS, see De Bleecker (2006).

PREVENTION/TREATMENT OF MYOPATHY

Various pharmacologic and therapeutic drugs have been tested to prevent or treat myopathy. Drugs effective in the treatment of neuromuscular signs of anti-ChE toxicity include (i) oximes that reactivate the phosphorylated AChE (Thiermann et al., 2005; Marrs and Vale, 2006), (ii) subparalyzing and paralyzing doses of *d*-tubocurarine (Clinton and Dettbarn, 1987; Patterson et al., 1987), (iii) atropine sulfate/atropine methyl nitrate (Patterson et al., 1987; Clinton et al., 1988), (iv) diazepam, and (v) creatine phosphate (Clinton et al., 1988). In several studies, reversible AChE-inhibiting carbamates, atropine, or anticonvulsants were given with oxime to achieve an optimal effect. Some of the drugs, including AChE reactivators, muscarinic and nicotinic ACh receptor blockers, NMDAR antagonists, anticonvulsants, and antioxidants, are described here briefly in terms of their prophylactic/therapeutic efficacy. It should be noted that some of these agents, however, produce toxicity when given for an extended period of time, thereby limiting their utility.

ACHE REACTIVATORS AND ACH RECEPTOR BLOCKERS

The standard treatment of nerve agent-induced muscle toxicity calls for the reactivation of the phosphorylated AChE with an oxime. Oximes such as obidoxime (bis(4-formylpyridiniomethyl) ether dioxime, also known as Toxogonin), pralidoxime (2-pyridine aldoxime methochloride/2-PAM) and a few others have been found very effective when given in combination with other drugs such as atropine, *d*-tubocurarine, and diazepam. Effectiveness of pretreatment with oximes varies with the chemical structures of the nerve agents and depends on the time after exposure. For example, it has been established that asoxime chloride (HI-6) effectively reactivates AChE inhibited by soman, while TMB-4 is known as one of the most efficient reactivators of tabun-inhibited enzyme. In *in vivo* studies, Jovanovic (1983) evaluated the effects of two bis-pyridinium oximes (BDB-27 and HGG-12) on neuromuscular blockades induced by nerve agents (sarin, soman, tabun, and VX) in rats, and effectiveness was compared with the two most potent oximes (HI-6 and TMB-4). It was found that BDB-27 was equal or superior to HI-6 in sarin, soman, and VX, and to TMB-4 in tabun-poisoned animals. The potency of HGG-12 was equal to HI-6 only in soman poisoning, but it was much less pronounced against neuromuscular blockades induced by the other nerve agents. In general, oxime therapy becomes progressively ineffective with time due to changes in the enzyme-inhibitor complex, which loses an alkyl radical. This so-called aged phosphorylated enzyme complex is resistant to oxime reactivation (Radic and Taylor, 2006). Thus, prevention of formation of the enzyme-inhibitor complex seems to be one of the preferred strategies in the treatment of myopathy.

The best pretreatment should include agents that prevent access of the nerve agent to AChE without affecting its activity, or a combination of drugs that would decrease the release of ACh from nerve terminals and

partially block access to the nicotinic and the presynaptic and postsynaptic muscarinic receptors. So far, timely administration of atropine sulfate or atropine methyl nitrate, in combination with an oxime (such as 2-PAM) or diazepam, is currently the treatment of choice for OP poisoning. Atropine modifies ACh receptor interaction at the NMJ postsynaptically by shortening the opening time of the ion channel, and possibly by lowering Na^+ conductance. Atropine sulfate has been observed to reduce motor activity and muscle necrosis caused by AChE inhibitors (Clinton and Dettbarn, 1987). Atropine methyl nitrate is four times more potent than atropine sulfate in its peripheral effects and has been confirmed to ameliorate peripheral motor activity caused by AChE inhibitors (Clinton et al., 1988). Atropine methyl nitrate (16 mg/kg, s.c.) failed to ameliorate the central effects of both soman and DFP due to its quaternary structure that hinders its passage across the blood–brain barrier (BBB; Clinton et al., 1988).

Earlier studies with *d*-tubocurarine and atropine indicated that nicotinic and muscarinic ACh receptor blockers can modulate the release of ACh induced by an inhibitor of AChE (Carlson and Dettbarn, 1988). The effects of these drugs, whether inhibiting or stimulating ACh release, are concentration dependent and determined by the frequency of nerve activity (Bowman, 1980; Wessler et al., 1987a,b). Therefore, drugs that reduce axonal hyperexcitability by decreasing the amount of ACh released from the nerve terminals without interfering with normal transmission, provide another pretreatment possibility. Protection against DFP-induced myopathy was achieved by using small concentrations of atropine sulfate (16 mg/kg, s.c.) or atropine methyl nitrate (16 mg/kg, s.c.) and *d*-tubocurarine (75 µg/kg, s.c.) that prevented fasciculations and muscle necrosis without interfering with normal neuromuscular function (Patterson et al., 1987). In this study, pretreatment agents acted presynaptically by preventing DFP-induced backfiring and muscle fasciculations, possibly by reducing the release of ACh. The protective drugs in the concentrations used had no significant effect on the normal characteristics of conduction and transmission. Carlson and Dettbarn (1988) showed that *d*-tubocurarine or atropine sulfate in subparalytic concentrations prevented the increases in MEPP frequency when given prior to an AChE inhibitor, or attenuated the increased frequency to normal when given after the increase of frequency. At a subparalytic dose *d*-tubocurarine (50 µg/kg, given intravenously) is known to suppress fasciculations by preventing repetitive firing of nerve terminals. Furthermore, *d*-tubocurarine and atropine sulfate reduced Ca^{2+} influx and ACh release (through presynaptic receptors), which is associated with repetitive activity, as seen during AChE inhibitor-induced antidromic firing.

NMDAR Antagonist

In a series of *in vivo* experiments, rats receiving a sublethal dose of soman (100 µg/kg, s.c.), sarin (110 µg/kg, s.c.), tabun (200 µg/kg, s.c.), VX (12 µg/kg, s.c.), or DFP (1.5 mg/kg, s.c.) developed seizures and severe muscle fasciculations within 15–20 min that lasted for about 4–6 h. Marked inhibition of AChE activity and necrotic lesions in skeletal muscles (the SOL, EDL, and diaphragm) became evident between 1 and 24 h postinjection and persisted for several days. Pretreatment of rats with the NMDAR antagonist memantine (18 mg/kg, s.c.), given with atropine sulfate (16 mg/kg, s.c.) 60 and 15 min prior to DFP or nerve agents, respectively, significantly attenuated AChE inhibition and prevented myonecrosis and muscle fasciculations, as well as other signs of behavioral toxicity. No muscle fasciculations were seen at any time (Gupta and Dettbarn, 1992).

Protection of AChE inhibition was greater when memantine was given prophylactically than therapeutically. It is noteworthy that memantine itself does not influence normal activity of AChE in non-OP-treated animals. Although the precise mechanism involved in reduction of AChE inhibition by memantine against nerve agents is yet to be elucidated, spontaneous reactivation as the cause of the remaining high-enzyme activity was ruled out since the enzyme activity was determined within a short period of time; i.e., 1 h after acute intoxication with soman, sarin, tabun, VX, or DFP (Gupta et al., 1986, 1987a,b, 1991; Gupta and Dettbarn, 1992), and AChE activity remained low when only atropine sulfate (16 mg/kg, s.c.) was used. Studies by McLean et al. (1992) revealed that memantine did not prevent inhibition of AChE by edrophonium, an anionic site inhibitor, or by decamethonium, a peripheral site inhibitor. Thus, memantine appears to bind to a different modulatory site to protect this enzyme's activity. The other mechanisms by which memantine might have attenuated AChEI's toxicity may include the following:

- Reduced reflex excitability of both flexors and extensors (Wand et al., 1977)
- Reduced high frequency of repetitive activation of peripheral nerves by reducing the permeability of Na^+ and Ca^{2+} in axonal membranes (Wesemann and Ekenna, 1982; Wesemann et al., 1983)
- Blockage of nicotinic ACh receptor-ion channel complex (Masuo et al., 1986)
- Prevention of neural hyperexcitability (McLean et al., 1992)
- Central muscle relaxation (Grossmann and Jurna, 1977)
- Reduced seizures by uncompetitive NMDAR blockage (Danysz et al., 1994; Carter, 1995; Parsons et al., 1999)
- Prevention of cellular energy depletion (Gupta and Goad, 2000).

Although not all of these mechanisms have been investigated in the protection of OP nerve agent-induced myopathy by memantine, it appears that memantine provides protection by multiple mechanisms. It needs to be mentioned that no significant change occurred in AChE activity in skeletal muscles of OP-untreated rats receiving memantine and atropine sulfate. Prophylactic administration of memantine and atropine sulfate also blocked the AChEI-induced increase in levels of citrulline and F_2-isoprostanes, markers of NO synthesis and lipid peroxidation, respectively (Milatovic et al., 2005). Memantine has the advantage of providing prophylactic benefits without producing sedation or any other side effects. Thus, memantine may prove to be a superior drug to many other agents.

Anticonvulsants and Anesthetics

The prevention and treatment of OP-induced myopathy are both complex since individual AChEIs differ in their major sites of action. As mentioned earlier, soman-produced muscle hyperactivity was generated mainly in the CNS, while DFP-induced hyperactivity arose approximately equally from the CNS and the peripheral nervous system (PNS) and NMJ. Clinton et al. (1988) reported that in the case of both soman (90 µg/kg, s.c.) and DFP (1.5 mg/kg, s.c.) poisoning, ketamine (25 mg/kg, s.c.) reduced centrally generated motor activity, while atropine methyl nitrate (16 mg/kg, s.c.) and sodium phenytoin (15 mg/kg, intravenously) had no significant effect. It was suggested that the effectiveness of ketamine may alter patterns of neuronal firing by reducing high-frequency neuronal discharges that are characteristic of seizures (MacDonald and McLean, 1986). Ketamine is known to act on sodium channels by producing a dose-dependent reduction of inward sodium current in a manner similar to that of local anesthetics, with a resultant suppression of high-frequency neuronal bursts, which are manifested as excess muscle activity seen with OP poisoning. Since ketamine and drugs in its class also have specific anticholinergic effects at both nicotinic and muscarinic (more potent at nicotinic than at muscarinic) receptors (Kloog et al., 1977), it may be a direct antagonist in the CNS against the cholinergic activation caused by AChEIs (Gupta et al., 1987a,b). Additionally, ketamine can inhibit excitation–contraction coupling within the muscle by altering Na^+ conductance at the muscle membrane (Marwaha, 1980a,b).

The anticonvulsant phenytoin did not reduce central motor activity induced by either soman or DFP (Clinton et al., 1988). Phenytoin suppresses sustained high-frequency neuronal firing through a membrane potential-dependent blockade of Na^+ channels with resulting inhibition of nonsynaptic events involved in epileptogenesis (MacDonald and McLean, 1986; Yaari et al., 1986). The failure of phenytoin to control the seizures induced by AChEIs suggests that they may not result from the same mechanisms of epileptogenesis as seizures produced by maximal electrical shock, against which phenytoin is most effective. Conventional anticonvulsant compounds have been reported to provide limited protection against nerve agent-induced seizures and muscle necrosis when given therapeutically (Lipp, 1972; Clinton et al., 1988). Sedation, tolerance, and abuse potential limit prophylactic use of benzodiazepine compounds, however.

In animal models, clonidine (alpha2 adrenergic agonist) has also been reported to prevent nerve agent-induced seizures (Buccafusco et al., 1988). Prophylactic use of clonidine may be limited by the marked ataxia and sedation produced by this drug in effective concentrations.

Antioxidants, Spin-trapping Agents, and Creatine

Scavenging or prevention of the ROS generation originated through various excitotoxicity mechanisms are of particular interest. An antioxidant such as the lipid-soluble vitamin E (α-tocopherol) is an excellent blocker of ROS production, as it extracts hydrogen and interrupts lipid peroxidation. Vitamin E mainly acts as a chain-breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage. There are reports demonstrating that vitamin E concentrates in the mitochondria, the major site for the generation of ROS, as well as energy metabolites. Therefore, vitamin E regulates ROS production, maintains oxidative phosphorylation in mitochondria, and accelerates the restitution of high-energy metabolites.

A synthetic spin-trapping agent such as phenyl-*N*-*tert*-butylnitrone (PBN) is capable of scavenging many types of free radicals. This compound is widely used to trap ROS in a variety of physical, chemical, and biological studies using electron magnetic resonance spectrometry. PBN is known to be concentrated in the mitochondria, where it reacts with ROS and forms stable adducts, thereby maintaining normal levels of energy metabolites. In addition, PBN has other pharmacological actions, such as (i) reversible Ca^{2+} channel blockade (Anderson et al., 1993), (ii) direct reversible interaction with AChE against phosphorylation by DFP (Zivin et al., 1999; Milatovic et al., 2000a,b), and (iii) protection of COX activity (Milatovic et al., 2001).

Pretreatment with PBN (75, 150, or 300 mg/kg, i.p.) 30 min prior to DFP injection (1.7 mg/kg, s.c.) in a dose-dependent manner protected AChE activity from inhibition, and prevented muscles from undergoing necrosis and rats from fasciculations (Milatovic et al., 2000a,b) (Table 40.9). The protective mechanism of PBN is proved

TABLE 40.9 Protective Effect of PBN Against DFP-Induced Fasciculations, AChE Inhibition, and Necrosis in Rat EDL

	Control	DFP[a]	PBN + DFP[b]	DFP + PBN[c]
Fasciculations	–	+	–	+
Necrotic fibers/cross-section of muscle	2 ± 2	82.55 ± 2.98*	0**	67.0 ± 4.59
AChE activity	12.17 ± 0.73 (100%)	3.70 ± 0.35* (30%)	10.85 ± 0.59** (89%)	2.97 ± 0.17 (24%)

Activity of AChE is expressed in terms of nmol/mg protein/min and number of necrotic fibers/1,000 muscle fibers. Ranking of fasciculations: – , absent; + , high frequency affecting all muscles. Values are means ± SEM of 5 muscles.
* Significant difference between control and DFP-treated rats ($p<0.01$).
** Significant difference between DFP-treated rats and PBN + DFP-treated rats ($p<0.01$).
[a]*DFP (1.7 mg/kg, s.c.) was given 1 h before sacrificing the rats.*
[b]*PBN (300 mg/kg, i.p.) was given 30 min before DFP (1.7 mg/kg, s.c.).*
[c]*PBN (300 mg/kg, i.p.) was given 20 min after DFP (1.7 mg/kg, s.c.) administration.*

to take place due to its ROS/RNS scavenging property (Gupta et al., 2000a, 2001b). Treatment with PBN 20 min after DFP exposure neither prevented fasciculations nor protected AChE activity. While the role of PBN as an antioxidant is well established, its prophylactic effect against excitotoxicity induced by an AChEI is due to its protection of AChE from critical inhibition (Milatovic et al., 2000a,b). Unlike PBN, vitamin E neither prevented DFP-induced muscle fasciculations nor protected AChE. Both vitamin E and PBN concentrate in the mitochondria, so they can regulate ROS production, maintain oxidative phosphorylation, and accelerate restitution of high-energy metabolites (Gupta et al., 2001a,b).

Since the major energy sources are ATP and PCr, an increase of both compounds in muscles through administration of creatine appears to sustain ATP levels under stress conditions. This is supported by the findings that rats pretreated intravenously with PCr showed reduced muscle necrosis that otherwise would have been seen following DFP treatment. PCr did not attenuate the DFP-induced muscle fasciculations that generated the necrosis (Clinton and Dettbarn, 1987).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Skeletal muscles are the target of a variety of chemicals, especially OP nerve agents. These agents modulate structural and functional properties of the muscles, and the toxic effects can range from minor chest pain, muscle cramps in the legs, complete paralysis, and even death. Both *in vivo* and *in vitro* data strongly implicate that OP nerve agents initially exert acute toxicity by excitotoxicity involving not only the cholinergic system, but also processes associated with noncholinergic mechanisms. These processes include the glutamatergic system, excess generation of free radicals (ROS/RNS), and alterations in antioxidants and the scavenging system, causing oxidative stress, lipid peroxidation, high-energy phosphate depletion, and muscle necrosis. Wide variations exist between slow and fast fiber-containing muscles, and they further appear to respond differently to each OP nerve agent. Available sensitive biomarkers are indicative of cytotoxicity and mitochondrial toxicity (Gupta et al., 2014). Myopathy induced by AChE inhibiting OP nerve agents is a serious concern, since it is untreatable in most circumstances. Instead, prevention seems a better option. More potent AChE reactivators need to be developed for better therapeutic efficacy against OP nerve agent-induced myopathy.

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CHAPTER

41

Reproductive Toxicity and Endocrine Disruption of Potential Chemical Warfare Agents

Timothy J. Evans

INTRODUCTION

Reproduction is a critical biological process in all living systems and is required for species survival. The immediate, short-term, toxicological concerns regarding chemical warfare and acts of agricultural and industrial terrorism are concerned primarily with human and animal incapacitation and mortality. Logically, most of the currently available literature on these aspects of warfare and terrorism focuses on these immediate adverse health effects and their sequelae. However, the entire scope of the effects of these toxic insults can involve severe emotional and financial distress and diminished prognosis for long-term survival of exposed human and animal populations as a result of toxicant-induced spontaneous abortions, congenital defects, and infertility. It is therefore important for individuals working in human and veterinary medicine, the military, public health, government regulatory agencies, industry, and agriculture, as well as public policy-makers, to be familiar with some of the potential adverse reproductive effects of these types of toxicants on exposed humans and animals. This chapter reviews what is currently understood about the potential effects of chemical warfare agents (CWAs), nuclear fallout, and hazardous industrial and agricultural wastes on human and animal reproductive function.

For the purposes of this chapter, the term "reproduction" is used primarily in reference to vertebrate species of animals (especially mammals) and is inclusive of "development" (Figure 41.1), which is sometimes treated as a separate topic in toxicology texts. This particular chapter emphasizes what is currently known about the adverse effects of known CWAs and selected environmental contaminants on male and female reproductive function, as well as xenobiotic-induced effects on the growth, maturation, and sexual differentiation of the embryo and fetus. Endocrine disruption is an extremely common mechanism of action for xenobiotics associated with impaired reproductive function and is discussed along with reproductive toxicity in this chapter. Efforts have been made to clarify the currently used terminology related to these topics and to provide the reader with a brief description of proposed mechanisms of action and observed reproductive outcomes associated with selected toxicants that might be relevant to chemical warfare and/or acts of terrorism.

Unfortunately, although there is relative lack of information documenting long-term reproductive effects of the types of toxicants covered in this book, space constraints still limit the amount of information that can be presented in this chapter. There are a number of recently published textbooks and book chapters that cover some of these and related subjects in greater detail and provide information that is complementary to what is presented in this chapter (Senger, 2003; Naz, 2005; Golub, 2006a,b; Gupta, 2006, 2007c; Evans, 2007; Capen, 2008; Foster and Gray, 2008; Rogers and Kavlock, 2008; Romano et al., 2008). This is especially true with respect to normal reproductive anatomy and function (Senger, 2003; Evans, 2007), and the reader is directed to these publications and the other references cited in this chapter to gain additional insight into specific areas of reproductive function and toxicology.

It is important to understand that the areas of toxicology involving the long-term effects of CWAs and environmental

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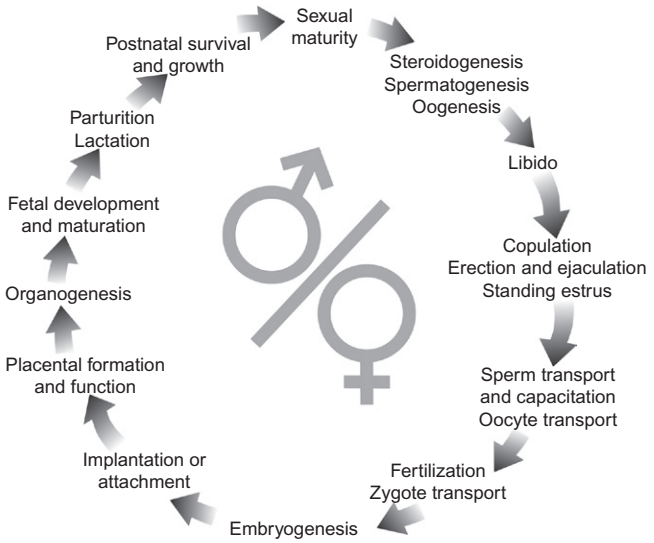

FIGURE 41.1 The multiple steps involved in reproductive development and function in both males and females are shown schematically to illustrate the complexity of reproduction in mammalian species and to demonstrate the various stages in the reproductive process that can be targeted for toxic insult. *Source: This figure was adapted, with permission, from Ellington and Wilker (2006) (modifications and artwork courtesy of Don Connor).*

contaminants, reproductive toxicity, in general, and endocrine disruption, in particular, are in continual flux. New data and exceptions to "classical" mechanisms of action are being reported on a regular basis, and there continues to be ongoing debate about the effects of chronic low-level exposures to toxicants available for or arising from military and terrorist activities and various aspects of normal as well as xenobiotic-induced abnormal reproductive functions. Every effort has been made to accurately represent what is currently understood about the topics of discussion in this chapter. Controversial topics or those currently still subject to debate within the scientific community have been noted whenever possible.

IMPORTANT DEFINITIONS AND CONCEPTS

Chemical Warfare Agents

For the purposes of this chapter, "chemical warfare agent" (CWA) is used as a fairly comprehensive term to refer to a diverse group of toxicants commonly discussed within the context of chemical incapacitation for crowd control during riots or death or incapacitation associated with military use or terrorism. The lachrymatory and irritant riot control agents include α-chlorbenzylidene malonitrile (CS), dibenz (*b,f*)-1:4 oxazepine (CR), ω-chloroacetophenone (CN), and oleoresin of capsicum (OC pepper spray) (Salem et al., 2008a). The CWAs currently of greatest interest and for which the greatest amount of data have been gathered are arsenicals, chlorine gas, phosgene and phosgene oxime, sulfur mustard, ricin, hydrogen cyanide and cyanide-related compounds, and organophosphate nerve agents (Wismer, 2007; Kikilo et al., 2008).

Environmental Contaminants Associated with Industrial or Agricultural Terrorism

For the sake of completeness, this chapter also discusses the adverse reproductive effects of potential toxicants, such as ionizing radiation, pesticides, and other organic environmental contaminants, as well as heavy metals, which are known to adversely affect reproductive function. Although all of these potential toxicants have been or are currently being released into the environment in generally low concentrations as a consequence of normal industrial and agricultural activities, the results of accidents, such as those that occurred in Chernobyl in the former Soviet Union (ionizing radiation), Bhopal, India (methyl isocyanate or MIC), Seveso, Italy, and Times Beach, Missouri (dioxins), and Minamata, Japan, and Basra, Iraq (methyl mercury), underscore the potential impact that large-scale industrial or agricultural terrorism-related releases of these contaminants could have on populations of humans and animals. In fact, these environmental contaminants have the potential to become "low-tech" CWAs in the hands of terrorists and less sophisticated military organizations.

Reproduction

As represented in Figure 41.1, reproduction in humans and domestic, wild, and laboratory vertebrate animals encompasses a wide range of physiological processes and associated behaviors and anatomical structures involved in the production of the next generation and the survival of a given species of animal (Senger, 2003; Evans, 2007). The physiological processes involved in reproduction generally include the following: gametogenesis (production of sperm or ova) and the prepubertal and peri-pubertal changes leading to its onset; release of gametes (i.e., sperm transport and maturation, penile erection and ejaculation of sperm (mammals), copulation between a male and a female of the same species (several vertebrate classes) and ovulation of oocytes); formation of the zygote (i.e., sperm storage, capacitation, and other processes leading to fertilization, or union, of a single sperm with an egg); embryonic and fetal development during the incubation process in egg-bearing vertebrates or, especially in the case of mammals, during pregnancy (gestation) (i.e., activities related to the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation

(mammalian species), neurulation, and organogenesis (including sexual differentiation)); "birth" of a single or multiple offspring (hatching in oviparous vertebrates); and, finally, in mammalian species, the initiation and maintenance of milk production (lactation) for the postpartum nutrition of offspring (Evans, 2007).

Reproductive Toxicity

For the purposes of this chapter, "reproductive toxicity" refers to any manifestations of xenobiotic exposure, including "endocrine disruption," reflecting adverse effects on any of the physiological processes and associated behaviors and/or anatomical structures involved in animal reproduction or development (Figure 41.1). This is a fairly broad definition that encompasses developmental toxicity, as well as any toxic effects of postpubertal exposures to xenobiotics on either male or female reproduction. "Developmental toxicity" refers to any adverse effect on the developing organism associated with either preconception parental exposures to toxicants or postconception xenobiotic exposures to the embryo, fetus, or prepubertal offspring, and adverse effects associated with developmental toxicity of xenobiotics might not necessarily be observed until after the affected individuals have reached sexual maturity (Hodgson et al., 2000; Eaton and Klaassen, 2001; Evans, 2007).

Teratogenesis

The term "teratogenesis" is derived from the Greek word for monster (*teras*) and is a form of developmental toxicity (Rogers and Kavlock, 2008). "Teratogenesis" refers specifically to developmental defects induced by toxicant exposures occurring between conception and birth (Hodgson et al., 2000; Eaton and Klaassen, 2001; Evans, 2007; Rogers and Kavlock, 2008). The types of abnormalities that are typically associated with teratogenesis include embryonic or fetal death, morphological, functional, and/or neurobehavioral abnormalities, and decreased growth rate and/or birth weight (Evans, 2007; Rogers and Kavlock, 2008).

With respect to teratogenesis, there are six basic tenets of teratology, first defined by J. G. Wilson in 1959, that need to be kept in mind whenever gestational exposure to a teratogenic xenobiotic is suspected or when a chemical is being evaluated for its teratogenic potential (Wilson, 1977; Evans, 2007):

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
2. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.
4. The final manifestations of abnormal development are death, malformation, growth restriction, functional disorder.
5. The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
6. Manifestations of deviant development increase in degree as dosage increases from no effect to the totally lethal level.

Mechanisms of Reproductive Toxicity and Teratogenesis

In general, normal reproduction and development require rapidly replicating and differentiating cells undergoing mitosis and, within the gonads, meiosis. There is a wide range of specific mechanisms of action by which xenobiotics can adversely affect reproductive function, including embryonic and fetal development. Many of these mechanisms are the same as those for toxicants affecting other organ systems and essentially involve some sort of toxicant-induced interference with the cell cycle, cellular dysregulation, and alterations in cellular maintenance, which, when possible, the body attempts to repair, either successfully or unsuccessfully (Gregus, 2008). Oxidative damage and interference with normal enzymatic reactions are two common mechanisms by which xenobiotics and, especially, some CWAs (Smith et al., 2008) can cause the dysregulation and altered maintenance of cells within various organs and tissues.

Teratogenesis can be associated with each of the following mechanisms of action: excessive cell death; interference with apoptosis; reduced cellular proliferation rate; failed interactions between cells; impaired morphogenetic movements; reduced synthesis of components essential for growth and development; mechanical disruption; and alterations in pH (Hood et al., 2002; Hood, 2006; Evans, 2007). Some teratogens are capable of more than one mechanism of action, and it is important to keep in mind that the observed developmental abnormalities associated with exposure to any given teratogen will, in large part, be dependent on the timing of the exposure to that xenobiotic during gestation. Familiarity with the timing of important developmental events in species of interest is critical in the diagnosis and prevention of teratogenesis, as is the design of experiments investigating the teratogenic potential of different chemicals in animal models (Evans, 2007).

Normal reproduction and development require signaling within and between a variety of diverse organs and, in sexual reproduction and mammalian pregnancy, critical communication even takes place between distinctly different organisms (i.e., male and female and mother and offspring, respectively) (Evans, 2007). It should be remembered that premature parturition or

spontaneous abortion can be induced by any circumstances that cause fetal or, potentially, maternal stress and initiate the cascade of endocrine and neural signaling events that would normally lead to parturition. Any sublethal intoxication or emotionally traumatic event in a pregnant woman or animal has the potential to threaten fetal survival.

The dependency of reproductive function on signaling pathways inclusive of gene transcription makes this physiological process especially prone to adverse effects associated with xenobiotic-induced disruption of or interference with cell-to-cell, organ-to-organ, and/or even animal-to-animal communication. Many of the mechanisms that interfere in some way with physiological signaling activity can be classified as forms of "endocrine disruption," but there is a great deal of overlap between the various different mechanisms for reproductive toxicity. The level of exposure to a particular toxicant is an important determinant of what toxic effects are observed, and xenobiotics that "disrupt" endocrine pathways can do so without interactions with endogenous receptors using mechanisms of action that can cause other forms of toxic insult at various dosages.

Reproductive Toxicants

Any xenobiotic associated with adverse effects on development of male or female reproductive function can be classified as a "reproductive toxicant" (Evans, 2007; Rogers and Kavlock, 2008). Even chemicals adversely affecting animal well-being have a potential negative impact on development and reproductive function. This chapter attempts to focus on toxicants that are available for or could arise from military and terrorist activities and specific mechanisms of actions that have a direct effect on the male and/or female reproductive tract or that target normal embryonic and/or fetal growth and maturation (Evans, 2007).

Teratogens

The subclass of reproductive toxicants capable of inducing teratogenesis is referred to as "teratogens." Some teratogenic chemicals induce their adverse effects indirectly on the fetus by altering maternal synthesis of essential nutrients or by other mechanisms that do not require their transport across the placenta. However, many teratogens directly affect fetal development by crossing the "placental barrier" and entering the fetal circulation. The passage of nutrients, hormones, and other endogenous, as well as exogenous, substances across the placenta has been traditionally thought of by some references as primarily a function of the intimacy (i.e., number of tissue layers) between the maternal and fetal circulations, especially with respect to maternal immunoglobulins that cross some types of placentation but not others (Evans, 2007). Although it is true that placental characteristics, such as thickness, surface area, carrier systems, and lipid protein characteristics can influence the passage of xenobiotics across the placenta and that the placenta is generally impermeable to chemicals with molecular weights greater than 1,000 Da, most xenobiotics have molecular weights less than 500 Da and cross the placenta by simple diffusion (Foster and Gray, 2008). It is currently thought that a potential teratogen's molecular size, degree of ionization, protein binding, and lipid solubility are the most important determinants of that chemical's ability to move from the maternal circulation across the placenta into the fetal circulation (Rozman and Klaassen, 2001; Senger, 2003; Evans, 2007; Foster and Gray, 2008). Some toxic xenobiotics can be actively transported by mechanisms intended for structurally similar endogenous molecules (Rozman and Klaassen, 2001), and there is some experimental evidence to suggest that transplacental transport of lead can mimic that of calcium (Evans et al., 2003; Evans, 2007).

Endocrine Disruption

"Endocrine disruption" is a developing multidisciplinary area of research involving aspects of both toxicology and endocrinology (McLachlan, 2001) and is a potential mechanism of action for many toxicants, especially those affecting reproduction. This term has been defined in a variety of different ways, depending on the circumstances and the intended audience. Endocrine disruption can also be defined fairly narrowly with respect to toxicant origin (synthetic versus naturally occurring), source or site of toxicant exposure (exposure from warfare-related or terrorism-related activities versus exposure from environmental contamination), xenobiotic mechanism of action (receptor agonism and/or antagonism versus other mechanisms independent of direct interactions between xenobiotics and receptors), and/or the timing of exposure (prenatal versus postnatal exposures) (Krimsky, 2000, 2001; Evans, 2007). However, the definition of endocrine disruption used in this and another book chapter previously written by the author (Evans, 2007) is fairly broad and encompasses the effects of any synthetic or naturally occurring xenobiotic that can affect the endocrine system of exposed individuals (i.e., the balance of normal hormonal functions) and, as a result of exposure, cause physiological alterations (Keith, 1997; Hodgson et al., 2000; Evans, 2007). Within the broad scope of this definition, reproduction, including prenatal and prepubertal development, certainly would be expected to be one of the physiological functions most profoundly affected by chemicals associated with chemical warfare or environmental contamination capable of endocrine disruption; however, adverse effects on other nonreproductive endocrine systems can also be associated with exposures to xenobiotics (Guillette, 2006; Evans, 2007). Thyroid function, glucocorticoid

metabolism, and other endocrine as well as enzymatic factors associated with adipogenesis have recently been shown to be susceptible to interference by several different classes of chemical compounds (Cooke and Naz, 2005; Newbold et al., 2005, 2006; Grün and Blumberg, 2006; Guillette, 2006; Evans, 2007; Capen, 2008).

Mechanisms of Endocrine Disruption

Although the imitation and/or inhibition of the actions of androgens and, especially, estrogens by xenobiotics is what was first referred to as endocrine disruption, both the multidisciplinary area of study and mechanism of action generally referred to as endocrine disruption have evolved over the years to encompass a wide range of specific mechanisms of action that can ultimately result in adverse effects on invertebrate and/or vertebrate animals (McLachlan, 2001; Evans, 2007). Endocrine disruption involves many mechanisms of action that can ultimately result in adverse effects on animal species. The mechanisms of action involved in endocrine disruption can include effects that are mediated directly by interactions between the xenobiotic and an endogenous hormone receptor (i.e., the xenobiotic functions as a ligand for an endogenous receptor and a receptor–ligand complex is formed), as well as those adverse effects that alter hormonal functions without direct interactions between the toxicant and an endogenous receptor (Keith, 1997; Evans, 2007; Capen, 2008). It should also be noted that a given xenobiotic can potentially disrupt the normal balance of hormonal function by more than one mechanism that is independent of direct interactions between the toxicant and an endogenous hormone receptor (Evans, 2007).

"Classic" endocrine disruption can involve imitation or mimicry of the interactions between cellular receptors and endogenous hormones (i.e., receptor agonism) and/or a blockade or inhibition of the formation of receptor–hormone complexes (i.e., receptor antagonism) (McLachlan, 2001; Evans, 2007), and both genomic and nongenomic physiological responses can be affected by this mimicry or blockade of endogenous hormone receptor–mediated activity (Thomas and Khan, 2005; Evans, 2007). Endocrine disruption can also be mediated by the complex interactions between the endogenous aryl hydrocarbon receptor (AhR) and its major agonists, which are xenobiotics belonging to the class of environmental contaminants referred to collectively as "halogenated aromatic hydrocarbons" or "polyhalogenated aromatic hydrocarbons" (PAHs) (Safe, 2005; Evans, 2007).

Endocrine disruption that is independent of interactions between xenobiotics and endogenous hormone receptors can occur in a variety of different ways, including alterations in the number of hormone receptor sites (upregulation or downregulation) or direct or indirect hormone modifications that alter hormonal function (Keith, 1997; Evans, 2007). Xenobiotics can change the rate of synthesis or destruction of endogenous hormones and can alter how hormones are stored, how they are released into and/or transported within the circulation, or even how they are eventually cleared from the body (Keith, 1997; Sikka et al., 2005; Evans, 2007; Capen, 2008). Any xenobiotic toxic to hormone-producing organs or tissues (e.g., testis and ovary) also has the potential to decrease hormone synthesis and thereby to indirectly cause endocrine disruption (Devine and Hoyer, 2005; Evans, 2007).

In addition to the aforementioned mechanisms of endocrine disruption, there has recently been increasing interest in the association between prenatal exposures to some hormonally active toxicants and the postnatal development of neoplasia (cancer) involving the reproductive tract, as well as the occurrence of transgenerational or vertically transmitted adverse reproductive effects (Crews and McLachlan, 2006). Either genetic mutations (i.e., alterations in the genotype or deoxyribonucleic acid (DNA) sequence) or epigenetic changes, such as DNA methylation of CpG nucleotides in the promoter regions of genes, which are heritable but nongenetic modifications in the properties of a cell (inherited phenotypic alteration without genotypic change), are possible explanations for these phenomena (McLachlan, 2001; Crews and McLachlan, 2006; Evans, 2007). Patterns of DNA methylation are generally established during development at the gastrulation stage (i.e., lineage-specific pattern in somatic cells) and after sex determination (i.e., germ line–specific lineage pattern in the gonad), and DNA methylation can facilitate genomic imprinting, which results in the expression of the allele from only one parent (i.e., monoallelic expression) (McLachlan, 2001; Anway and Skinner, 2006).

Endocrine-Disrupting Chemicals, Endocrine Disruptors, and Hormonally Active Agents

Any reproductive toxicant capable of endocrine disruption can also be considered an endocrine-disrupting chemical (EDC) or an endocrine disruptor. Another term frequently used with respect to endocrine disruption, especially regarding xenobiotics that interact with endogenous hormone receptors, is hormonally active agent (HAA). In most instances, "EDC," "endocrine disruptor," or "HAA" can be used interchangeably to discuss the actions of a given xenobiotic (Evans, 2007).

THE REPRODUCTIVE TOXICITY OF SELECTED TOXICANTS

It should be remembered that the use of CWAs will invariably be associated with wars, acts of terrorism,

revolutions, and civil unrest, all of which do not occur in a vacuum and will be concurrent with emotional stress, famine, and other conditions leading to reproductive failure. As a result, information is relatively lacking with respect to specific adverse reproductive effects, as well as endocrine disruption, related to acute and, particularly, low-level, chronic exposures to many potential CWAs. In addition, much of the information that does exist regarding the long-term reproductive and teratogenic effects of these types of weapons is, unfortunately, somewhat contradictory. Although it is possible that exposures to some CWAs might not generally be associated with serious adverse reproductive effects, the discrepancies between the results of different studies are probably, in part, due to variability in individual responses to specific toxicants and the complexity of analyses of human epidemiological data. In addition, differences in dosing regimens, routes of exposure, and the animal models utilized for *in vivo* studies can confound comparisons of experimental results. The relative insensitivity of many reproductive endpoints might also contribute to the impression that some chemical weapons do not adversely affect reproductive function.

The embryo and fetus, without a developed blood–brain barrier and with only rudimentary DNA repair mechanisms and hepatic detoxifying and metabolizing capabilities, are especially susceptible, as compared with adults, to the adverse effects of low-level exposures to xenobiotics (Newbold et al., 2006; Evans, 2007). There has recently been increasing concern within the regulatory, public health, and scientific communities about the effects of prenatal exposures to potential reproductive toxicants, especially those capable of endocrine disruption, on humans and animals. To increase our understanding of the long-term adverse reproductive effects associated with warfare and acts of terrorism, public knowledge of the use of CWAs or awareness of massive releases of reproductive toxicants associated with confirmed acts of terrorism should result in increased surveillance for phenotypic abnormalities in the most susceptible populations of humans and animals exposed during embryonic, fetal, and early postnatal development. Based on these epidemiological observations, carefully designed experiments mimicking "real life" exposures can be performed in multiple laboratory animal species, using sensitive biomarkers of toxic insult to reproductive development and function, to further elucidate the adverse reproductive effects of xenobiotic exposures likely to be associated with military and/or terrorist activities.

Our relative lack of understanding of the effects of CWAs and other xenobiotics on reproductive function can also be attributed to the complexity of the entire reproductive process and the mechanisms by which it is regulated. It should be evident from Figure 41.1 that maximum reproductive efficiency, including normal embryonic and fetal development, is dependent on the structural and functional integrity of multiple organs and tissues, as well as various physiological processes and signaling pathways within and (with respect to sexual reproduction and pregnancy) between individuals. Putative reproductive toxicants can affect one or several different steps in the reproductive process, depending on the physiological timing, duration, and level of exposure (Evans, 2007; Foster and Gray, 2008).

In "real life," humans and animals can be exposed to some toxicants both prenatally and postnatally. Many organic xenobiotics have the potential to bioaccumulate within exposed individuals, possibly affecting future generations by way of genetic and epigenetic effects. However, reproductive endpoints, such as conception rates and sperm counts, are relatively insensitive, and subtle toxicant-induced changes in reproductive efficiency can be overlooked or missed (Evans, 2007).

Much of the evidence for the adverse reproductive effects of selected toxicants will be based on cases involving wildlife exposures to environmental contaminants or on the experimental results of research exposing laboratory animals to large pharmacological doses of potential toxicants. When available, data are presented from accidental or intentional human and domestic animal exposures to toxicants associated with riot control and chemical warfare or with environmental catastrophes during which incidences of infertility, spontaneous abortion, and teratogenesis have been traced over the course of a number of years.

From an epidemiological perspective, it can be extremely challenging to determine the exact cause of reproductive abnormalities in humans and animals. Questions will often remain regarding whether the observed poor reproductive outcomes associated with acute exposures to toxicants are due to direct effects of these chemicals on reproductive function or are secondary to toxicant-induced systemic disease and its accompanying stress (e.g., spontaneous abortions and preterm births in intoxicated pregnant women or animals). There are multiple factors, including exposures to mixtures of toxicants and other concurrently occurring causes of reproductive failure, that need to be taken into consideration in heterogeneous populations in which exposures to toxic agents are not uniform between individuals. This is especially true in instances when there is a significant time interval between exposure to potential toxicants and the observed reproductive outcomes.

The Reproductive Toxicity of Riot Control Agents

The major lachrymatory and irritant riot control agents include α-chlorbenzylidene malonitrile (CS),

dibenz (*b,f*)-1:4 oxazepine (CR), ω-chloroacetophenone (CN), and oleoresin of capsicum (OC pepper spray) (Salem et al., 2008a). Exposure of pregnant women or animals to these compounds could be expected to be associated with maternal and/or fetal stress, which could potentially lead to the induction of premature parturition (Evans, 2007). Although the riot control agents CS and CN are both alkylating agents with at least the potential to adversely affect embryonic and fetal development, neither of these chemicals has yet been found to be embryotoxic or teratogenic (Sanford, 1976; Salem et al., 2008a). Limited studies performed with laboratory animals suggest that CR is neither embryotoxic nor teratogenic and that OC, with the possible exception of a slightly reduced crown–rump length, is not associated with any adverse effects on reproductive function (Salem et al., 2008a).

The Reproductive Toxicity of CWAs

Given current global politics and the "War on Terrorism," pertinent information regarding many newer potential CWAs is very likely to be unavailable for public review. To be as complete as possible, some of the more historical and currently available CWAs are discussed in the context of their primary, immediate, adverse effects on humans and animals. By organizing this discussion in that manner, the potential adverse reproductive effects of "new" CWAs can be anticipated based on the similarities of those toxicants to existing chemical weapons. For simplicity, CWAs are broadly classified as vesicants (i.e., blistering agents), inhibitors of protein synthesis (e.g., ricin), inhibitors of cellular respiration or "blood agents" (e.g., hydrogen cyanide and cyanide-related compounds), and nerve agents (i.e., organophosphate compounds).

Vesicants

Arsenicals

Although arsenicals are discussed in this chapter with respect to their use in chemical warfare, the metalloid element arsenic, which is classified as a carcinogen, is also a potential environmental contaminant. Arsenicals continue to have important industrial and agricultural uses, and arsenic-containing feed additives, pesticides, and wood preservatives are all still readily available. In general, arsenic binds to sulfhydryl groups, with the activities of thiol-containing enzymes, including those involved in cellular energy production, frequently being adversely affected (Wismer, 2007). Increased capillary permeability is also associated with many acute intoxications involving arsenic.

It is important when discussing the toxicity of arsenicals to distinguish between the effects of organic and inorganic forms of arsenic. Several organic arsenicals, including lewisite (dichloro (2-chlorovinyl) arsine or Agent L), diphenylcyanoarsine, and diphenylchloroarsine, have been used as vesicants and systemic toxicants (Ishii et al., 2004; Wismer, 2007). Like other arsenicals, Lewisite has been associated with fetal death in laboratory animals; however, lewisite is reported not to be teratogenic (Wismer, 2007). In contrast, a degradation product of diphenylcyanoarsine and diphenylchloroarsine, diphenylarsinic acid, has recently been associated with abnormal brain development in humans (Ishii et al., 2004). Inorganic forms of arsenic, particularly arsenite and arsenate, have been associated with neoplasia, estrogenic activity, and testicular and ovarian toxicity, as well as congenital neural tube, skeletal, and gonadal abnormalities, in laboratory rodents (Golub, 2006b). Epidemiological evidence in human populations has suggested that acute arsenic exposures with sublethal, maternal toxicity, and subchronic exposures to elevated arsenic concentrations in drinking water have been associated with increased incidences of spontaneous abortions, stillbirths, and preterm deliveries (Golub, 2006b).

Chlorine Gas

Chlorine (Cl_2) is one of the more commonly produced chemicals in the United States, and chlorine gas is a potent oxidant that is very irritating and, potentially, corrosive (Wismer, 2007; Kikilo et al., 2008; Smith et al., 2008). Chlorine gas is used as a pulmonary and choking agent, and exposure is frequently associated with moderate to severe painful irritation of the eyes and respiratory tract (Wismer, 2007). Such stressful sublethal exposures in late-gestational women or animals might be expected to be associated with the induction of premature parturition and, possibly, spontaneous abortion. Oxidative stress can definitely have adverse effects on reproductive function, but the chronic disease usually associated with chlorine gas exposure is primarily related to the ocular and respiratory systems (Smith et al., 2008). The limited information available regarding the reproductive effects of chlorine gas indicates that it is teratogenic (Wismer, 2007).

Phosgene and Phosgene Oxime

The most important industrial use of phosgene (Agent CG or carbonyl chloride) is in the production of isocyanates (Kikilo et al., 2008), and MIC exposure is discussed with respect to its accidental release in Bhopal, India. Phosgene is classified as a choking agent, and it acylates sulfhydryl, amine, and hydroxyl groups. Phosgene oxime (Agent CX), a halogenated oxime, is a nonpersistent chemical blistering agent, which, like phosgene, interacts with sulfhydryl and amine groups (Wismer, 2007). Other than possible adverse reproductive outcomes related to maternal and/or fetal stress or

increased dermal absorption of concurrently used CWAs, phosgene oxime is unlikely to have direct adverse effects on reproductive function.

Sulfur Mustard

Sulfur mustard (*bis*-(2-chloroethyl) sulfide; mustard gas, Agents HD, H, or HS) and analogs, such as 2-chloroethyl ethyl sulfide, are vesicants that can damage cells by alkylation of macromolecules (i.e., DNA, ribonucleic acid (RNA), and proteins), oxidative stress, glutathione depletion, and inflammation (Watson and Griffin, 1992; Dacre and Goldman, 1996; Wismer, 2007; Smith et al., 2008). Similar to ionizing radiation and a variety of other radiomimetic alkylating agents, such as busulfan, cyclophosphamide, and nitrogen mustard, sulfur mustard and its analogs can target rapidly dividing cells in multiple organs, including the testes and ovaries, as well as the developing embryo and fetus (Wismer, 2007; Foster and Gray, 2008; Hurst and Smith, 2008; Rogers and Kavlock, 2008). Consistent with DNA alkylation, as well as possibly other mechanisms of action, men exposed to sulfur mustard gas have been reported to have lower sperm counts and testosterone concentrations for several weeks after respiratory exposure, and Iraqi use of mustard gas has been associated with alterations in the newborn sex ratio and an increase in some birth defects (Pour-Jafari, 1994; Azizi et al., 1995; Wismer, 2007).

However, despite the epidemiological and laboratory animal evidence indicating that sulfur mustard is a teratogen and reproductive toxicant in humans and animals (Pour-Jafari, 1994; Azizi et al., 1995; Wismer, 2007), contradictory research data involving several different routes of exposure have suggested no adverse sulfur mustard–related reproductive effects, especially at levels of exposure not associated with maternal intoxication (Watson and Griffin, 1992; Dacre and Goldman, 1996; Wismer, 2007). Although based on its mechanisms of action it appears that exposures to sulfur mustard should be associated with several potential adverse reproductive outcomes, including maternal and/or fetal stress-induced premature parturition, additional mustard gas inhalation experiments should be performed in rodent and nonrodent animal models to confirm this suspicion.

Inhibitors of Protein Synthesis

Ricin

The seeds of the ubiquitous castor bean plant (*Ricinus communis*) contain high concentrations of a highly toxic, relatively stable, heterodimeric, glycoprotein toxin called ricin, which is a type 2 ribosome-inactivating protein (RIP) (Burrows and Tyrl, 2001; Millard and LeClaire, 2008; Salem et al., 2008b). The inhibition of protein synthesis by ricin and related type 2 RIPs has been associated with endothelial toxicity and, depending on the route of exposure, severe gastrointestinal or respiratory disease and death (Millard and LeClaire, 2008). Although it would be anticipated that acute sublethal ricin intoxication would be associated with spontaneous abortion or preterm delivery secondary to maternal and/or fetal stress, ricin has also been shown to have direct adverse effects on reproductive function in female rabbits, causing spontaneous abortion and inhibiting ovulation and implantation in this species (Salhab et al., 1999). Because of the availability of the raw ingredients and the relative ease of its extraction, ricin has the potential to be a "low-tech" alternative for terrorist attacks targeting public water supplies (Salem et al., 2008b). Additional research is needed to gain a better idea of the adverse reproductive effects that would be anticipated with sublethal and/or chronic exposures to ricin and related toxins (Millard and LeClaire, 2008).

Inhibitors of Cellular Respiration ("Blood Agents")

Hydrogen Cyanide and Cyanide-Related Compounds

With respect to their use in chemical warfare and, most likely, acts of terrorism, hydrogen cyanide (Agent AC, hydrocyanic acid (liquid form), or prussic acid), cyanogen halides, and other cyanide-related compounds are frequently described as "blood agents" (Wismer, 2007; Kikilo et al., 2008). The major mechanism of action associated with acute cyanide intoxication is the formation of a stable complex with the ferric iron (Fe^{3+}) in cytochrome oxidases, resulting in cytotoxic hypoxia from the inhibition of cellular respiration, oxygen utilization, and energy production (Wismer, 2007; Ballantyne and Salem, 2008). As a result of the rapid lethality of this class of compounds, very few studies have been conducted to investigate the adverse reproductive effects of hydrogen cyanide and cyanide-related compounds (e.g., cyanogen halides, cyanides, and nitriles). However, there is evidence to suggest that cyanide exposure in laboratory animals and livestock exposures to plants containing cyanogenic glycosides can be associated with embryonic and fetal death, as well as teratogenesis (Burrows and Tyrl, 2001; Wismer, 2007; Ballantyne and Salem, 2008). In addition, it would be anticipated that sublethal cyanide-induced hypoxia could cause enough maternal and/or fetal stress to result in spontaneous abortion or preterm deliveries.

Nerve Agents

Organophosphate Nerve Agents

The development of easily disseminated nerve agents as chemical weapons has been of interest to both military strategists and terrorist organizations because of the ability of these chemicals to rapidly incapacitate and kill opposing forces as well as civilian populations. The major chemical nerve agents, tabun (Agent GA), sarin (Agent GB), soman (Agent GD), cyclosarin (Agent GF),

and Agent VX, are extremely toxic and even very brief exposures to these nerve agents can be lethal (Watson et al., 2006; Wismer, 2007; Kikilo et al., 2008). These chemicals are classified as organophosphorus, organophosphate, or "OP" compounds, and their mechanism of action involves the competitive and irreversible inhibition of acetylcholinesterase (AChE) (Watson et al., 2006; Gupta, 2007a; Wismer, 2007). Commonly used OP and carbamate pesticides also inhibit AChE, and these chemicals, although generally less potent than those designed for use as CWAs, usually have longer lasting effects and the potential to be used as low-tech chemical weapons.

There have been some discrepancies between the results of various studies evaluating the reproductive toxicity of different OP compounds. Although decreased libido has been observed in men after acute exposures to both OP nerve agents and insecticides, this "reproductive" effect is most likely related to the neurobehavioral effects, such as posttraumatic stress disorder, associated with acute exposures to these chemicals (McDonough and Romano, 2008). Some nerve agents have been associated with postimplantation morbidity and mortality in laboratory animals (Wismer, 2007), but rats and rabbits exposed to soman did not apparently experience fetal toxicity or prenatal mortality, even with maternal illness (Wismer, 2007). Similarly, low-level exposures to other nerve agents, as well as some insecticides, have not consistently resulted in impaired fertility and developmental abnormalities (McDonough and Romano, 2008). However, adverse reproductive effects have recently been reported by several authors in association with OP insecticide exposures (Sikka and Gurbuz, 2006; Joshi et al., 2007; Peiris-John and Wiskremasinghe, 2008). Chlorpyrifos exposure has resulted in decreased sperm counts and testosterone concentrations as well as testicular degeneration in laboratory animals (Joshi et al., 2007). Similar abnormalities have also been observed in humans after low-level exposures to OP insecticides (Sikka and Gurbuz, 2006; Peiris-John and Wiskremasinghe, 2008). It has been reported that OP insecticides can cause disturbances in the feedback loops within the hypothalamic–pituitary–adrenal axis, thereby affecting the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Sikka and Gurbuz, 2006), and some members of this class of pesticides have even been reported to have anti-androgenic activity (Kitamura et al., 2006). In addition, exposures to OP insecticides can cause oxidative stress, leading to increased apoptosis within the testes (Sikka and Gurbuz, 2006).

As mentioned previously, maternal and/or fetal stress associated with sublethal exposures to OP insecticides could lead to an increased incidence of spontaneous abortions or preterm births in intoxicated pregnant women and animals. Because of lower levels of detoxifying enzymes (i.e., paraoxonase), the fetus appears to be more susceptible to OP intoxication than adults, and developmental neurotoxicity and growth restriction have been associated with low-level prenatal exposures of humans to OP insecticides (Desaiah, 1998; Eskenazi et al., 2008; Peiris-John and Wickremasinghe, 2008). In addition to the maternal and fetal effects, OP insecticides can also have direct toxic effects on the placenta, possibly involving (depending on the species) AChE inhibition within the placental cholinergic system (Pelkonen et al., 2006).

Because the effects of OP insecticides are generally longer lasting and more diverse than those of OP nerve agents, extrapolations between OP insecticides and nerve agents need to take into consideration the toxicokinetic and toxicodynamic differences between these two classes of OP compounds (McDonough and Romano, 2008). Given their frequent occupational use and the duration of the toxic effects of OP insecticides, it is probably more likely that adverse reproductive outcomes will be investigated and observed with exposures to these compounds than with exposures to nerve agents specifically designed for immediate incapacitation without environmental persistence. Depending on the circumstances, acute or low-level exposures to OP nerve agents might not be associated with overt effects on reproductive function, especially when direct toxicant-induced effects on reproductive performance are of secondary importance to neurological and/or psychological concerns and the reproductive endpoints being assessed are relatively insensitive. Duration, route, and amount (i.e., dose) of nerve agent exposure, the developmental period during which exposure occurs, and the reproductive endpoints and animal species being evaluated are factors that will need to be taken into consideration in the design of future studies investigating the direct effects of nerve agents on fertility and embryonic and fetal development.

The Reproductive Toxicity of Environmental Contaminants Resulting from Acts of Terrorism

Not all acts of chemical warfare will necessarily involve weapons specifically designed for that purpose. Especially in instances of terrorism or military attacks involving nongovernmental militias or governments with limited weapons resources, chemical warfare can be low-tech and make use of toxic chemicals in commercially available products or those hazardous materials present at manufacturing, processing, or storage facilities. Ionizing radiation can be released into the environment through strategic military use of nuclear weapons or from nuclear accidents involving municipal power plants, nuclear-powered aircraft carriers, and submarines, or the explosion of misplaced, misused, or stolen nuclear "weapons of mass destruction." In addition, many potential reproductive toxicants, including

pesticides, are routinely used in various manufacturing and agricultural processes. Acts of terrorism or military strikes on industrial or agricultural complexes have the potential to greatly increase the exposure of humans and animals to a large number of different toxicants in the air, water, soil, and food chain.

Depending on the circumstances, both short-term and long-term (i.e., immediate and delayed) morbidity and mortality can be observed in environmental disasters. In these types of catastrophic events, illness and death can be due to the direct effects of toxicants, or they can arise secondarily from other factors associated with the environmental release of xenobiotics. Related conditions, such as famine, can accompany disasters and negatively impact reproductive function in humans and animals. The very real or, in some cases, imagined or exaggerated threats to human and animal welfare from environmental contaminations can be associated with instances of mass fear, panic, and emotional incapacitation. These psychological stresses can affect reproductive function and, especially, gestational length and fetal survival in humans and animals. Emotional responses are naturally heightened with toxic exposures involving pregnant women and children.

Ionizing Radiation

Ionizing radiation can target rapidly dividing cells in multiple organs, including the testes and ovaries, as well as the developing embryo and fetus (Cockerham et al., 2008; Foster and Gray, 2008; Rogers and Kavlock, 2008). Exposure of males to ionizing radiation can result in diminished spermatogenesis and testosterone production by the testes, with increased secretion of LH and FSH by the anterior pituitary (Cockerham et al., 2008). Consistent with these effects, Ukrainian workers involved in the clean-up of radioactive materials after the Chernobyl nuclear accident had increased ultramorphological sperm abnormalities (Fischbein et al., 1997; Cockerham et al., 2008). Similar to the radiation-induced endocrine effects observed in the testes, ovarian steroid production is reduced by exposure to ionizing radiation (Cockerham et al., 2008). Depending on the timing and dose of the radiation exposure, ionizing radiation can cause pubertal failure, ovarian failure, or premature menopause in women. Clusters of Down syndrome cases in Belarus 9 months after the explosion at the Chernobyl nuclear power plant suggest a radiosensitive phase of oogenesis in mammals at the time of ovulation and conception (Zatsepin et al., 2007). High radiation exposure in late-gestational women or pregnant animals has the potential to cause spontaneous abortion or preterm births associated with maternal and/or fetal radiation sickness and stress. Depending on the stage of development and the dose of radiation, exposure of the conceptus, embryo, or fetus to ionizing radiation can result in lethality or morphologic abnormalities (Cockerham et al., 2008), and observations in humans and animals after the Chernobyl incident are consistent with these developmental effects (Østerås et al., 2007; Peterka et al., 2007). In addition, anxiety associated with exposures of pregnant women to ionizing radiation from the Chernobyl nuclear accident reportedly led to increased incidences of spontaneous abortions in several European countries, even in instances when the exposure was minimal (Cordero, 1993).

Pesticides and Other Organic Contaminants

Pesticides and other organic contaminants are ubiquitous in both industrial and agricultural settings. Acts of terrorism have the potential to increase the exposure of humans and animals to these types of xenobiotics in the environment by targeting industrial and agricultural complexes. The massive release of pesticides, in particular, has the potential to be a readily available means of inciting fear and inducing morbidity and mortality in humans and animals. In fact, as mentioned previously, carbamate and OP insecticides have the same basic mechanism of action as the previously discussed AChE-inhibiting nerve agents, and MIC, an intermediate in the production of carbamate insecticides, contains a cyanide moiety.

There have been many well-documented instances of reproductive abnormalities in species of wildlife living in environments contaminated by a wide range of industrial and/or agricultural chemicals (McLaclan, 2001; Hess and Iguchi, 2002; Guillette, 2006; Jobling and Tyler, 2006; McLaclan et al., 2006; Evans, 2007). Wildlife populations are very likely sentinels for exposure to reproductive toxicants because of the contamination of the aquatic habitats in which many of them live and the bioaccumulation of some organic chemicals in predators (Hess and Iguchi, 2002). There is also recent evidence to suggest that domestic animals can act as potential sentinels for human exposure to endocrine disruptors and that hyperthyroidism in cats might be associated with exposure to polybrominated diphenyl ethers (PBDEs) (Dye et al., 2007).

Based on the observations of reproductive toxicity (including endocrine disruption) in wildlife and domestic animals, as well as ongoing concerns about reproductive dysgenesis in human populations and the observed effects of industrial accidents involving MIC and dioxins, there has been increasing interest in the effects of prenatal exposures of humans to suspected endocrine disruptors and other reproductive toxicants. However, when impaired reproductive function is discovered in adults, it is difficult to comment with complete certainty on the relative contributions of prenatal versus postnatal exposures to reproductive toxicants. There is a wide array of pesticides and other organic environmental

contaminants that have the potential to adversely impact reproductive function. Specific epidemiological or laboratory studies suggesting adverse reproductive effects of exposures to these xenobiotics are discussed with respect to observed abnormalities in male and female reproductive function, as well as embryonic and fetal development.

Adverse Effects of Pesticides and Other Organic Contaminants on Male Reproductive Function

"Androgenization" or a state of indeterminate sexual development encompasses both feminization and demasculinization in males and, similar to the testicular dysgenesis syndrome described in humans, has been observed in populations of various vertebrates, including fish, amphibians, reptiles, birds, and mammals (Edwards et al., 2006; Evans, 2007). Adult and immature amphibians exposed to the herbicide atrazine and hatchling, juvenile, and adult male alligators originating from a lake previously contaminated with dichlorodiphenyltrichloroethane (DDT) and other persistent bioaccumulated pesticides have been reported to exhibit varying patterns of androgenization (Hayes et al., 2006; Milnes et al., 2006; Evans, 2007). Although still somewhat controversial, there is evidence to support the observation that sperm counts in men within some industrialized regions of the world have been decreasing over the past several decades (Swan et al., 2000; Jørgensen et al., 2006; Skakkebæk et al., 2006). The findings of epidemiological studies have suggested a relationship between decreased anogenital distance and prenatal exposures of male infants and phthalates used as plasticizers, as well as a correlation between reduced semen quality in men within certain regions of the United States and the metabolites of several economically important herbicides (Swan et al., 2003a,b, 2005). In addition, a recently completed epidemiological study in Italy has demonstrated a significant relationship between postnatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and abnormal semen and endocrine parameters in men (Mocarelli et al., 2008).

The dicarboximide fungicides, vinclozolin and procymidone, and/or their metabolites inhibit the binding of androgens to nuclear androgen receptors and can demasculinize and feminize the prenatally exposed male fetus or induce important alterations in prepubertally or peripubertally exposed offspring (Monosson et al., 1999; Gray et al., 2006; Evans, 2007). While still subject to debate among scientists, vinclozolin has also been reported to be capable of inducing epigenetic modifications that facilitate the occurrence of transgenerational or vertically transmitted reproductive abnormalities (Anway et al., 2005; Anway and Skinner, 2006). Linuron, *p,p′*-DDE, prochloraz, PBDEs, and selected OPs can function as androgen receptor antagonists (Gray et al., 2006; Kitamura et al., 2006), and AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone and disrupt testosterone signal transduction pathways (Jana et al., 1999; Sikka et al., 2005; Mocarelli et al., 2008).

The testes have xenobiotic biotransformation capabilities within both Leydig and Sertoli cells (Thomas and Thomas, 2001). Although many toxicants and/or their metabolites are capable of producing relatively nonspecific effects, such as oxidative stress, there are a number of pesticides and other organic compounds that target specific cell populations within the testes. Several toxicants targeting Sertoli cells, including diethylhexyl phthalate and 2,5-hexanedione (a metabolite of *n*-hexane), have age-specific and species-specific effects (Thomas and Thomas, 2001; Creasy and Foster, 2002; Foster and Gray, 2008). Tri-*o*-cresyl phosphate, an industrial chemical used in lacquers and varnishes that is associated with some organophosphate insecticides, inhibits LH-induced steroidogenesis in the Leydig cells but, after Leydig cell–mediated conversion to its active metabolite, causes morphological abnormalities in Sertoli cells (Thomas and Thomas, 2001; Creasy and Foster, 2002; Evans, 2007).

As mentioned previously with regard to sulfur mustard, a variety of radiomimetic alkylating agents, including busulfan, cyclophosphamide, and nitrogen mustard, can target rapidly dividing mitotic or meiotic germ cells in the testes. In some instances, xenobiotics can target a specific population of germ cell precursors, such as spermatogonia spermatocytes or round or elongate spermatids (Creasy and Foster, 2002; Evans, 2007; Foster and Gray, 2008). TCDD appears to adversely affect several populations of spermatozoal precursors and alters the sex ratio in favor of female offspring (i.e., decreased viability of Y chromosome–bearing sperm) (Thomas and Thomas, 2001; Ishihara et al., 2007; Foster and Gray, 2008; Mocarelli et al., 2008).

Adverse Effects of Pesticides and Other Organic Contaminants on Female Reproductive Function

A wide range of agricultural and industrial chemicals has estrogenic and/or anti-estrogenic activities (Evans, 2007), and some of the synthetic xenobiotics most commonly discussed with respect to these activities include DDT, polychlorinated biphenyls, and TCDD (McLachlan, 2001). Effluents from industrial and agricultural activities have been shown to have androgenic activities and are associated with masculinization of female fish (Orlando et al., 2004; Gray et al., 2006; Evans, 2007).

The effects of toxicants on specific cell types within the ovaries are not as well-understood as they are in the testes (Thomas and Thomas, 2001). Many female reproductive toxicants do not actually target particular cell lines but, rather, disrupt the endocrine milieu of the tubular genitalia or cause changes in ovarian structures

secondary to alterations in the hypothalamic–pituitary–gonadal axis (Yuan and Foley, 2002). Like the testes, the ovaries also have some xenobiotic biotransformation capabilities, and oxidative damage can adversely affect ovarian structure and function (Thomas and Thomas, 2001; Yuan and Foley, 2002). Phthalates and TCDD can delay or decrease ovulations and, like sulfur mustard and ionizing radiation, some of the alkylating agents reported to adversely affect rapidly dividing germ cells within the testes can also adversely affect primordial follicles within the ovary (Thomas and Thomas, 2001; Devine and Hoyer, 2005). Several PAHs (i.e., BaP, 3-methylcholanthrene (3-MC) and DMBA) and 1,3-butadiene appear to target oocytes in preantral follicles, and DMBA can adversely affect antral follicular development (Devine and Hoyer, 2005; Evans, 2007).

Adverse Effects of Pesticides and Other Organic Contaminants on Embryonic/Fetal Development

As has been emphasized previously, the developing fetus undergoing phenotypic sexual differentiation is particularly susceptible to the adverse effects of various agonists and antagonists of estrogen and androgen receptors (Hess and Iguchi, 2002; Evans, 2007). A large number of xenobiotics, including many pesticides (e.g., carbamates, OPs, organochlorines, and pyrethroids) and other potential organic environmental contaminants, have been recognized as potential teratogens in humans and animals (Desaiah, 1998; Evans, 2007; Rogers and Kavlock, 2008). Alkylating agents with radiomimetic activity, such as busulfan, cyclophosphamide, and nitrogen mustard, cause teratogenesis by targeting rapidly replicating cells (Rogers and Kavlock, 2008). TCDD has been found to be teratogenic both in laboratory animals exposed experimentally (Kransler et al., 2007; Aragon et al., 2008) and in humans exposed after an accidental release in Seveso, Italy, in 1976 (Alaluusua et al., 2004). Accidental exposure to MIC in Bhopal, India, in 1984 resulted in a significant increase in spontaneous abortions and neonatal mortality in humans (Varma, 1987; Varma and Mulay, 2006), and these epidemiological data were corroborated by the results of a subsequent rodent experiment (Varma, 1987). In addition, some xenobiotics can cause fetal death and spontaneous abortion by having direct toxic effects on the placenta, rather than on the fetus itself (Pelkonen et al., 2006; Gupta, 2007c).

Heavy Metals

Heavy metals are routinely used in various manufacturing processes and are contained within many products commonly used by humans. Acts of terrorism have the potential to increase the environmental exposure of humans and animals to heavy metals by targeting industrial complexes and sewage treatment facilities. Because a number of heavy metals have the potential to affect different stages of reproductive function by different mechanisms of action, the adverse effects of metals on male and female reproduction and embryonic and fetal development are discussed separately.

Adverse Effects of Heavy Metals on Male Reproductive Function

Excessive cobalt can potentially interfere with normal spermatogenesis and even generalized hypoxia related to increased blood viscosity that affects the testes (Thomas, 1995; Evans, 2007). Chromium and vanadium have also been associated with adverse reproductive effects, and cis-platinum exposure has been associated with the death of spermatocytes and spermatids, as well as disruption of Sertoli cell tight junctions (Thomas, 1995; Thomas and Thomas, 2001; Evans, 2007). Exposure of male laboratory animals to organotin compounds has been associated with reduced testicular size, alterations in testicular morphology, and impaired spermatogenesis (Ema and Hirose, 2006; Evans, 2007).

Lead and cadmium are ubiquitous heavy metals and have been associated with testicular toxicity and impaired fertility in a number of species. Divalent lead is known to interact with physiological processes involving calcium and generally has an affinity for sulfhydryl groups (Evans, 2007). Lead is reported to be toxic to germ cells as well as Leydig cells and can suppress anterior pituitary secretion of LH and FSH (Thomas and Thomas, 2001; Evans, 2007). Lead also appears to be able to adversely affect the ability of spermatozoa to fertilize ova; however, this effect, like others associated with lead exposure, appears to be dependent on age and individual variations in susceptibility, adaptation, and reversibility (Sokol, 2006; Evans, 2007). Like lead, cadmium is thought to adversely affect male reproduction by inhibition of spermiation as well as by interactions with the hypothalamic–pituitary–gonadal axis and adverse effects on the endothelium of the testicular and epididymal vasculature (Thomas, 1995; Creasy and Foster, 2002; Akinloye et al., 2006; Evans, 2007). Cadmium can also alter the junctional complexes between adjacent Sertoli cells and disrupt the integrity of the blood–testis barrier (Thomas and Thomas, 2001; Akinloye et al., 2006; Evans, 2007).

Adverse Effects of Heavy Metals on Female Reproductive Function

The ovaries do not appear to be as sensitive to the toxic effects of heavy metals as do the testes (Thomas, 1995; Evans, 2007). The neuroendocrine function of the hypothalamic–pituitary–gonadal axis appears to be targeted by lead in females as well as in males (Evans, 2007). Anterior pituitary release of FSH and LH and

ovarian steroidogenesis can be inhibited by cadmium (Thomas, 1995; Hoyer, 2006; Evans, 2007). Exposure of female laboratory animals to organotin compounds has been associated with reductions in follicular development and size as well as the formation of corpora lutea, and it has recently been suggested that tributyltin and cadmium might also have estrogenic activities (Ema and Hirose, 2006; Golub, 2006b; Evans, 2007).

Adverse Effects of Heavy Metals on Embryonic/Fetal Development

Several heavy metals have been identified as teratogens and possible abortifacients in humans and animals, and the adverse effects of prenatal lead exposure on the developing nervous systems of both human and laboratory animal species have been well-documented (Rice, 1998; Evans et al., 2003; Rogers and Kavlock, 2008). Prenatal exposure to organotins has been associated with pregnancy loss and impaired ossification in rodents (Ema and Hirose, 2006). The outcomes of an industrial accident in Japan and misuse of contaminated grain in Basra, Iraq, clearly demonstrated the developmental neurotoxicity of organic mercury (i.e., methyl mercury and related compounds) in humans and animals (Cordero, 1993; Chang and Guo, 1998; Golub, 2006b). Other heavy metals, including cadmium, have been associated with placental toxicity as well as developmental neurotoxicity (Hastings and Miller, 1998; Gupta, 2007b), and it has been recently reported that cadmium and other metals or metalloids might also have estrogenic effects (Golub, 2006b).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Reproduction is a critical biological process required for financially viable livestock production as well as long-term survival of human and animal populations. Toxicant-induced spontaneous abortions, congenital defects, and infertility can have devastating effects on humans, domestic animals, and wildlife species. There is growing global concern about all of the potential adverse effects, including those on reproduction, of exposures to CWAs, and other xenobiotics resulting from military or terrorist activities. The information presented in this chapter is intended to familiarize the reader with terminology and concepts pertinent to reproductive toxicity, including endocrine disruption, and to provide an overview of what is understood about the adverse reproductive effects of selected toxicants. It is hoped that the information and references provided in this chapter will assist readers in making informed decisions in their interpretation of experimental or epidemiological data and their preparation for future experiments or clinical investigations.

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CHAPTER

42

Liver Toxicity of Chemical Warfare Agents

Shashi K. Ramaiah and Atrayee Banerjee

INTRODUCTION

Xenobiotic-induced liver injury has become the most frequent cause of acute liver failure in humans in the United States and around the world, exceeding all other causes combined (Watkins and Seef, 2006). Owing to its detoxification mechanisms, the liver protects the individual against xenobiotic-induced injury. Certainly, the liver toxicity caused by chemical warfare agents (CWAs) is a potential area of concern.

Chemical-induced liver injury is encountered in a variety of circumstances. Some natural toxins, such as the peptides of *Amanita phalloides*, the pyrrolizidine alkaloids, the toxin of the cycad nut, and other plant toxins, are hazards posed by the environment. Some mycotoxins are ingested unknowingly because of feed contamination due to climatic conditions that favored fungal growth. Other circumstances of exposure to hepatotoxins include the contamination of water supply with cyanobacterial toxins, which led to the tragic death of 60 patients in a hemodialysis clinic in Brazil in 1996 (Jochimsen et al., 1998).

Research in the last decade has focused on elucidating different mechanisms for chemical-induced liver injury. Investigators have attempted to understand the basis for such hepatic injury. The goal of this chapter is to provide a basic understanding of liver pathophysiology and to introduce the general concepts of liver injury. The chapter also describes a few examples of CWAs that can inflict liver damage.

STRUCTURAL ORGANIZATION OF THE LIVER

The hepatic lobule and hepatic acinus are relatively well accepted models to describe the structure and functional aspects of the liver. Histologically, the *hepatic lobule* is a hexagonal region of the liver parenchyma around the central vein. Typically, six portal triads, consisting of branches from the portal vein and hepatic artery as well as bile ductules, border the edge of the lobule. Cords of hepatocytes are arranged radially around the central vein, and blood sinusoids form between them. The hepatic parenchyma is divided into three zones based on proximity to the central vein. The area closest to the central vein is termed *centrilobular*, the area near the portal triads is *periportal*, and the area between the centrilobular and periportal parenchyma is termed *midzonal*.

Alternatively, *hepatic acinus* is defined as the structural and functional unit in the liver based on the hepatic microcirculation. In simple terms, the hepatic acinus is defined as a parenchymal mass organized around the portal triad. Within the acinus, blood drains from the portal area via the sinusoids into the central hepatic vein. The acinus is arbitrarily divided into zone 1, which corresponds to the periportal zone of the hepatic lobule; zone 2, which corresponds to the midzonal parenchyma; and zone 3, which corresponds to the centrilobular zone. The majority of blood supply to the liver is from the portal vein. Approximately 60–80% of the blood originates from branches of the portal vein and supplies nutrients and toxins from the gastrointestinal tract while 20–40% of the blood originates from the hepatic artery, supplying oxygen (Treinen-Moslen, 2001). The blood from the portal vein and hepatic artery is mixed in the penetrating vessels, which then enter the sinusoids. Blood flows sequentially through zone 1, zone 2, and zone 3 before draining via the central vein. Because of this preferential blood circulation, hepatocytes in zone 1 receive blood that is 9–13% oxygenated, whereas zone 3 is relatively hypoxic—the blood is 4–5% oxygenated and nutrient-depleted (Treinen-Moslen, 2001).

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In addition to hepatic parenchymal cells, which are hepatocytes, hepatocyte stem cells, termed *oval cells*, are reported to be located in the canals of Hering, where bile canaliculi from the hepatic cords converge on bile ductules of the portal triad. It is postulated that new hepatocytes travel down hepatic cords to replace the aging and damaged hepatocytes from zone 3.

Sinusoids lined by specialized endothelium are blood channels located between hepatocyte cords. The endothelial lining of the sinusoids is discontinuous and has fenestrae to facilitate the movement of fluid and molecules less than 259 kDa in size (Watkins, 1999; Treinen-Moslen, 2001; Plumlee, 2004). This material enters the space of Disse, which is located between the endothelium and the hepatocytes. Within the space of Disse, hepatocytes contact free and protein-bound molecules, which may be absorbed by diffusion or active transport.

Hepatic Functional Capacity

The liver contributes to a plethora of functions. Liver filters the blood drained from the gastrointestinal tract via the portal vein for xenobiotics, endotoxins, ammonia, and other bacteria-derived products (Treinen-Moslen, 2001; Plumlee, 2004). The liver is directly involved in glucose homeostasis (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004); cholesterol synthesis and uptake (Treinen-Moslen, 2001); synthesis of proteins such as clotting factors, albumin, and very-low-density lipoprotein (Treinen-Moslen, 2001; Plumlee, 2004); storage of glycogen, lipids, minerals, and vitamins (Plumlee, 2004); metabolism and excretion of hemoglobin breakdown products (Plumlee, 2004); steroid hormones (Brown, 2001); and drug metabolites.

The hepatic zones have remarkable regiospecificity and metabolic diversity to accommodate the numerous functions of the hepatocytes. In addition to differences in oxygen gradients (zone 3 hepatocytes are oxygen-depleted compared to zone 1 hepatocytes), hepatocytes of zone 3 in particular are rich in drug-metabolizing enzymes. Zone 3 hepatocytes are involved in glycolysis and lipogenesis (Plumlee, 2004), and zone 1 hepatocytes are mitochondria-rich (Treinen-Moslen, 2001; Plumlee, 2004). Functions of zone 1 hepatocytes include bile salt extraction, fatty acid oxidation, gluconeogenesis, and protein synthesis (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). Zone 1 hepatocytes have the highest levels of glutathione (GSH; Treinen-Moslen, 2001).

Bile secretion is a major function of the liver. Bile is composed of bile salts, bilirubin, GSH, phospholipids, cholesterol, proteins, organic anions, metals, and conjugated xenobiotics (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Bile salts and bilirubin enter bile canaliculi via active transport through hepatocyte membranes. Canaliculi are dynamic structures located between hepatocytes and formed by hepatocyte membranes (Treinen-Moslen, 2001; Plumlee, 2004). Energy-dependent transport exists for certain hormones, drugs, and other xenobiotics. These include a group of multiple drug resistance p-glycoproteins that transport lipophilic cationic drugs, estrogens, phospholipids, and canalicular multiple organic anion transporters involved in the movement of molecules conjugated to GSH, glucuronide, and sulfate. Metal and mineral transport is important for mineral homeostasis and occurs through facilitated diffusion and receptor-mediated endocytosis across the sinusoidal membrane. Lysosomes are involved in storage and export of metals and minerals into canaliculi.

Canaliculi enter canals of Hering in the portal triad and lead to intrahepatic bile ducts, which coalesce to form the hepatic bile duct. The bile duct empties the bile into the gallbladder, which then is released into the duodenum. Bile that is excreted into the small intestine enhances nutrient uptake, protects enterocytes from oxidation, and facilitates the excretion of xenobiotics and endogenous waste in the feces (Treinen-Moslen, 2001).

Hepatic Cellular Components

In addition to the hepatocytes and the hepatocytic stem cells (oval cells), which are parenchymal in origin, there are four types of nonparenchymal cells present within the liver. The nonparenchymal cells include the endothelial cells lining the sinusoids; bile duct epithelium; Kupffer cells, which are resident macrophages; the *Stellate* cells, also called *Ito cells* or *fat-storing cells*; and the pit cells or large granular lymphocytes. In the rat, hepatocytes represent about 60% of the total cell number and 80% of hepatic tissue volume. Nonparenchymal cells in the rat are estimated to constitute about 30% of total cellular population, but comprise only 6–7% of tissue volume due to their small size relative to hepatocytes (Dahm and Jones, 1996).

Kupffer cells represent 80% of the fixed macrophages in the body. These cells are mostly located within the sinusoidal lumina in close association with endothelial cells. Kupffer cells function as phagocytes, ingesting foreign material which may arrive through the portal circulation (Treinen-Moslen, 2001; Plumlee, 2004), as well as apoptotic or necrotic hepatocytes. Kupffer cells have other immune functions in that they act as antigen-presenting cells and secrete various cytokines. Kupffer cells may store minerals and are also involved in the pathogenesis of a variety of liver diseases induced by toxins such as ethanol (Laskin, 1990; Thurman et al., 1998).

Stellate cells are located within the sinusoids and store fat and vitamin A (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). In the event

of liver injury, stellate cells may become activated to a myofibroblast-like phenotype (Plumlee, 2004; Maddrey, 2005). Activated stellate cells produce collagen and play a role in the pathogenesis of hepatic fibrosis.

Pit cells are natural killer cells that have anti-neoplastic actions (Treinen-Moslen, 2001; Plumlee, 2004). They are also involved in granuloma formation (Plumlee, 2004).

FACTORS INFLUENCING HEPATIC TOXICITY

Preferential Hepatic Uptake

As mentioned previously, the liver has a dual blood supply. The hepatic artery delivers material from the systemic circulation and the portal blood flow delivers directly from the gastrointestinal system. The portal system is involved in the *first pass effect*, where the nutrients and xenobiotics that are absorbed from the stomach and intestines are filtered through the liver before reaching the systemic circulation (Treinen-Moslen, 2001). The space of Disse allows close contact between circulating plasma, plasma proteins, and hepatocytes, allowing rapid diffusion of lipophilic compounds across the hepatocyte membrane. Some compounds are specifically taken up by sinusoidal transporters, including phalloidin from several species of mushrooms in the genus *Amanita*, microcystin produced by the cyanobacteria *Microcystis aeruginosa*, and bile acids.

Liver cells have the potential to accumulate high levels of metals and vitamins, which can lead to toxic injury. Excessive vitamin A storage in stellate cells acutely leads to the activation and proliferation of these cells (Treinen-Moslen, 2001), while chronic high levels can lead to hepatic fibrosis and portal hypertension, precipitating increased fibrosis (Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004; Maddrey, 2005). The liver is also responsible for iron homeostasis. There is a receptor mediated uptake of iron from the sinusoids and sequestration in storage proteins such as ferritin. High levels of iron cause lipid peroxidation of zone 1 hepatocytes (Treinen-Moslen, 2001). A common example of hepatic accumulation of metals is copper-mediated liver toxicity noted in certain breeds of dogs (e.g., Bedlington terriers, Dobermans, and Dalmatians) where copper is stored within lysosomes of hepatocytes, resulting in progressive accumulation of copper resulting in liver necrosis (Rolfe and Twedt, 1995).

Xenobiotic Metabolic Bioactivation

Most xenobiotic agents absorbed by the small intestine are highly lipophilic. Renal excretion is the primary mechanism of xenobiotic removal, but kidney excretion of lipophilic compounds, which are frequently protein-bound in the circulation, is poor (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins, 1999). Such lipophilic compounds must be metabolized to increase their water solubility for excretion (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman, 1999). Microsomal enzymes within the liver add functional groups or conjugate xenobiotics to water-soluble molecules to facilitate excretion. While these reactions often function in the detoxification of compounds, there is significant potential for toxification (Zimmerman, 1999). Examples of phase I reactions include oxidation, reduction, and hydrolysis. Phase I enzymes, which are predominantly located in zone 3 of the hepatic lobule, may produce reactive metabolites.

Many hepatic enzymes are present in the smooth endoplasmic reticulum of the hepatocyte. When liver tissue is homogenized, the endoplasmic reticulum breaks down into small vesicles known as *microsomes*; thus, these enzymes are termed *microsomal enzymes*. As a rule, microsomal enzymes require oxygen and reduced nicotinamide adenine dinucleotide (NADPH) to function (Dahm and Jones, 1996; Brown, 2001). Most phase I enzymes contain heme, giving them a red coloration, and they absorb light at a wavelength of 450 nm. Most cytochrome P450s act as mixed function oxidases. Genes for cytochrome P450s are highly conserved in mammals. There are three gene families, CYP1, CYP2, and CYP3, and more than 36 cytochrome P450 isoenzymes have been identified in animals (Dahm and Jones, 1996; Watkins, 1999).

Oxidation is the major phase I reaction produced by the group of cytochrome P450s. Important substrates for CYP450s include steroid hormones and lipid-soluble drugs (Brown, 2001). Oxidative reactions frequently lead to the formation of highly reactive epoxides. These toxic metabolites are usually detoxified rapidly by phase II conjugation or other mechanisms, such as microsomal epoxide hydrolases (Watkins, 1999; Piñeiro-Carrero and Piñeiro, 2004).

Non-cytochrome P450 enzymes may also be involved in oxidative reactions. One such enzyme is alcohol dehydrogenase, whose substrates include vitamin A, ethanol, and ethylene glycol. Aldehyde dehydrogenase is another enzyme. Most reduction reactions also involve microsomal enzymes, with the exception of ketone reduction. Nitro compounds are reduced to amines, and volatile anesthetics undergo dehalogenation by microsomal enzymes. Hydrolysis reactions are involved in the metabolism of compounds with amide bonds or ester linkages, as in the conversion of aspirin to salicylate (Brown, 2001).

Phase II/Conjugation Reactions

Phase II enzymes may be cytosolic or microsomal (Dahm and Jones, 1996; Brown, 2001). Phase II enzymes

are predominantly involved in conjugating phase I metabolites or xenobiotics with functional groups. Phase II metabolites are rarely reactive, but there are a few exceptions, such as the glucuronide of the nonsteroidal anti-inflammatory drug diclofenac and the GSH conjugate of α-naphthothiourea (ANTU). Phase II enzymes conjugate a polar group to the substrate at a hydroxyl group, carboxyl group, amino group, or sulfhydryl group produced through the actions of phase I microsomal enzymes. Polar molecules that are added to the substrate include glucuronic acid, sulfate derived from sulfuric acid ester, acetate, GSH, methyl groups derived from methionine, and amino acids such as glycine and cysteine. These polar groups significantly increase water solubility of the substrate facilitating rapid renal or biliary excretion.

Glucuronidation is the most common phase II reaction in humans, though it is deficient in the neonate (Sturgill and Lambert, 1997; Brown, 2001; Piñeiro-Carrero and Piñeiro, 2004). Substrates for glucuronidation usually include steroid hormones, thyroxin, and bilirubin, as well as many drugs, including salicylates and acetaminophen. Glucuronyl transferases are microsomal enzymes that catalyze the transfer of glucuronide from uridine 5′-diphosphate (UDP; Watkins, 1999). UDP may be depleted in patients overdosed with acetaminophen or other drugs that undergo this detoxification pathway. Products of glucuronidation may be excreted in the bile or urine. Those excreted in the bile may undergo hydrolysis in the intestine, which leads to reabsorption of the parent compound in a phenomenon called *enterohepatic cycling* (Brown, 2001). Similar to CYP 450 enzymes, some agents can also induce glucuronyl transferases, such as phenobarbital (Sturgill and Lambert, 1997).

Sulfation is the primary conjugation reaction for substrates with phenol groups or aliphatic alcohols (Sturgill and Lambert, 1997; Brown, 2001). These reactions are catalyzed by sulfotransferases in the cytoplasm. Agents that undergo sulfation include acetaminophen, morphine, ascorbic acid, and endogenous compounds like chondroitin, heparin, and some steroids. The pool of available sulfates may become saturated in drug overdoses.

Drugs with amine and hydrazine groups may be conjugated to acetate (Sturgill and Lambert, 1997). Sulfonamides often undergo acetylation (Brown, 2001). *N*-acetyltransferase is an enzyme in the cytoplasm involved in acetylation reactions.

GSH and cysteine both have sulfhydryl groups that readily bind many phase I metabolites (Brown, 2001). GSH is a free-radical scavenger that prevents membrane damage from reactive metabolites. These reactions may be spontaneous or catalyzed by GSH peroxidases, which are selenium-dependent enzymes. Because these enzymes are cytosolic, damaged membrane phospholipids must be released by phospholipase A2 for detoxification. GSH is also involved in the reduction and recycling of other antioxidants, such as vitamins E and C (Dahm and Jones, 1996). When oxidized, GSH forms a dimer, which must be reduced by GSH reductases, which are NADPH-dependent enzymes. GSH may be depleted in the acetaminophen-overdosed or fasting person (Dahm and Jones,1996; Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004). *N*-acetylcysteine is frequently used to replenish GSH.

Phase III Reactions

In addition to phase I and II biotransformation enzymes, studies suggest the involvement of hepatic transporter systems involved in drug efflux from hepatocytes as a means for the liver to rid itself of foreign chemicals. These are termed *phase III transporter systems*. Several transporter families that mediate uptake of chemicals into liver and excretion of chemicals from liver into blood, bile, or both have been cloned and identified. In general, the organic anion transporting polypeptide family (Oatps), along with organic cation transporter 1 (Oct1) and organic anion transporter 2, mediate the uptake of a large number of xenobiotics from the blood to the liver. Conversely, multidrug resistance proteins (Mdrps), multidrug resistance-associated proteins (Mrps), and breast cancer resistance protein (Bcrps) mediate the efflux of xenobiotics from the liver into bile or blood (Klaassen and Slitt, 2005).

Pathologic Manifestations of Hepatic Injury

Hepatic Steatosis/Fatty Liver

Hepatic steatosis is the accumulation of fat droplets within the hepatocytes. Steatosis is usually a common response noted with a variety of liver toxicants and represents a potentially reversible injury to hepatocytes (Treinen-Moslen, 2001). Grossly, the affected liver will be swollen, with rounded edges, friable, and light brown to yellow in color. Compounds that produce prominent steatosis include the antiepileptic drug valproic acid and the antiviral agent fialuridine. Other toxins that may cause hepatic steatosis include aflatoxin and white or yellow phosphorus. Although steatosis has been considered benign and reversible, there are recent reports that suggest the progression of the steatosis stage to steatohepatitis, fibrosis, and cirrhosis (Ramaiah et al., 2004). Recently, there is a syndrome noted in obese individuals, who are often type 2 diabetics, called nonalcoholic fatty liver disease (NAFLD), where hepatocytes are markedly steatotic and there is a marked inflammatory component (Diehl, 2005). Other disorders that result in fatty livers include hepatotoxic chemicals such as thioacetamide,

ethanol, and carbon tetrachloride. It should be noted, however, that several endocrine abnormalities result in steatosis, thus assigning the cause to a specific etiology should be done with caution.

Steatosis is termed *microvesicular* if the fat droplets are small and do not completely displace the nucleus. Microvesicular steatosis likely indicates a slow lipid accumulation (Bastianello et al., 1987; Plumlee, 2004) and may indicate a deficiency in mitochondrial β-oxidation of fatty acids. It is a relatively severe form of steatosis and has been associated with certain toxins, including aflatoxin in primates and dogs (Bastianello et al., 1987; Zimmerman, 1999) and valproic acid in humans (Sturgill and Lambert, 1997; Zimmerman, 1999). In contrast, macrovesicular steatosis describes hepatocytes containing large, usually single fat droplets that displace the hepatocyte nucleus to the periphery of the cytoplasm. Macrovesicular steatosis indicates an imbalance between fatty acid uptake and secretion of very-low-density lipoproteins. This may be due to increased triglyceride mobilization, decreased fatty acid oxidation, decreased synthesis of very-low-density lipoproteins, or other metabolic anomalies (Sturgill and Lambert, 1997; Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004).

Steatohepatitis

Steatohepatitis is the accumulation of lipids and the presence of inflammatory cells within hepatic parenchyma. Steatohepatitis is usually the next stage of steatosis if untreated (Lieber, 1994; Bautista, 2002; French, 2003). The inflammatory cells are usually neutrophils and macrophages. Conditions usually associated with steatohepatitis are alcoholic liver disease, NAFLD, and endotoxemia secondary to intestinal disease. Any toxic compounds that cause steatosis can also result in steatohepatitis if the condition is left untreated. Steatohepatitis may progress to fibrosis/cirrhosis and hepatocellular carcinoma if the inciting cause is not removed or treated (Diehl, 2002).

Apoptosis Versus Necrosis

Two forms of cell death are described within hepatocytes: apoptosis and necrosis. As with other organs, apoptosis is often called *programmed cell death* and is a normal physiologic process. Individual cells are affected (Dahm and Jones, 1996), cell death is not associated with inflammation, and normal architecture of the hepatic parenchyma is maintained, allowing regeneration (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Apoptotic cells undergo cell shrinkage and nuclear condensation and pyknosis, but mitochondrial function (Piñeiro-Carrero and Piñeiro, 2004) is maintained and the cell membrane remains intact (Zimmerman, 1999). Apoptotic cells are occasionally seen in the centrilobular area but are rapidly phagocytosed by macrophages and other hepatocytes (Plumlee, 2004). Apoptosis may be induced by xenobiotics due to oxidative stress (Piñeiro-Carrero and Piñeiro, 2004), decrease in apoptotic suppressors, or enhanced expression of apoptosis genes (Dahm and Jones, 1996).

Necrosis is the predominant form of cell death in most hepatotoxic insults. The term *necrotic* is used to describe dead and dying cells, which are often identified by homogeneous eosinophilic cytoplasm on hematoxic and eosin stained liver sections with variable loss of nuclear and cellular detail. Degenerative changes to the hepatocyte may precede necrosis. During necrosis, cells lose osmotic homeostasis and there is swelling of hepatocytes and organelles on an ultrastructural basis (Dahm and Jones, 1996; Treinen-Moslen, 2001), both of which can be observed microscopically. Energy production fails due to loss of calcium homeostasis (Dahm and Jones, 1996; Zimmerman, 1999). Eventually, the cell membrane ruptures, and leakage of cell contents occurs.

Necrosis is often initiated by damage to membranes, either the plasma membrane of the cell or the membranes of organelles, particularly mitochondria (Zimmerman, 1999). Cell membrane damage is often caused by membrane phospholipid peroxidation. Plasma membrane damage interferes with ion regulation, calcium homeostasis, energy production, and decrease in the ability of that organelle to sequester calcium. Inhibition of protein synthesis is an alternative mechanism that may cause cell necrosis. Toxins that act in this way include phalloidin and related mushroom toxins, which inhibit the action of ribonucleic acid (RNA) polymerase, and therefore mRNA synthesis (Piñeiro-Carrero and Piñeiro, 2004).

Hepatic Pigment Accumulation

Various substances may accumulate within hepatocytes or Kupffer cells. These substances may be visible by microscopy as pigment. Occasionally, these pigments lend a grossly visible tint to the liver. Bile pigment may accumulate in canaliculi and bile ducts, particularly in zone 3, leading to a yellow to green color (Plumlee, 2004; Zimmerman, 1999). Iron, in the form of hemosiderin, is stored in the liver as a yellow-brown pigment that may be visualized using Pearl's Prussian blue. Copper may be yellow-brown and is visualized using rhodenase. Lipofuscin may be present within hepatocytes as a senile change. This yellow-brown pigment represents lipid accumulation within lysosomes.

Hepatic Cholestasis

Cholestasis may be transient or chronic (Treinen-Moslen, 2001) and may be subdivided into canalicular cholestasis and cholangiodestructive cholestasis. Canalicular cholestasis can be produced by drugs or other chemicals that damage the bile canalicular structure

and function. A key component of bile secretion involves several adenosine triphosphate (ATP)–dependent export pumps, such as the canalicular bile salt transporter, that moves bile salts and other transporters that export bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some of the drugs bind these transporter molecules, resulting in the arrest of bile formation or movement within the lumen of the canalicular system (Klaassen and Slitt, 2005). Secondary bile injury can result if there is cholestasis due to the detergent action of bile salts on the biliary epithelium or hepatocytes in areas of cholestasis. Cholestasis can occur simply as a result of physical obstruction of canaliculi within the liver parenchyma (intrahepatic) or outside the liver (extrahepatic). Causes of cholestasis may include hepatobiliary tumors, endotoxemia, hepatocyte swelling, and intraductal crystals such as calcium salts of plant saponins. Disruption of actin filaments within the hepatocyte may cause cholestasis by preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments such as phalloidin, those that affect cytoskeletal assembly such as microcystin, and those that affect calcium homeostasis and cellular energy production can generate this type of energy.

Cholangiodestructive cholestasis is caused by bile duct obstruction, which may be intrahepatic or extrahepatic. Bile duct injury may lead to the sloughing of epithelial cells into the lumen, cell edema, and inflammation, which may contribute to obstruction (Treinen-Moslen, 2001; Plumlee, 2004). Chronic lesions associated with cholangiodestructive cholestasis typically include bile duct proliferation and periductular fibrosis. Vanishing bile duct syndrome, characterized by a loss of bile ducts, has been seen in chronic cholestatic disease in humans (Zimmerman, 1999; Treinen-Moslen, 2001) and has been produced experimentally in dogs (Uchida et al., 1989).

Hepatic Fibrosis/Cirrhosis

Fibrosis usually results from chronic inflammation, which can be the result of continuous exposure to a variety of hepatotoxic chemicals, such as organic arsenicals, vinyl chloride, or high doses of vitamin A (Zimmerman, 1999), chronic ethanol ingestion, and NAFLD. Fibrosis usually occurs around the portal area, in the space of Disse, and around the central veins. This results in loss of liver architecture and function. The hepatocytes are replaced with fibrous material, and thus there is hepatocyte loss. Periportal fibrosis may lead to portal hypertension.

Cirrhosis

Hepatic cirrhosis is typically the end stage of liver disease. Cirrhosis describes an irreversible change (Treinen-Moslen, 2001) characterized by accumulation of excessive collagen deposition in the form of bridging fibrosis, which disrupts the hepatic architecture. Cirrhosis may be micronodular or macronodular, depending on the amount of fibrosis and tissue regeneration. Liver transplantation is the only solution to restore adequate liver function in human medicine.

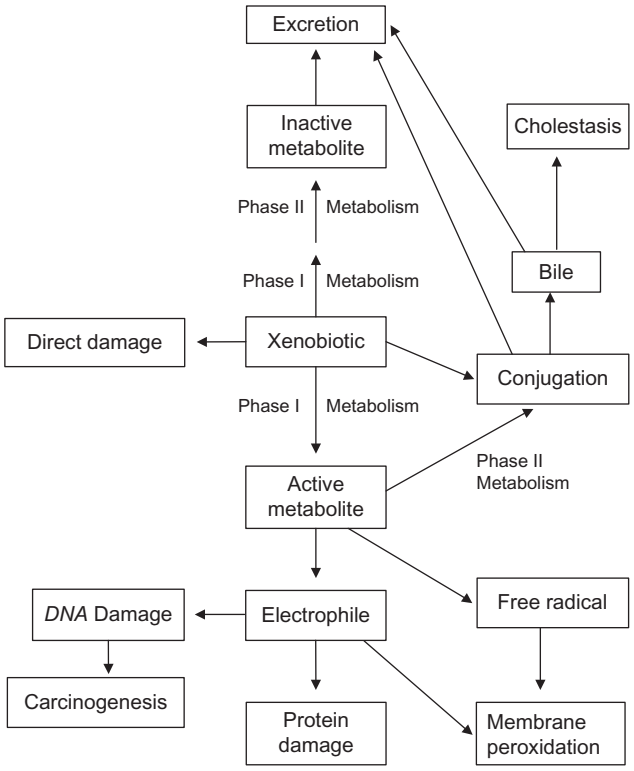
Pathomechanisms of Hepatic Injury

Mechanisms of liver injury have been divided into two categories: intrinsic and idiosyncratic. Intrinsic injury may lead to steatosis, necrosis, cholestasis, or a mixed form of damage, often with minimal inflammation (Sturgill and Lambert, 1997). Intrinsic liver injury is a predictable, reproducible, dose-dependent reaction to a toxicant (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004). A threshold dose exists for xenobiotics causing intrinsic liver injury. There is commonly a predictable latent period between the time of exposure and clinical evidence of liver injury. This type of liver injury accounts for the vast majority of toxic liver injury and is often caused by reactive products of xenobiotic metabolism, most commonly electrophiles and free radicals. A few drugs cause intrinsic liver injury without bioactivation. An abbreviated summary of mechanisms of intrinsic liver injury is illustrated in Figure 42.1.

Idiosyncratic responses are, by contrast, unpredictable responses to a drug or other toxicant. They are rare, non-dose-dependent, and often associated with extrahepatic changes (Sturgill and Lambert, 1997; Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004; Shenton et al., 2004). Idiosyncratic drug reactions often occur after sensitization, followed by reexposure to a drug. There is a usually a delay of 1–5 weeks (and occasionally several months) between the time of the first dosing and the time that clinical signs become evident, but onset is expedited with rechallenge (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins, 1999). Hepatic changes associated with idiosyncratic drug reactions include necrosis, cholestasis, or both; and there is often an inflammatory response involving macrophages and eosinophils. Extrahepatic clinical signs may include pyrexia, rash, and peripheral eosinophilia. Some idiosyncratic drug reactions resemble serum sickness. Some of the mechanisms of liver injury are described in more detail next.

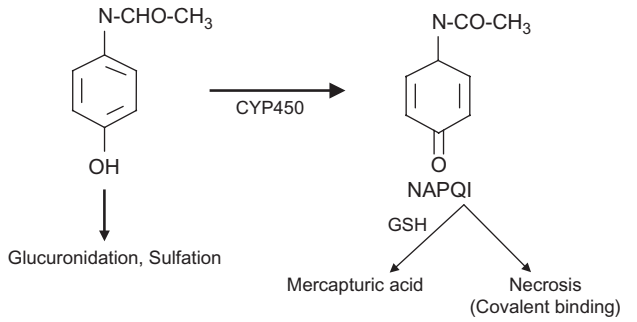
Oxidative Stress and Free Radicals with Classic Examples

Free radicals are generated within hepatocytes in several ways, such as ionizing radiation, oxidative metabolism by cytochrome P450, reduction and oxidation (redox) reactions that occur during normal metabolism, transition metals such as iron and copper, and from



Source: Adapted from Gupta, R. (Ed.), 2007. Veterinary Toxicology, first ed. Academic Press, p. 158.

FIGURE 42.1 Multiple metabolic pathways involved in the mediation of hepatic injury for any compound. The liver is central to the metabolism of xenobiotic (and some endogenous) compounds, which produces water-soluble products amenable to urinary or biliary excretion. Some compounds undergo metabolic activation to produce free radicals, electrophiles, or other toxic products that may induce hepatic injury.



Source: Adapted from Gupta, R. (Ed.), 2007. Veterinary Toxicology, first ed. Academic Press, p. 155.

FIGURE 42.2 Metabolism and mechanism of acetaminophen toxicity. Bioactivation of acetaminophen by P450 enzymes results in the formation of the reactive intermediate (NAPQI), which forms covalent protein adducts with GSH, which then is converted to mercapturic acid. When the amount of the reactive metabolite formed exceeds the GSH available for binding, the excess metabolite binds to tissue molecules, resulting in centrilobular hepatic necrosis.

nitric oxide generated by a variety of inflammatory cells. The reactive species that are generated result in lipid peroxidation of membranes, oxidative modification of proteins, and lesions within DNA (Crawford, 1999).

Free radicals have unpaired electrons, making them highly reactive. They may be formed by one electron oxidation or reduction reactions, leading to cationic or anionic radicals, respectively (Dahm and Jones, 1996). Alternatively, hemolytic bond scission leads to neutral radical formation. Oxygen free radicals result from metabolic processes, leukocytic respiratory burst, or the effects of ionizing radiation. Hydroxyl radicals, superoxide radicals, and hydrogen peroxide are major reactive oxygen species. The free radical nitric oxide (NO), an important cell-signaling agent released by leukocytes, may react with superoxides to form peroxynitrite.

Free radicals cause peroxidation of phospholipids within the plasma membrane of the cell, as well as the membranes of the mitochondria and endoplasmic reticulum. The radicals act by removing a proton (H^+) from a methylene carbon within a polyunsaturated fatty acid, forming a lipid free radical. This lipid free radical then may abstract a proton from a neighboring polyunsaturated fatty acid, generating more lipid free radicals. It is estimated that this can occur 4–10 times per initiation. Effects of lipid peroxy radicals on the cell membrane include increased permeability, decreased fluidity, and inactivation of membrane proteins (Dahm and Jones, 1996). Additionally, mitochondrial membranes lose polarity (Watkins, 1999).

Lipid peroxy radicals can react with metal ions stored within the hepatocyte, generating more lipid radicals. It is estimated that propagation by this mechanism can occur in 4–10 steps per initiation (Dahm and Jones, 1996). The most frequent mechanism of free radical production leading to hepatocellular injury involves phase I metabolism of xenobiotics and the cytochrome P450 system. Phase I metabolism may lead to bioactivation of the substrate to a high-energy reactive intermediate molecule in preparation for phase II conjugation reactions. However, in circumstances such as overdosage, phase I products may accumulate. Lesions produced by these compounds are mostly centrilobular because the cytochrome P450s responsible for metabolism are mostly situated in the centrilobular areas of the liver.

The classic examples of this process are cell death resulting from carbon tetrachloride and acetaminophen toxicosis (Figure 42.2). Acetaminophen has a hydroxyl group that can undergo immediate phase II conjugation reactions. Indeed, at therapeutic doses, 90% of this substrate undergoes glucuronide or sulfate conjugation in humans (Court and Greenblatt, 1997; Sturgill and Lambert, 1997). These are major metabolic pathways in most species, but the glucuronyl transferase

deficiency in cats in part explains the sensitivity of felines to this drug.

Acetaminophen in itself is not considered toxic. Cellular injury is caused by the unstable metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI; Figure 42.2). Under normal conditions in humans, 5% of a dose of acetaminophen is oxidized to NAPQI, which is rapidly neutralized by conjugation with GSH (Sturgill and Lambert, 1997; Maddrey, 2005; Figure 42.2). Toxic levels of NAPQI may accumulate under certain conditions, as when large amounts of substrate are available for metabolism due to either large ingestions or inadequate glucuronidation. Metabolism of acetaminophen to NAPQI is increased in individuals who regularly consume alcohol or take medications that induce microsomal enzymes (e.g., antiepileptic therapy). The hepatic pool of GSH becomes overwhelmed and depleted, permitting the accumulation of NAPQI. Possible additional risk factors that lower the threshold for hepatotoxicity have been identified; they include fasting and malnutrition, which deplete GSH reserves (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004).

The reaction that produces NAPQI generates superoxide anions as a by-product. Interactions of NAPQI with other cellular molecules also generate reactive oxygen species, leading to oxidative stress on hepatocytes (Dahm and Jones, 1996; Zimmerman, 1999). The role of calcium and Kupffer cell activation have been implicated as contributing factors for acetaminophen-induced liver injury by producing reactive nitrogen species (Treinen-Moslen, 2001).

NAPQI also acts as an electrophile, targeting the mitochondria in particular. This reactive metabolite forms covalent adducts with cellular molecules, particularly proteins with thiol groups. Other targets in addition to mitochondrial proteins include plasma membrane proteins involved in calcium homeostasis and adenine nucleotides (Dahm and Jones, 1996; Sturgill and Lambert, 1997).

Disruption of Calcium Homeostasis

Calcium ions (Ca^{2+}) are important for the mediation of hepatic injury. Cytosolic-free calcium is maintained at relatively low concentrations compared to the extracellular levels. The majority of intracellular calcium is sequestered within the mitochondria and endoplasmic reticulum. Membrane associated calcium and magnesium ATPases are responsible for maintaining the calcium gradient (Farrell et al., 1990). Significant and persistent increases in the intracellular calcium result from nonspecific increases in permeability of the plasma membrane, mitochondrial membranes, and membranes of the smooth endoplasmic reticulum. Calcium pumps in the mitochondrial membrane require NADPH; thus, depletion of available NADPH can cause the release of calcium from mitochondria (Cullen, 2005).

Elevated cytoplasmic calcium activates a variety of enzymes, with membrane-damaging effects. The major enzymes that are involved in activation by calcium include ATPases, phospholipases, proteases, and endonucleases. Thus, increased calcium causes increased mitochondrial permeability and induction of apoptosis and necrosis. Calcium is required for maintenance and function of the cytoskeleton as well (Dahm and Jones, 1996; Delgado-Coello et al., 2006).

Although cell injury results in increased calcium, which causes a variety of damaging effects, the cause-and-effect relationship of calcium to cell damage is not known. The chemicals that cause liver damage by this mechanism include quinines, peroxides, acetaminophen, iron, and cadmium.

Inhibition of Mitochondrial Function

Mitochondria function in the process of producing energy, in the form of ATP, for the cell by oxidative phosphorylation. Hepatocytes are highly metabolically active and require a continuous supply of ATP. Hepatocytes active in detoxification or replacement of damaged tissue have even greater ATP requirements (Dahm and Jones, 1996). Compounds that may disrupt mitochondrial oxidative phosphorylation include bile acids and amiodarone. Mitochondria are also critical to modulation of cell redox status, osmotic regulation, pH control, and cytosolic calcium homeostasis and cell signaling. Mitochondria are important targets for virtually all types of injurious stimuli, including hypoxia and toxins. Mitochondria are targeted by oxidants, electrophiles, lipophilic cations, and weak acids. Damage is often precipitated by increases in cytosolic calcium.

Hepatic injury is frequently accompanied by morphological changes in mitochondria. Mitochondrial changes evident as structural abnormalities include greatly increased size and the development of crystalline inclusions. These changes are usually regarded as pathologic, reflecting as either a protective or degenerative response to injury. Mitochondrial damage may result in the formation of high-conductance channels (the so-called mitochondrial permeability transition) in the inner mitochondrial membrane. This is an irreversible change, and because membrane potential is critical for mitochondrial oxidative phosphorylation, it constitutes a death blow to the cell.

Oxidative phosphorylation produces reactive oxygen species (Watkins, 1999). These are deactivated by antioxidants present within the mitochondrion. GSH is present within mitochondria as a scavenger for peroxides and electrophiles. Synthesis of GSH requires ATP

and takes place outside the mitochondrion. A greater than 90% depletion in GSH reserves decreases the ability of the mitochondrion to detoxify reactive oxygen species produced by oxidative phosphorylation. GSH *S*-transferase, the enzyme required for recycling of GSH, may become overwhelmed by toxicants and reactive metabolites (Dahm and Jones, 1996).

Xenobiotics may cause cell death by their effects on mitochondrial DNA. Some antiviral dideoxynucleoside analogs can disrupt mitochondrial DNA synthesis through the inhibition of DNA polymerase gammas, leading to depletion of mitochondria and consequent hepatocyte death.

Chemicals that damage mitochondrial structure, enzymes, or DNA synthesis can disrupt beta oxidation of lipids and oxidative energy production within hepatocytes. Prolonged interruption of beta oxidation leads to microvesicular steatosis, which can progress to macrovesicular steatosis. This sequence of events has been noted with both alcoholic and nonalcoholic steatohepatitis. The role of mitochondria has been extensively studied with NAFLD, a major issue in human medicine. Alcoholic steatosis and other forms of hepatic steatosis have been linked to impairment of ATP homeostasis, and mitochondrial abnormalities have been reported in a growing body of literature.

There are several drugs that inhibit beta oxidation of fatty acids in mitochondria leading to lipid accumulation, such as aspirin, valproic acid, and tetracyclines.

Disruption of Cytoskeleton

Changes in intracellular calcium homeostasis produced by active metabolites of xenobiotics may cause disruption of the dynamic cytoskeleton. There are a few toxins that cause disruption of the cytoskeleton through mechanisms independent of biotransformation. Microcystin is one of these toxins. Microcystin is produced by the cyanobacterium *M. aeruginosa*. Similar toxins are produced by other species of cyanobacteria. The hepatocyte is the specific target of microcystin, which enters the cell through a bile-acid transporter. Microcystin covalently binds to serine/threonine protein phosphatase, leading to the hyperphosphorylation of cytoskeletal proteins and deformation of the cytoskeleton (Treinen-Moslen, 2001).

Phalloidin and related toxins found in some mushrooms, including *A. phalloides*, act by binding tightly to actin filaments and preventing cytoskeletal disassembly (Treinen-Moslen, 2001).

Cholestatic Mechanisms

Sinusoidal transporters and canalicular transporters are involved in the movement of bile salts from the sinusoids into the canaliculi. Within the hepatocyte, transcytosis is mediated by cytoskeletal transport mechanisms. Bile is moved within the canaliculi through actions of the hepatocyte cytoskeleton, causing contraction of the canalicular lumina (Treinen-Moslen, 2001). Xenobiotics acting on any of the above systems may influence bile transport and secretion.

Most chemicals that cause cholestasis are excreted in the bile, including the mycotoxin sporodesmin, which concentrates 100-fold in the bile (Treinen-Moslen, 2001). α-naphthylisothiocyanate (ANIT) is a hepatotoxicant that damages bile duct epithelium and hepatocytes. The drug is used experimentally in rodents as a model of intrahepatic cholestasis. A single dose of ANIT induces acute cholangitis; prolonged exposure causes bile duct hyperplasia and biliary fibrosis. Although the biochemical and histological features of ANIT toxicity are well documented, the mechanism by which ANIT causes liver injury remains uncertain. ANIT does not cause liver damage *in vivo* until it appears in bile (Jean and Roth, 1995).

This drug is initially detoxified in hepatocytes by conjugation with GSH. ANIT–GSH complexes are secreted into bile, but they are unstable and rapidly dissociate, which exposes biliary cells to high concentrations of the parent compound, which presumably causes direct cytotoxicity. The reappearance of ANIT in bile also leads to enterohepatic cycling, reuptake of the drugs in the intestine, and repetitive rounds of GSH conjugation and secretion. This not only delays elimination of the drug, but also depletes GSH progressively from hepatocytes and leads to hepatocellular damage. In addition, ANIT is known to cause hepatotoxicity by neutrophil- and platelet-dependent mechanisms (Jean and Roth, 1995).

Idiosyncratic Reactions

Many idiosyncratic drug reactions are believed to be immune-mediated. Neoantigens may result from adducts formed from the interaction of reactive drug metabolites with cellular proteins. These neoantigens may be processed by Kupffer cells or other antigen-presenting cells, transported to the cell surface, and presented as antigens. Cell- and antibody-mediated immune response may cause severe liver damage. Various drugs are believed to cause immune-mediated idiosyncratic reactions in humans, including halothane, diclofenac, phenytoin, and sulfonamides (Sturgill and Lambert, 1997; Watkins, 1999; Zimmerman, 1999; Treinen-Moslen, 2001).

Liver injury can be a result of both direct cytotoxicity and antibody-dependent cellular toxicity. Alcoholic liver disease is another example of possible immune-mediated damage. Acetaldehyde, produced by the metabolism of ethanol, forms adducts with hepatic proteins similar to halothane, resulting in higher antibody titers, to which some of the liver damage following ethanol ingestion may be attributed (Ramaiah et al., 2004). However,

the role of immune-mediated liver damage following ethanol ingestion is minimal compared to other known mechanisms of alcohol liver damage.

WARFARE AGENTS AFFECTING LIVER

Fungal and Plant Toxins

Microcystins

The microcystins are hepatotoxic products of freshwater blooms of cyanobacteria of *Microcystis*, *Anabena*, and *Oscillatoria* species (Chen et al., 1993; Luu et al., 1993), with *M. aeruginosa* being the most common. Nearly 60 microcystin cyclic hepatopeptides have been identified, of which microcystin-LR, also known as the *fast death factor*, is the most common, and the toxin of choice to be weaponized (Craig et al., 1993; Rinehart et al., 1994). This toxin has been reported to be responsible for the deaths of wild animals and agricultural livestock (Carmichael, 1988). A potential threat to the health of humans has been recognized in countries where water supplies are contaminated with cyanobacteria (Yu, 1989; Gkelis and Zaoutsos, 2014). In 1996, microcystin-LR was also implicated in the death of 50 Brazilian dialysis patients (Jochimsen et al., 1998).

This potent mammalian liver toxin (Carmichael, 1988) acts by altering the hepatocyte cytoskeleton actin filaments, leading to disruption of the structural integrity of the sinusoids. This causes massive necrosis in the liver leading to cell death. The toxic effects of microcystin-LR have been reported due to the presence of 3-amino-9-methoxy-10-phenyl-2,6,8 trimethyl deca,4,6 dienoic acid (ADDA), which is believed to be essential for its functioning. In addition, microcystins are known to be potent and specific inhibitors of catalytic subunits of protein phosphatases 1 and 2A (Cohen and Cohen, 1989; Honkanen et al., 1990; Yoshizawa et al., 1991), and activate the enzyme phosphorylase b. Microcystin administered intraperitoneally in mice caused disruption of bile flow in less than 10 min, and death within a few hours due to hypovolemic shock induced by interstitial hemorrhage following liver necrosis (Carmichael, 1988). Death was also reported within hours of administration of aerosol (LD_{50}, 67 μg/kg body weight) in mice. In humans, microcystin ingestion leads to diarrhea, vomiting, weakness, and pallor, with death occurring in a few hours if a lethal dose is taken. For further details, see chapter 31.

Aflatoxins

Aflatoxins were first isolated more than 40 years ago after the outbreak of disease and deaths in turkeys. These toxins are produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* at temperatures between 24°C and 35°C and humidity exceeding 7% (Williams et al., 2004). These fungi are known to infect a variety of crops, such as peanuts, walnuts, pecans, pistachios, and corn.

Aflatoxins are bisfuran polycyclic compounds, and depending on the characteristic blue or green fluorescence produced under ultraviolet light, these compounds are known as aflatoxin B_1, B_2, G_1, and G_2, respectively. Although these compounds have been recognized as potent mutagens and carcinogens, they are still not as toxic as botulinum or ricin. Before the first Gulf War, aflatoxins were not recognized as biological warfare agents; however, they were weaponized by Iraq for missile delivery, and at present, they can be considered as agents of biological warfare (Marshall, 1997; Zilinskas, 1997).

Of the several aflatoxins, aflatoxin B_1 (AFB_1) is the most prevalent and the most potent. AFB_1 is converted to an unstable metabolite (the 8,9-epoxide), is highly electrophilic, and forms covalent adducts with RNA, DNA, and proteins (Roebuck and Maxuitenko, 1994). In addition, aflatoxin M_1, a metabolite of AFB_1, has been found in the milk of cows fed AFB_1.

AFB_1 is acutely toxic in all species tested thus far, with an LD_{50} ranging from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse (Wogan, 1973). Acute exposure to AFB_1 has been reported to cause hepatic lesions with edema, biliary proliferation, and parenchymal cell necrosis. In addition, aflatoxin B_1 poisoning has been reported to cause jaundice, rapidly developing ascites, and portal hypertension, with high mortality resulting from massive gastrointestinal bleeding. In children, aflatoxin produces a condition called *Reye syndrome*, resulting in disturbed consciousness, fever, convulsions, and vomiting (Palmgren and Ciegler, 1983).

Ricin

Ricin, a potent plant toxin, was discovered by Peter Hermann Stillmark in 1889 (Flexner, 1897) as the first plant lectin. It was extracted from the seeds of castor plants (*Ricinus communis*) and has been considered a CWA since World War I. Although ricin is less toxic than botulinum or anthrax, easy availability and lack of a specific antidote make it a weapon of choice, and it has been included in Schedule 1 of the Chemical Weapons Convention (CWC). To date, more than 750 cases of intoxication in humans have been reported (Rauber and Heard, 1985). Being a ribosome-inactivating protein, a single molecule of ricin reaching the cytosol can kill that cell as a consequence of protein synthesis inhibition (Eiklid et al., 1980). Ricin is also very stable and extremely toxic by several routes of exposure, including ingestion and inhalation; however, compared to oral exposure, inhalation of ricin has been reported to be more harmful. In humans the lethal dose has been reported to be 5–10 μg/kg body weight.

In addition to pulmonary toxicity, ricin has been reported to be a major hepatotoxicant (Kumar et al., 2003). Studies by Muldoon et al. (1992) reported that humans exposed to ricin experience increased hepatic transaminase and lactate dehydrogenase activities. Furthermore, mice treated with ricin experienced significant oxidative stress, resulting in hepatic GSH depletion and lipid peroxidation (Muldoon and Stohs, 1991). Elevation of lipid peroxidation is reported to originate mainly from the damaged Kupffer cells, which are believed to be the target of this toxin in the liver (Skilleter et al., 1981). The high sensitivity of Kupffer cells has been ascribed to the ability of ricin to bind to the mannose receptors present on these cells (Skilleter et al., 1981; Magnusson and Berg, 1993). For further details on ricin toxicity, see chapter 27.

Abrin

Abrin, a potent toxin, is extracted from the seeds of the rosary pea (*Abrus precatorius*). Due to its easy availability and preparation, this toxin is an attractive option for weaponizing in poor countries, and thus has also been included in the Schedule 1 of the CWC. The mechanism of action of abrin is very similar to that of ricin; however, in mice, abrin is 75 times more toxic than that of ricin (0.04 µg/kg for abrin is equivalent to 3 µg/kg of ricin). Similar to ricin, inhalation of abrin is found to be more toxic than ingestion. However, abrin ingestion has reported to be toxic to the liver, unlike ricin. At the cellular level, abrin is a potent toxalbumin known to cause cell death by inhibiting protein synthesis (namely, type 2 ribosomal inhibitory protein). Further, abrin is also known to induce endothelial cell damage leading to an increase in cell permeability, fluid and protein leakage, and tissue edema.

Bacterial (Anthrax)

Anthrax is a disease caused by the spores of the bacterium *Bacillus anthracis*. It is a recognized biological warfare agent. In the United States, anthrax was deliberately spread through the postal system in 2001 via letters sent to several legislators and other public figures with powder containing anthrax. This resulted in 22 cases of anthrax infection. Inhalation and cutaneous exposure are the main routes of infection. Recent studies have revealed that laboratory animals infected with anthrax have developed significant liver injury. Studies by Moayeri et al. (2003) reported that mice infected with *B. anthracis* developed extensive liver necrosis and pleural edema. Liver lesions in these animals ranged from small hemorrhagic infarcts to large areas of centrilobular coagulative necrosis. A significant increase in alanine aminotransferase and aspartate aminotransferase was also observed in these animals. Liver dysfunction was also indicated by decreased serum albumin levels in these animals in response to toxin exposure over time. For further details on anthrax toxicity, see chapter 29.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In spite of significant advances in human knowledge about the mechanisms of liver damage, scientists seem to have a long way to go before all the mechanisms of toxicity in liver for a given chemical are completely established. In the meantime, new platforms (such as multiplex, multicolored flow cytometry and mesoscale discovery) and new "omics approaches" have provided additional tools for researchers to obtain breakthroughs in the area of liver toxicity. Clearly, current reports on the number of CWAs targeting the liver are limited, and with further research and case studies, these numbers will continue to grow. With the changing political global climate and the potential for CWAs, target organ toxicities, especially the liver, will likely gain attention.

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CHAPTER

43

Renal System

Sharon M. Gwaltney-Brant

INTRODUCTION

The kidney and urinary system are responsible for the maintenance of homeostasis by regulating the body's fluid and electrolyte balance, eliminating waste products, maintaining acid–base balance, secreting regulatory peptides and hormones, and metabolizing and excreting endogenous compounds and xenobiotics. The kidney receives 20–25% of cardiac output, putting it at risk for exposure to high levels of blood-borne toxicants. Besides exposure to directly toxic compounds, the kidney can be exposed to toxicants through metabolic activation of xenobiotics by renal tubular epithelial enzyme systems (e.g., cytochrome P450 enzymes). In its excretory capacity, the kidney and urinary tract are exposed to progressively higher levels of toxicants because they are concentrated within the renal/urinary tubules. Loss of renal function because of toxic kidney injury can result in severe systemic derangement and death of the individual; therefore, in theory, toxicants that target the kidney might be considered to be potential agents of chemical or biological warfare. However, if the goal of a chemical warfare agent is to rapidly incapacitate or kill civilians or opposing personnel, then a primary nephrotoxicant would make a poor choice of weapon because of the long delay in clinical effects from the time of exposure. Once renal damage has occurred, it may take many hours or up to a few days before signs of renal insufficiency develop to the point at which the victim is effectively incapacitated. Renal injury occurring subsequent to a chemical or biological attack is generally a matter of collateral damage rather than targeted injury, and many of the chemical agents used in warfare and terrorism have minimal effects on the kidney or urinary system. The renal effects that do develop will be seen in the individuals who survive the acute chemical attack and live long enough for evidence of renal insufficiency to develop.

ANATOMY AND PHYSIOLOGY

Functional Anatomy

In mammals, the kidneys are paired organs that reside ventrolateral to the lumbar vertebrae and musculature. Mammalian kidneys are bean-shaped to horseshoe-shaped with uniform to multilobulated external surfaces, depending on species, and a medial indented hilar region from which renal artery, renal vein, lymphatics, nerves, and ureter emerge. On sagittal section, the kidney displays two distinct regions, the outer cortex and the inner medulla. The renal cortex corresponds to approximately 80% of total renal mass, and the normal cortex:medulla ratio is 1:2 to 1:3 in most species (Maxie and Newman, 2007). The medulla is divided into ray-shaped sections known as renal pyramids that have their bases at the corticomedullary junction and their apices (papillae) that empty into a renal calyx or pelvis at the hilar area. The number of renal pyramids in the kidney varies with species. Uni-pyramidal or uni-papillate kidneys have a single renal papilla into which renal lobes empty, whereas multi-pyramidal or multi-papillate kidneys have two or more papillae. From the hilar region, urine is channeled to the distal urinary bladder via the ureter, and from the bladder, urine is voided from the body via the ureter.

Renal blood flow originates from the renal arteries, which are direct branches off of the aorta. Renal arteries progressively branch to form interlobar arteries, arcuate arteries, interlobular arteries, and afferent arterioles, the latter of which provide blood to the glomerulus. The kidney receives up to 20–25% of cardiac output, with the cortex receiving the majority (90%) of the blood flow, and the medulla (6–10%) and papilla (1–2%) receiving considerably less direct blood flow (Schnellmann, 2008). Thus, blood-borne toxicants are delivered in higher amounts to the cortex, whereas the medulla and papilla

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are exposed to higher luminal levels of toxicants that concentrate in the urine. Because of the relative sluggishness of medullary and papillary filtrate transport, these areas are also exposed to intraluminal toxicants for prolonged periods of time.

The functional unit of the kidney is the nephron, which comprises the renal corpuscle (Bowman's capsule and the glomerulus), proximal tubule, loop of Henle, and distal tubule (Figure 43.1). The number of nephrons per kidney varies with species size, ranging from approximately 10,000 in mice compared with approximately 1,000,000 in humans and 7,000,000 in elephants (Braun, 2008). High hydrostatic pressure from afferent arterioles results in ultrafiltration of plasma within the branching and anastomosing capillaries that form the glomerulus. Six to nine nanometer fenestrae within the glomerular basement membrane (GBM) form the "sieve" through which the plasma filtrate passes. Filtrate moves through Bowman's capsule and flows into the proximal renal tubule, which has three anatomically and functionally different segments. The S_1 segment is the most proximal segment that consists of the convoluted portion of the proximal tubule, and it contains epithelial cells with tall brush borders, well-developed lysosome systems, and numerous basally located mitochondria. The S_2 segment extends from the end of the convoluted tubule to the beginning of the straight segment; its epithelial cells have shorter brush borders, fewer mitochondria, and fewer lysosomes than S_1 cells. The S_3 segment consists of the remaining distal straight segment of the proximal tubule and extends into the outer reaches of the medulla. The S_1 and S_2 segments have higher oxygen consumption, sodium/potassium ATPase activity, and gluconeogenic capacity, whereas the S_3 segment has higher transport capabilities for secretion of certain compounds (e.g., organic acids) and is the primary site for metabolic activation of some toxicants (Castro et al., 2008).

The proximal tubule functions in the passive reabsorption of water and active reabsorption of sodium and potassium (via sodium/potassium ATPase pumps), as well as other solutes, including calcium, phosphorus, bicarbonate, glucose, amino acids, proteins, and various xenobiotics (Rouse and Suki, 1985). Each segment of the proximal tubule has a different range of capacity for reabsorption of various solutes; for instance, the S_1 segment reabsorbs a higher percentage of bicarbonate, glucose, amino acids and low-molecular-weight proteins, whereas the S_2 segment reabsorbs more calcium and phosphorus. Ultimately, the proximal tubule reabsorbs

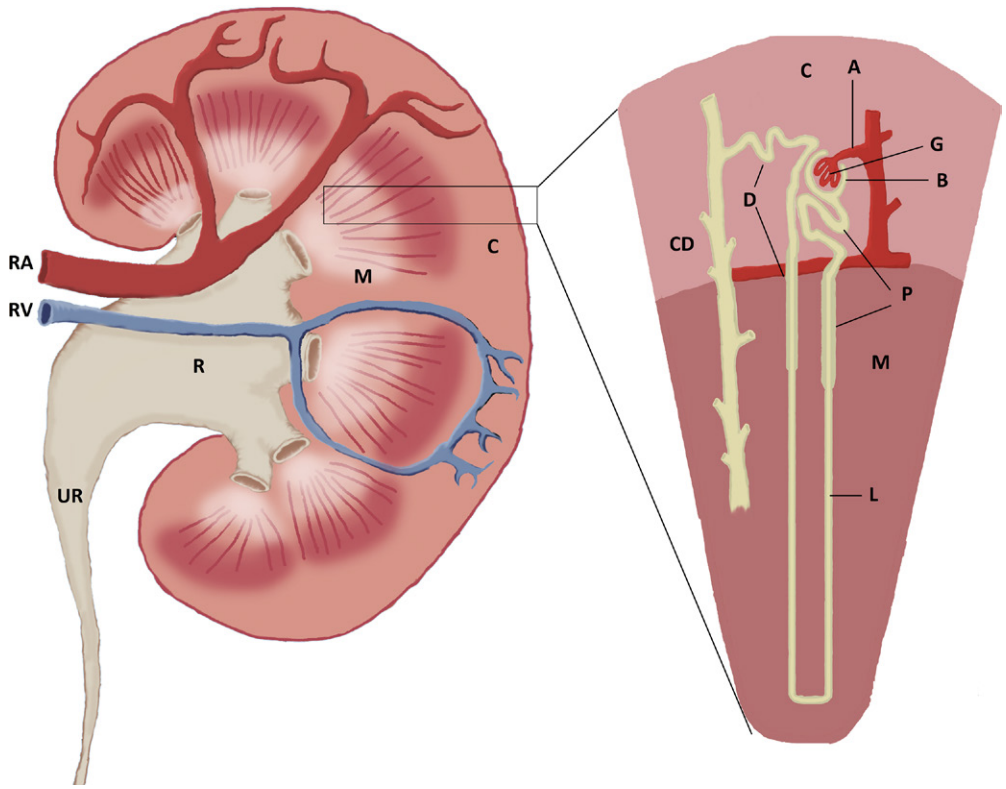

FIGURE 43.1 Diagrammatic view of the kidney showing the relationship of the cortex (C), medulla (M), renal pelvis (R), renal artery (RA), renal vein (RV), and ureter (UR). Enhanced view of microanatomy of the classical nephron (A, afferent arteriole; G, glomerulus; B, Bowman's capsule; P, proximal tubule; L, loop of Henle; D, distal tubule; CD, collecting duct).

60–90% of solute and water that was filtered through the glomerulus. Excretory functions of the proximal tubule include the active secretion of weak organic anions and cations. The absorptive and secretory capacities of the various proximal tubule segments can be induced or inhibited because of pathological or physiological alterations in filtrate composition.

Leaving the proximal tubule, filtrate enters the loop of Henle, which is composed of a thin-walled descending limb and a thick-walled ascending limb that extends to the level of the outer medullary region. Countercurrent exchange mechanisms within the loop result in reabsorption of approximately 20% of filtered water and 25% of filtered sodium and potassium (Schnellmann, 2008). The loop resides largely within the poorly oxygenated renal medulla and contains ATPases with high oxygen demand, making the loop of Henle especially susceptible to hypoxic injury (Brezis et al., 1984). The macula densa sits at the junction of the ascending loop of Henle and the proximal aspect of the distal tubule, in close proximity to the nephron's selfsame afferent arteriole, permitting feedback between macula densa and arteriole. The macula densa "reads" the intratubular solute concentrations and provides feedback to the afferent arteriole, resulting in vasoconstriction or vasodilation that decreases or increases, respectively, the glomerular filtration rate (GFR), thus regulating fluid loss and urine production. The final segment of the distal tubule along with the collecting duct function to reabsorb most of the remaining intraluminal electrolytes and water as needed to regulate the volume and composition of the urine. Collecting ducts progressively intersect and anastomose toward the renal papilla and ultimately empty into the renal calyx, renal pelvis, or ureter, depending on species. Peristaltic action of the ureter propels urine toward the urinary bladder for temporary storage and elimination via the urethra.

In addition to its role in regulation of waste excretion and water/electrolyte balance, the kidney secretes a variety of hormones and regulatory peptides vital for normal systemic homeostasis. Secretion of erythropoietin by renal peritubular interstitial cells promotes red blood cell formation; significant chronic renal disease is often associated with anemia because of decreases in erythropoietin secretion. Renin secreted from the juxtaglomerular cells increases systemic and renal blood pressure and aldosterone release. Prostaglandins and prostacyclin are produced by a variety of renal cells and aid in regulation of renal vascular tone, mesangial contractility, and processing of water and electrolytes by the renal tubules.

Biotransformation

In addition to its other functions, the kidney also plays an important role in xenobiotic metabolism. Renal tubular epithelium contains a variety of biotransformation enzymes including cytochrome P450 monooxygenases, flavin-containing monooxygenases, reductases, hydrolases, UDP-glucuronosyltransferases, sulfotransferases, glutathione-*S*-transferases, cysteine conjugate β-lyase, methylases, and acetylases (Rankin and Valentovic, 2005). Although the majority of biotransformation reactions result in inactive metabolites that are then eliminated via the urine, some biotransformation reactions result in the formation of reactive intermediates or metabolites that can cause nephrotoxicity. For example, the blue-green algal toxin cylindrospermopsin requires bioactivation by cytochrome P450 enzymes for it to become genotoxic (Zegura et al., 2011). Biotransforming enzymes are not evenly distributed throughout the kidney, but rather tend to be concentrated in specific sites of the renal tubular epithelium, thereby resulting in a particular pattern of injury depending on which enzyme system was involved in the formation of toxic intermediates.

The cytochrome P450 superfamily is composed of a large number of member enzymes and is divided into four gene families (CYP1, CYP2, CYP3, and CYP4). Individual enzymes (e.g., CYP1E2) are named by family (e.g., CYP1), subfamily (e.g., E) and individual (e.g., 2) designations. This superfamily of enzymes is of major importance in biotransformation of xenobiotics throughout the body and is most prominently expressed in the liver. Cytochrome P405 expression in the kidney is approximately 10% of that of the liver (Cummings et al., 1999). The highest concentrations of P450 in the kidney are in the renal cortex, with smaller amounts in the medulla (Rankin and Valentovic, 2005). There is considerable variability in the amount and activity of individual P450 enzymes within the kidney depending on species, age, and gender. Along the nephron, cytochrome P450 expression is highest within the epithelium of the S_2 and S_3 segments of the proximal tubules, so xenobiotics that are bioactivated by P450 to toxic metabolites will preferentially cause damage to those nephron segments first. Flavin-containing monooxygenases catalyze the oxidation of nitrogen-containing, sulfur-containing, phosphorus-containing, selenium-containing, and other nucleophilic heteroatoms-containing compounds in xenobiotics (Cashman and Zhang, 2002). Six isoforms of flavin monooxygenases (FMO1, FMO2, FMO3, FMO4, FMO5, and FMO6) have been identified and the expression of these monooxygenases within tissues varies depending on species, gender, age, and tissue. Glutathione-*S*-transferases catalyze the conjugation of electrophilic sites in xenobiotics with the tripeptide L-γ-glutamyl-L-cysteinylglycine, thus protecting macromolecules from injury from electrophiles (Rankin and Valentovic, 2005). Glutathione-*S*-transferase activity is highest in the proximal tubular cells, although distal tubules do express some activity as well.

TOXIC RESPONSES OF THE URINARY SYSTEM

Acute Renal Failure

Acute renal failure is the sudden decrease in renal function resulting in retention of nitrogenous wastes, and it is a common manifestation of acute nephrotoxic injury (Schnellmann, 2008; Langston, 2010). "Acute kidney injury" (AKI) is the term that has been suggested as most appropriate for use when discussing renal injury, because it encompasses all phases of kidney insult, ranging from minor elevations of serum chemistry renal values to anuric renal failure (Karajala and Kellum, 2010).

The primary manifestation of AKI is decreased GFR resulting in excess accumulation of nitrogenous wastes in the blood (azotemia). Decreased GFR can result from prerenal, renal, or postrenal etiologies. Decreased cardiac output, hypovolemia, and renal vasoconstriction are common prerenal events that can decrease GFR. Postrenal causes of reduced GFR include obstruction of renal tubules or of the lower urinary tract by casts or crystals. Renal tubular injury, glomerular injury, interstitial renal disease (e.g., inflammation, neoplasia), and renal vascular compromise are all primary renal factors that can lead to decreased GFR. In humans, 20–80% of AKI is attributable to prerenal factors, 10–45% is attributable to primary renal factors, and postrenal factors cause 5–15% of AKI (Langston, 2010). Of primary renal factors, ischemia/reperfusion and nephrotoxicosis are thought to be responsible for more than 90% of AKI cases in humans (Schnellmann, 2008).

Damage to kidneys by nephrotoxicants occurs through a variety of different mechanisms, including: direct injury to renal tubular epithelium leading to epithelial cell necrosis with sloughing and obstruction of tubules by cellular debris (tubular casts); detachment of lethally injured cells from the basement membrane resulting in back-leakage of filtrate across the exposed basement membrane and adherence of detached cells to sub-lethally injured cells still attached to the basement membrane causing lumen obstruction; renal vasoconstriction resulting in hypoxia and ischemic necrosis of renal structures; damage to the glomerular filtration barrier; and impairment of renal healing and repair (Counts et al., 1995). Most nephrotoxic agents cause injury at the level of the renal tubules, and many toxicants will target specific tubule segments.

Clinical effects of AKI generally do not become apparent until GFR is approximately 40% of normal and nitrogenous wastes have accumulated (Khan et al., 2013). Depending on the individual and the dose of toxicant received, this may take several hours to a few days after an acute toxic insult, making nephrotoxicants a poor choice of chemical weapon if the goal is to rapidly disable an opponent. The clinical signs of acute renal injury and/or failure can include polydipsia, nausea or vomiting, lethargy, anorexia, weakness, dehydration, and polyuria/oliguria/anuria. More severe cases may have halitosis, oral ulceration, abdominal (renal) pain, palpably enlarged kidneys, and cardiac arrhythmias. Clinical laboratory abnormalities indicative of AKI include elevations in blood urea nitrogen (BUN) and serum creatinine (azotemia), hyperphosphatemia, hyperkalemia or hypokalemia, and metabolic acidosis. *Uremia* is the term used when azotemia is accompanied by typical clinical signs of AKI. Advanced cases of uremia may present with gastrointestinal ulceration, anemia, peripheral neuropathy, encephalopathy, metastatic mineralization, and cardiac dysfunction.

The ability of the kidney to heal after an acute toxic insult is dependent on several factors, including the dose and type of toxicant, the amount of functional kidney remaining, the presence and severity of secondary uremic conditions (e.g., soft tissue mineralization), and the degree of medical intervention and supportive care provided during the acute crisis. Mild to moderate renal tubular injury with retention of tubular basement membrane has a reasonable prognosis for tubular regeneration provided that supportive care is administered until tubules have had a chance to recover. In uncomplicated acute tubular injuries, regeneration of epithelial cells generally begins approximately 7–10 days after the renal insult; in mild cases, full recovery of architecture may occur within 2–3 weeks, with longer recovery periods being required for more severe renal injury (Maxie and Newman, 2007). In situations in which nephrons have been fully obstructed by cellular debris or crystals, or if basement membrane integrity is lost, regeneration may be incomplete, resulting in long-term renal insufficiency and/or progression to chronic renal failure (CRF).

Chronic Renal Failure

The kidneys have a large compensatory capacity to adapt to injury resulting in loss of functional renal mass, with potential increases in GFR by 40–60% in nephrons not directly affected by the insult (Schnellmann, 2008). Biochemical evidence of renal injury generally is not detectable using current renal parameters (i.e., BUN, serum creatinine) until 50–70% decreases in GFR occur. For these reasons, significant renal injury may occur before any clinically detectable evidence of renal insufficiency develops. CRF is a common result of long-term exposure to toxicants, and many of the alterations found when CRF is finally diagnosed are related to secondary compensatory changes triggered by the initial injury. Toxicant-induced nephron loss causes a decrease in overall GFR, which triggers an increase in blood flow and pressure to surviving nephrons in an attempt to

re-establish normal whole-kidney GFR. Increased glomerular pressure can contribute to degenerative changes such as glomerular sclerosis, tubular atrophy, and interstitial fibrosis, which, in turn, further the progression of renal injury (Brenner et al., 1982). Intraglomerular hypertension results in glomerular hypertrophy, hyaline deposition within glomerular capillary walls, mesangial dysfunction, microaneurysm formation, and thrombosis attributable to endothelial injury (Polzin, 2010). As the glomerulus expands, focal denudation of the glomerular basement membrane occurs, allowing leakage of larger proteins into the glomerular filtrate (proteinuria). Other factors such as cytokines expressed by inflammatory cells, elaboration of reactive oxygen species (ROS), lipid accumulation, increased extracellular matrix deposition, and tubulointerstitial injury contribute to the progression toward CRF (Schnellmann, 2008).

Clinical effects associated with CRF include uremia, gastrointestinal disorders (uremic gastritis, uremic enterocolitis), polyuria, polydipsia, nocturia, dehydration, atrial hypertension, peripheral neuropathy, uremic encephalopathy, myopathy, platelet dysfunction, cachexia, and hypokalemia (Polzin, 2010). Edema caused by retention of sodium and water, renal secondary hyperparathyroidism, and anemia caused by decreased erythropoietin synthesis are common manifestations of CRF. Immunosuppression is a potential complication of CRF in humans, and studies of dogs with CRF have shown impaired immunological function as well (Kravola et al., 2010). Patients with CRF may show few outward signs of illness until late in the course of their disease; in some instances, acute decompensation may occur during periods of physical or emotional stress (Hosseininejad and Hosseini, 2008). Lesions of CRF include kidneys that are small and irregular in shape with uneven capsular surfaces. On cut section, pale streaks (fibrosis) may be seen within the interstitium and the parenchyma may be gritty on cutting because of mineralization and/or crystal deposition.

Patterns of Toxic Injury

Identification of the target site of action of nephrotoxicants can assist in determining the functional impact and potential mechanism of toxicity. Compounds that are directly toxic to cells may cause injury to the glomerulus or the S_1 segment of the proximal tubule as they first enter the nephron. Conversely, direct-acting toxicants may be dilute in the initial ultrafiltrate and may not cause injury until they reach the more distant nephron, where they may reach toxic concentrations as water is gradually reabsorbed and/or the pH changes. Some toxicants require bioactivation to exert their toxic effects and may cause site-specific injury to the segments of the nephron where those bioactivation processes are most active. Figure 43.2 illustrates the distribution of lesions within the nephron and lower urinary tract that can be induced by chemical warfare agents.

Glomerular Injury

The glomerular capillaries are the first component of the nephron to be exposed to blood-borne toxicants. The glomerular cells, matrix, and mesangium are susceptible to toxic injury by several different mechanisms, including direct injury to cellular components, formation of oxygen-derived free radicals, disruption of extracellular substrates (e.g., basement membranes), immune-mediated injury, and disruption of renal hemodynamics (Khan et al., 2013). Direct injury or injury secondary to reactive oxygen intermediates can result in endothelial loss, glomerular podocyte injury, and necrosis of mesangial cells and substrate (mesangiolysis). Further damage could occur secondary to cytokines released by inflammatory cells responding to the site of injury. Alterations in the GBM secondary to cellular injury can result in disruption of the glomerular filtration barrier, leading to proteinuria. Deposition of an immune-mediated reaction to various toxicants (e.g., mercurial, gold salts) can result in thickening of the GBM, disrupting the glomerular filtration barrier and leading to membranous glomerulonephropathy.

Proximal Tubular Injury

Tubular injury is the most common pattern of renal injury induced by toxicants and the proximal tubule is most frequently affected by nephrotoxicants (Schnellmann, 2008). Damage to the proximal tubule may occur because of direct damage from toxicants, metabolic activation of toxicants, ischemia-reperfusion, or physical or chemical disruption of endothelium and/or basement membrane. The S_1 segment is the most vulnerable to injury from toxicants that exert direct injury because the epithelium in this area is exposed to the toxicant first. The proximal convoluted tubule epithelium is actively involved in endocytosis of various compounds that bind to the brush border, sequestering the compounds in phagolysosomes. When this process is overwhelmed by the presence of certain toxicants (e.g., uranyl ion), loss of phagolysosome membrane integrity occurs, resulting in lysosomal leakage and cell injury or necrosis (Khan et al., 2013). This type of injury is most commonly associated with the S_1 and S_2 segments of the proximal tubule. In contrast, the straight segment (S_3) is most susceptible to injury by metabolic activation, transporter-associated accumulation, and ischemia-reperfusion.

Various metabolic derangements, such as severe acid–base imbalances, induced by toxicants can result

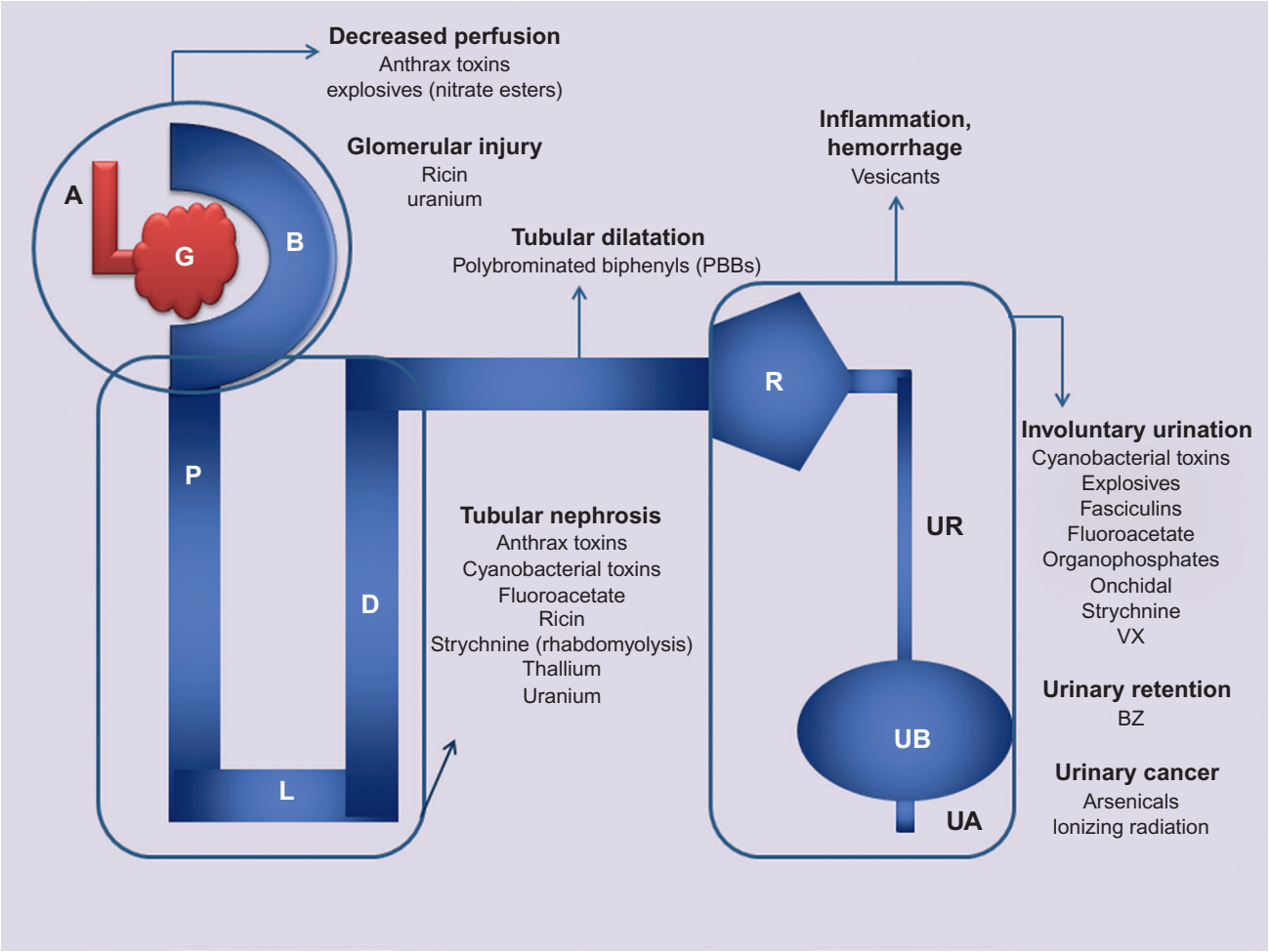

FIGURE 43.2 Cartoon of the nephron and lower urinary tract showing sites of injury caused by various chemical and biological warfare agents (A, afferent arteriole; G, glomerulus; B, Bowman's capsule; P, proximal tubule; L, loop of Henle; D, distal tubule; C, collecting ducts; R, renal pelvis; UR, ureter; UB, urinary bladder; UA, urethra).

in injury to renal tubules. Prolonged muscle activity from tremors or convulsions (e.g., from nerve agents) may result in rhabdomyolysis with myoglobinemia causing renal tubular damage (Hamilton et al., 1989). Similarly, intravascular hemolysis that occurs during a chemical warfare toxidrome can result in hemoglobinuric nephropathy with resulting renal tubular injury. Decreased cardiac output, hypovolemia, and anemia can result in ischemia of renal tissue and precipitate tubular injury; renal ischemia can also occur secondarily to toxicosis from agents that alter oxygen delivery to tissues (e.g., cyanide).

In humans and dogs, the renal tubules and interstitium are related in such a way that significant injury to one results in a reaction in the other; for this reason, the terms "tubulointerstitial disease" and "chronic interstitial nephritis" are often applied to lesions found in the kidneys of these species (Khan et al., 2013). The latter term acknowledges the fact that, in advanced tubulointerstitial disease, it is difficult to determine which injury came first, that to tubules or to interstitium.

Distal Nephron/Renal Papillary Injury

Toxic injury to the distal nephron is relatively uncommon, and injury to this area generally manifests as decreased urine concentrating ability or as defects in acid secretion resulting in metabolic acidosis (Khan et al., 2013). Injury to the renal papilla is most commonly seen with toxicants that impede blood flow to this normally poorly perfused area. Direct injury to renal papillae from renally excreted irritants (e.g., vesicants such as cantharidin) may also occur.

Lower Urinary Tract

Toxicants that cause injury to the ureters, urinary bladder, and urethra tend to be those that directly interact

with the urothelium (Cohen, 2013). Direct cytotoxicity to urothelium by vesicants such as cantharidin can result in necrosis, ulceration, and hemorrhage. Ionizing radiation and genotoxic chemicals can damage DNA within the regenerative layer of the urothelium, inducing mutations and increasing the potential for development of urinary tumors. Not all toxic effects on the lower urinary tract are related to cellular injury. Psychotropic or neurotoxic agents can affect neuromuscular control of the bladder, resulting in the involuntary retention or voiding of urine.

TOXIC EFFECTS OF CHEMICAL WARFARE AGENTS

Very few compounds selected as agents of chemical warfare have significant and direct effects on the urinary system, and any injury to the kidney can be considered an unintended consequence of most chemical weapons. Indirect renal injury can occur because of a variety of effects of chemical weapons, including hemodynamic abnormalities causing decreased renal perfusion (e.g., shock, hypotension), acid–base derangements, or induction of pigmenturia attributable to rhabdomyolysis or hemolysis.

Vesicants

Vesicants are agents that produce chemical burns on body surfaces. Phosgene oxime, an urticariant, is not a true vesicant because it does not produce blisters, but is included in this section. Through their action on the skin, respiratory tract, and eyes, these agents tend to incapacitate rather than kill, although systemic involvement and death can occur with severe exposures or because of secondary complications such as bacterial infections, shock, or multi-organ failure.

The biochemical mechanisms of injury from vesicant agents are poorly understood. Mustards (bis-(2-chloroethyl) sulfide) are thought to act as alkylating agents that form highly reactive electrophiles that combine with nucleophilic sites on cellular macromolecules to form stable adducts that interfere with normal function of cells and disrupt the epidermal–dermal junction (Hurst et al., 2008). Once tissue injury has been established, activation of massive local inflammatory response leads to vesicle development. Depletion of glutathione may also contribute to the vesicant injury through loss of protection against oxygen-derived free radicals and lipid peroxidation, resulting in a "snowballing" effect of cellular damage. The biochemical mechanism of action of the arsenical lewisite (b-chlorovinyldichloroarsine) may involve interactions with enzymatic sulfhydryl groups, resulting in inhibition of the pyruvate dehydrogenase complex, leading to inactivation of carbohydrate metabolism. As with mustards, depletion of glutathione resulting in increased lipid peroxidation and ROS generation may also play a role in lewisite-mediated cellular injury. Phosgene oxime may act by direct necrotizing effects of chlorine, by enzymatic inactivation of target cells, and/or by activation of macrophages, recruitment of neutrophils, and production of ROS, although the exact mechanism remains obscure (Hurst et al., 2008).

Renal lesions with vesicant exposures are not commonly encountered and, in many cases, may be nonspecific secondary effects attributable to multiorgan failure associated with fatal exposures. Mustards have been reported to cause hemorrhagic nephritis, oliguria, anuria, albuminuria, and casts in mortally injured victims (Papirmeister et al., 1991). Microscopic lesions associated with sulfur mustard victims included calcified and hemoglobin-containing intratubular casts (Alexander, 1947). Although lewisite is an arsenical vesicant, renal lesions typical for inorganic arsenic toxicosis have not been reported in laboratory species or human patients exposed to lewisite (Hurst et al., 2008). No specific renal lesions have been reported with exposure to phosgene oxime. For further details on vesicants toxicity, readers are referred to Chapter 8.

Nerve Agents

Nerve agents classically include the organophosphorus (OP) compounds (e.g., soman, sarin, cyclosarin, tabun, and VX) originally developed as insecticides, but repurposed and refined for use as warfare agents as their extreme toxicity was recognized (Sidell et al., 2008). Although developed and stockpiled for use in Germany during World War II, nerve agents were not used on the battlefield until 50 years later during the Iran–Iraq War. Since then, nerve agents have been used both in battle and in terrorist attacks, such as the 1995 release of sarin on commuter trains in Japan that killed 12 people. Onchidal, an anticholinesterase mollusc toxin, has similar clinical effects as the OP nerve agents and is considered here and under the term "nerve agent" (Abramson et al., 1989).

Nerve agents act by binding and inhibiting acetylcholinesterase (AChE), an enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh). The cholinergic system is the only known system to terminate the action of a neurotransmitter via enzymatic cleavage. When anticholinesterase agents such as OPs and onchidal disable AChE, ACh accumulates within the synapse, resulting in continued stimulation of cholinergic pathways and development of the classic nicotinic and muscarinic signs (Anadon et al., 2009; Watson et al., 2009). Urinary system signs associated with anticholinesterase poisoning include involuntary urination because of cholinergic stimulation of bladder musculature. Acute renal injury has occasionally

been reported after anticholinesterase poisonings and may be attributed to hemodynamic dysfunction leading to shock and hypovolemia during the cholinergic crisis, which, in turn, results in decreased renal perfusion (Bloch-Shilderman and Levy, 2007). Because the mechanism of anticholinesterase poisoning is biochemical, few characteristic gross lesions would be expected beyond nonspecific organ congestion and presence of increased bronchial secretions (Meerdink, 2004). Histopathologic evidence of skeletal muscle necrosis, myocardial hemorrhage and necrosis, and neuronal necrosis within the hippocampus, cerebral cortex, amygdala, and thalamus have been reported after severe OP intoxication (Gupta et al., 1987a,b, 1991; Gupta and Crissman, 2013).

Depleted Uranium

Depleted uranium (DU) is previously enriched uranium that has had its radioactivity largely spent and, as such, is a very weak alpha and gamma emitter (Gwaltney-Brant, 2013). Lesions produced by depleted uranium are thereby related to the metal itself rather than any emitted radiation. Uranium is poorly absorbed orally, and absorption via inhalation requires uranium to be soluble and of small size (<10 μm). Once absorbed, uranium is converted to uranyl ion that complexes with citrate, bicarbonate, or plasma proteins and is distributed via the blood, ultimately accumulating in bone and in the proximal tubules of the kidney. In the proximal tubular epithelium, uranyl ion is cleaved from its complex and causes damage to renal tubules and glomeruli, resulting in proteinuria, glucosuria, aminoaciduria, and, with larger exposure levels, acute renal failure. Glomerular lesions include endothelial swelling and necrosis, glomerular sclerosis, and disruption of glomerular fenestrae. Tubular epithelium becomes vacuolated and necrotic, and over time a mosaic of degenerating and regenerating tubules and glomeruli can be seen along with interstitial inflammation and fibrosis. On removal of uranium exposure, tubules and glomeruli may fully regenerate, provided that fibrosis has not developed. For more details on DU toxicity, see Chapter 33.

Thallium

Thallium is a heavy metal that has historically been used as a pesticide and as a therapeutic agent, and has been used illegally as an agent for suicide attempts and intentional poisonings of small groups of people (Thompson, 2009). Thallium is rapidly absorbed via most routes and is widely distributed throughout the body, although the highest levels occur in the skin. Thallium substitutes for potassium in many cellular enzymatic and transport systems, causing widespread disruption of cellular function. Clinically, the classic syndrome of thallium poisoning involves gastroenteritis, polyneuropathy, and alopecia. Renal injury may occur secondarily and is characterized by elevations in BUN and urine protein levels. Histopathological lesions in the kidney include necrosis of epithelium of loop of Henle, degeneration and necrosis of proximal convoluted tubules, and stromal edema. On electron microscopy, degenerative changes were seen in mitochondria, microvilli, and endoplasmic reticulum (Danilewicz et al., 1979). For further details on thallium toxicity, see Chapter 14.

Ricin

Ricin is a lectin isolated from the seeds of the castor bean plant (*Ricinus communis*) that acts as a ribosome-inactivating protein, enzymatically depurinating adenine residues of 28S ribosomal RNA and irreversibly arresting protein synthesis (Lapadula et al., 2013). The toxicity of ricin has been known for centuries, and more than 750 human cases of intoxication have been reported (Poli et al., 2007). Its potency, worldwide availability, and ease of production have made ricin a biological warfare agent of interest in many countries, but its widespread use as such has not been reported. Ricin has been used as an agent of political assassination, with the most famous incident being the murder of Georgi Markov, a Bulgarian dissident who died a few days after a ricin-impregnated bead was injected into his thigh via a specially modified umbrella (Papaloucas et al., 2008). The fact that the ricin had to be injected into Markov underscores one of the drawbacks in its use as an agent of warfare or terrorism: the toxicity of ricin is largely route-dependent (Poli et al., 2007). Dermal absorption of ricin does not occur to any significant degree; ingestion results primarily in gastrointestinal injury, which is largely survivable if patients receive appropriate therapy. Parenteral administration via injection is not feasible for large-scale use, and efficient inhalational administration requires refinement in manufacturing and delivery methods that make it less desirable as an efficient warfare or terrorism weapon (Schep et al., 2009).

Clinical signs and pathologic lesions in ricin toxicosis are largely route-specific, with ingestion causing primarily gastrointestinal signs (vomiting, diarrhea, abdominal pain) and with inhalation resulting in respiratory signs such as wheezing, pneumonia, and pulmonary edema (Poli et al., 2007). At larger oral or inhaled dosages or with parenteral administration (i.e., injection), more serious systemic effects can develop, including hemorrhagic diarrhea, fever, vascular collapse, hypotension, dehydration, cyanosis, hypovolemic shock, and death after 3 or more days. Liver failure and kidney failure have been reported in human cases with survival beyond several days. Systemic lesions after ricin ingestion by humans include gastrointestinal

ulceration and hemorrhage, diffuse nephritis, and necrosis of liver, spleen, and lymph nodes. Renal lesions associated with ricin toxicosis resemble lesions of hemolytic-uremic syndrome, which is most commonly associated with verocytotoxin-producing *Escherichia coli* (Taylor et al., 1999; Korcheva et al., 2005). In rodents exposed intratracheally to lethal levels of ricin, initial renal injury occurs in the glomerulus, which shows leukocytosis, accumulation of proinflammatory RNA transcripts, substantial damage to 28S rRNA, and accumulation of fibrin and fibrinogen, resulting in glomerular thrombotic microangiopathy (Wong et al., 2007). Renal tubular degeneration and necrosis develop subsequent to the glomerular injury. Mice exposed to sublethal levels of ricin did not have development of histopathological lesions and 28S rRNA damage within the kidney. In dogs with naturally occurring fatal ricin toxicosis, membranous glomerulonephritis devoid of fibrin deposits and renal tubular degeneration and necrosis have been reported (Roels et al., 2010). For details on ricin toxicity, see Chapter 27.

Anthrax Toxins

Anthrax is a zoonotic disease caused by *Bacillus anthracis* that has a long association in human history. Inhalational anthrax, also known as woolsorter's disease because of its association with English woolsorters, is of concern because of its ability to be spread via aerosol route and its high mortality in humans lacking rapid access to appropriate treatment. A 2001 bioterrorist attack in the United States resulted in the deaths of 5 out of 11 individuals who had development of inhalational anthrax. In addition to inhalational anthrax, other forms of anthrax include gastrointestinal anthrax, cutaneous anthrax, and meningitis, with the latter occurring as a complication attributable to bacteremia from any of the other forms of the disease (Purcell et al., 2007).

B. anthracis produces two major toxins, lethal toxin (LT) and edema toxin (ET), which play essential roles in its virulence (Sweeney et al., 2010). As with many bacterial toxins, anthrax toxins are binary in structure, possessing a cell-binding, pore-forming subunit that gains entry into cells and an enzymatic component that produces the toxic effect. LT is a zinc endopeptidase that cleaves mitogen-activated protein kinases, whereas ET is a potent calmodulin-dependent adenyl cyclase. Although ET is less potent than LT on a molar basis, it produces death more rapidly and is primarily responsible for the renal effects of anthrax (Fioved et al., 2005). ET causes decreased renal perfusion as well as possible direct cytotoxic effects on renal tubular epithelium, leading to increases in BUN and serum creatinine. Histopathologic renal lesions of anthrax include degeneration and necrosis of cortical tubular epithelial cells. For further details on anthrax toxicity, see Chapter 29.

Cyanobacterial Toxins

Blue-green algae produce a variety of toxins, including anatoxins, microcystins, nodularins, and cylindrospermopsin (Patocka et al., 2011; Puschner and Roegner, 2012). Anatoxin-a and anatoxin-a(s) are not directly nephrotoxic, but their effects on the nervous system (nicotinic and anticholinesterase, respectively) can result in loss of voluntary bladder control and involuntary urination. Microcystins and nodularins are primarily hepatotoxins that cause apoptosis and necrosis of hepatocytes through interference with cytoskeletal structures after acute exposure. Death from acute microcystin toxicosis is attributable to liver failure, with any renal injury generally attributed to terminal multi-organ failure. Chronic administration of microcystins to rats resulted in nephrotoxicity caused by disruption of cytoskeletal structures of the renal tubular cells (Milutinovic et al., 2003). Nodularins have been reported to produce renal lesions after acute exposure (Simola et al., 2012). The lesions were described as radiating streaks of acute tubular necrosis primarily affecting proximal tubular epithelium, with multifocal extensions of necrosis into more distal tubules and collecting ducts. In both microcystin and nodularin toxicosis, decreased renal perfusion may also play a role in the renal lesions that develop. Cylindrospermopsin binds to DNA, causing DNA damage, inhibiting protein synthesis, and inducing oxidative damage to cells (Solter and Beasley, 2013). As with microcystins and nodularins, the liver is the primary target of cylindrospermopsin, with renal injury thought to be a combination of direct toxic injury as well as ischemic injury attributable to cardiovascular compromise in terminal stages of toxicosis. Renal lesions include acute degeneration and necrosis of proximal and distal convoluted tubules. In addition to cytotoxicity, cylindrospermopsin can be bioactivated to a genotoxic compound by cytochrome P450 enzymes (Zegura et al., 2011). The genotoxic nature of cylindrospermopsin also makes it a concern as a potential human and animal carcinogen. For further details, readers are referred to Chapter 31 on cyanobacterial (blue-green algae) toxins.

Other Agents

Many potential agents of chemical warfare have little to no direct injurious effect on the kidney or urinary system, but instead may cause indirect renal injury and/or urinary dysfunction. Nitrate esters used as explosives can cause profound hypotension with the potential to result in renal ischemia secondary to decreased renal perfusion (Gahagan and Wismer, 2012). Metabolic acidosis, proteinuria, glucosuria, and myoglobinuria were reported in a survey of five human cases of cyclonite

(C-4) plastic explosive ingestion, and chronic renal insufficiency developed in a dog that recovered from seizures caused by ingestion of cyclonite (Kuccukardali et al., 2003; Fishkin et al., 2008). Chronic or repeated exposure to the riot control agent chloropicrin (PS, nitrochloroform) has been reported to cause kidney injury, but the mechanism or nature of renal injury has not been fully described (Salem et al., 2008). Cattle fed 25 g of polybrominated biphenyls per day for 33–60 days had development of degeneration and necrosis of cells of the collecting ducts and convoluted tubules, whereas those fed 250 mg per day had no development of signs or lesions of toxicosis (Moorhead et al., 1978). Toxic inhalants such as phosgene, chlorine, and hydrogen cyanide do not appear to cause direct injury to the kidney, nor do they appear to cause chronic or long-term renal effects (Tuorinsky and Sciuto, 2008).

Agents that cause neuromuscular dysfunction resulting in fasciculations, tremors, or seizures include compounds such as nerve agents, strychnine, fasciculins, and fluoroacetate. Although none of these has a direct toxic effect on the kidney, all have the potential to induce involuntary urination during the period of intoxication, and myoglobinuria or hemoglobinuria secondary to severe muscle injury from prolonged tremor or convulsion can cause acute renal tubular degeneration and necrosis. Incapacitants and psychotropic agents such as LSD also can result in loss of voluntary bladder control without causing overt lesions within the urinary system (Ketchum and Salem, 2008). Similarly, agents that produce neuromuscular blockade, paralysis, or unconsciousness such as tetrodotoxin or botulinum toxin can cause loss of voluntary bladder control, but cause no direct action on the kidney (Williams et al., 2007; Dembek et al., 2007). Conversely, the antimuscarinic nerve agent 3-quinuclidinyl benzilate (BZ) can cause urinary retention necessitating urethral catheterization to empty the bladder (Barreuto and Nelson, 2006).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Despite the fact that some nephrotoxic agents (e.g., ethylene glycol) have been used in cases of malicious poisoning of individuals or in cases of suicide, in general, the urinary system makes a poor target for chemical warfare agents whose goal is to rapidly and consistently disable, incapacitate, or kill people on a large scale. Although renal dysfunction can lead to debilitating illness and death of the individual, barriers to the use of nephrotoxins as chemical weapons include delay between exposure and development of clinical signs, difficulty in ensuring delivery of appropriate doses to the targets, and individual variation in response to nephrotoxic agents. Given these barriers, it is not surprising that agents that have been developed to be used for chemical warfare tend to target other organ systems that can accomplish the goal of rapid incapacitation of the opponent more efficiently than primary nephrotoxicants.

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CHAPTER

44

The Immune System as a Target for Chemical Warfare Agents

Kavita Gulati and Arunabha Ray

INTRODUCTION

A chemical warfare agent (CWA) is a substance that is intended for use in military operations to kill, seriously injure, or incapacitate people because of the severe pathophysiological changes induced by them in various body systems. A United Nations (UN) report from 1969 defines CWAs as "chemical substances, whether gaseous, liquid, or solid, which might be employed because of their direct toxic effects on man, animals, and plants." However, the Chemical Weapons Convention (CWC) defines chemical weapons as including not only toxic chemicals, but also ammunition and equipment. Toxic chemicals are stated to be "any chemical which, through its chemical effect on living processes, may cause death, temporary loss of performance, or permanent injury to people and animals." Normally, they are either liquids or solids.

CWAs have been used in war since time immemorial. In 600 BC, Hellebore roots were used successfully by the Athenians to contaminate water supplies during the siege of Kirrha. Spartans ignited pitch and sulfur to create toxic fumes during the Peloponnesian War in 429 BC. The uses of CWAs on the battlefield reached a peak during World War I, and the French were the first to use ethylbromoacetate. It was followed by *o*-dianisidine chlorosulphonate, chloroacetate, chlorine, phosgene, hydrogen cyanide, diphenylchloroarsine, ethyldichloroarsine and methyldichloroarsine, and sulfur mustard, resulting in nearly 90,000 deaths and over 1.3 million casualties (Eckert, 1991). CWAs were most brutally used by the Germans in gas chambers as one way of carrying out the mass genocide of Jews during World War II, and they have been used intermittently both in war, as in the Iraq–Iran war, and in terrorist attacks, such as the one carried out in the Tokyo subway stations in 1995. It is estimated that nearly 100,000 U.S. troops may have been exposed to CWAs during Operation Desert Storm in the first Gulf War (Chauhan et al., 2008). In 2013, Syrian armed forces used organophosphate (OP) nerve agents and other CWAs against its own people.

CWAs have been widely condemned since they were first used on a massive scale during World War I. However, as already indicated, they are still stockpiled and used in many countries, as they are cheap and relatively easy to produce and can cause mass casualties. Although the blood agent creatine kinase (CK) is extremely volatile and undergoes rapid hydrolysis, the degradation of three types of vesicant CWAs, sulfur mustard, nitrogen mustard, and lewisite results in persistent products. For example, sulfonium ion aggregates formed during hydrolysis may be persistent and may retain vesicant properties. The nerve agents include the V agent *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), as well as three G agents (tabun, sarin, and soman). VX gives rise to two hydrolysis products of possible concern: EA 4196, which is persistent; and EA 2192, which is highly toxic and possibly persistent under certain limited conditions (Small, 1984). Thus, their long-term persistence in the body may lead to alterations in the immune system of the exposed population. This chapter describes the immunotoxicity of CWAs and gives an insight into the probable mechanisms of such effects.

Immunotoxicity deals with immune dysfunction resulting from exposing an organism to a xenobiotic and explores the mechanisms underlying these effects in a biological system. The immune dysfunction may take the form of immunosuppression or, alternatively, exaggerated immune reaction such as allergy, autoimmunity, or

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any number of inflammatory-based diseases or pathologies. Immunotoxicity adversely affects the functioning of both local and systemic immune systems, which are activated on exposure to toxic substances, including CWAs. Observations in humans and animal studies have clearly demonstrated that a number of environmental and industrial chemicals can adversely affect the immune system. Immunosuppression may lead to the increased incidence or severity of infectious diseases or cancer, since the immune system's ability to respond adequately to invading agents is suppressed. Toxic agent-induced immunostimulation can cause autoimmune diseases, in which healthy tissue is attacked by an immune system that fails to differentiate self-antigens from foreign antigens. For example, the pesticide dieldrin induces an autoimmune response against red blood cells, resulting in hemolytic anemia.

Although immunotoxicology is a relatively new field, a considerable amount of data has accumulated during the past few years on the immunotoxicity of certain xenobiotics. The majority of the research thus far has focused on environmental contaminants. Thus, from the defense point of view, considerable work is still required to investigate the immunotoxicity of several chemicals, and some bacterial and fungal toxins that may be potential CWAs. Furthermore, there are several chemicals used in the defense industry to which industrial workers may be constantly exposed. These chemicals, following low-level exposure to humans and animals, may cause immunological alterations. Thus, studies on such chemicals are being conducted to understand the potential risks of such exposure on the host's defense, as well as the cellular and molecular mechanisms of such immunomodulatory action.

THE IMMUNE SYSTEM

The immune system is composed of several organs, cells, and noncellular components, which act in an interrelated manner to protect the host against foreign organisms and chemical substances. The immune system participates in the mechanisms responsible for the maintenance of homeostasis, and an altered immune system reflects the adverse changes in both internal and external microenvironments. The immune system protects organisms against pathogens or other innocuous substances like pollens, chemicals, indoor molds, potential food allergens, and environmental agents, and it acts as layered defenses of increasing specificity. Most simply, physical barriers (e.g., skin) prevent pathogens and xenobiotics from entering the organism. If they breach these barriers, the innate immune system provides an immediate but nonspecific response. However, if pathogens successfully evade the innate response, there is a third layer of protection (i.e., the adaptive immune system), which is activated by the innate response. Here, the immune system adapts during an infection to improve its recognition of the pathogen, and its response is then retained after the pathogen or xenobiotic has been eliminated. This immunological memory allows the adaptive immune system to respond faster, and with a stronger attack, each time the same insult is encountered (Kindt et al., 2007).

The immune system protects organisms from infection with layered defenses of increasing specificity. The layered defense includes mechanical, chemical, and biological barriers, which protect organisms from toxic substances. Skin, a mechanical barrier, acts as the first line of defense against infection. In the lungs, coughing and sneezing mechanically eject pathogens and other irritants from the respiratory tract, while mucus secreted by the respiratory and gastrointestinal tract traps and entangles microorganisms and other toxins (Boyton and Openshaw, 2002). Chemical barriers also protect against infection. The skin and respiratory tract secrete antimicrobial peptides such as β-defensins. Enzymes such as lysozyme and phospholipase A2 in saliva, tears, and breast milk are also antibacterials (Hankiewicz and Swierczek, 1974; Moreau et al., 2001). In the stomach, gastric acid and proteases serve as powerful chemical defenses against ingested pathogens.

Innate Immune System

The innate immune system defends the host from infection and toxicants in a nonspecific manner. This means that the cells of the innate system recognize a threat and respond in a generic way but do not confer long-lasting or protective immunity to the host. The innate immune response was initially dismissed by immunologists, as it was thought to provide a temporary holding of the situation until a more effective and specific adaptive immune response develops. It is now clear that the innate immune system plays an important role as a dominant system of host defense in most organisms (Litman et al., 2005). The major function of the innate immune system is to recruit immune cells to sites of infection and inflammation. Inflammation is one of the first responses of the immune system to infection or irritation through the production of cytokines. These cytokines released by injured cells serve to establish a physical barrier against the spread of infection. Several chemical factors are produced during inflammation (e.g., histamine, bradykinin, serotonin, leukotrienes, and prostaglandins), which sensitize pain receptors, cause vasodilation of the blood vessels, and attract phagocytes. The inflammatory response is characterized by redness, heat, swelling, pain, and possible dysfunction of the organs or tissues involved. The fluid exudate contains mediators

for four proteolytic enzyme cascades: the complement system, the coagulation system, the fibrinolytic system, and the kinin system. The exudate is carried by lymphatics to lymphoid tissue, where the toxin of a foreign organism can initiate an immune response.

The activation of the complement cascade helps to identify the invading substance, activate cells, and promote clearance of dead cells by specialized white blood cells (WBCs). The cascade is composed of nine major components, designated C1 to C9, which are plasma proteins synthesized in the liver, primarily by hepatocytes. These proteins work together to trigger the recruitment of inflammatory cells. One of the main events is the splitting of C3, which gives rise to various peptides. One of them, C3a (anaphylatoxin), can stimulate mast cells to secrete chemical mediators; and another, C3b (opsonin), can attach to the surface of a foreign body and facilitate its ingestion by WBCs. C5 is a powerful chemotactic of white cells and causes release of mediators from mast cells. Later components from C5 to C9 assemble in a sequence at the surface of bacteria/xenobiotics and lead to their lysis, ridding the body of neutralized antigen–antibody complexes. The main events of this system can also be directly initiated by the principal enzymes of the coagulation and fibrinolytic cascade, thrombin and plasmin, and by enzymes released from WBCs. Further, an innate immune system leads to the activation of an adaptive immune system.

Adaptive Immune System

The adaptive immune system is composed of highly specialized systemic cells and processes that eliminate pathogenic challenges and provide the ability to recognize and mount stronger attacks each time the same pathogen is encountered. Antigen specificity requires the recognition of specific "nonself" antigens during a process called *antigen presentation.* The ability to mount these immune responses is maintained in the body by "memory cells." The cells of the adaptive immune system are special types of leukocytes, B cells and T cells, which constitute about 20–40% of WBCs. The peripheral blood contains 20–50% of circulating lymphocytes and the rest move within the lymphatic system (Kindt et al., 2007). B cells and T cells are derived from the same pluripotential hematopoietic stem cells in the bone marrow, and are indistinguishable from one another until after they are activated. B cells play a large role in the *humoral immune response*, whereas T cells are intimately involved in *cell-mediated immune (CMI) responses*. B cells derive their name from the bursa of Fabricius, an organ unique to birds, where the cells were first discovered. However, in nearly all other vertebrates, B cells and T cells are produced by stem cells in the bone marrow (Kindt et al., 2007). T cells are named after the thymus, where they develop and through which they pass. In humans, approximately 1–2% of the lymphocyte pool recirculates each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues. Both B cells and T cells carry receptor molecules that recognize specific targets.

T cells express a unique antigen-binding molecule, the T-cell receptor (TCR), on their membrane. There are two well-defined subpopulations of T cells: T helper (T_H) and T cytotoxic (T_C) cells. They can be distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces. T cells displaying CD4 generally function as T_H cells whereas those displaying CD8 function as T_C cells. T cells recognize a nonself target, such as a pathogen, only after antigens have been processed and presented in combination with a self-receptor called a major histocompatibility complex (MHC) molecule. T_C cells only recognize antigens coupled to class I MHC molecules, while T_H cells only recognize antigens coupled to class II MHC molecules.

B cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph, known as *humoral immunity.* Like the TCR, B cells express a unique B-cell receptor (BCR), which in this case is an immobilized antibody molecule. The BCR recognizes and binds to only one particular antigen. A critical difference between B cells and T cells is how each cell "sees" an antigen. T cells recognize their cognate antigen in a processed form (as a peptide in the context of an MHC molecule), while B cells recognize antigens in their native form. Once a B cell encounters its cognate (or specific) antigen [and receives additional signals from a helper T cell (predominantly of the Th2 type)], it further differentiates into an effector cell, known as a *plasma cell.*

Plasma cells are short-lived cells (2–3 days) that secrete antibodies that circulate in blood plasma and lymph, and these cells are responsible for humoral immunity. Antibodies (or immunoglobulin, Ig) are large, Y-shaped proteins used by the immune system to identify and neutralize foreign objects. In mammals, there are five types of antibody: IgA, IgD, IgE, IgG, and IgM. Differing in biological properties, each has evolved to handle different kinds of antigens. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade. About 10% of plasma cells will survive to become long-lived, antigen-specific memory B cells (Lu and Kacew, 2002). Already primed to produce specific antibodies, these cells can be called upon to respond quickly if the same foreign body reinfects the host. This is called *adaptive immunity* because it occurs during the lifetime of an individual as an adaptation to infection with that pathogen and prepares the immune system for future challenges.

A number of animal models have been developed and validated to detect chemical-induced direct immunotoxicity. Several compounds, including certain drugs, have been shown in this way to cause immunosuppression or skin allergic responses. This chapter discusses the various mechanisms of immunotoxicity by which a compound affects different cell types and interferes with immune responses, ultimately leading to immunotoxicity as well as sensitizing capacity.

TARGETS OF IMMUNOTOXICITY

Effects on Precursor Stem Cells

The bone marrow is an organ with precursor stem cells that are responsible for synthesizing peripheral leukocytes. All leukocyte lineages originate from these stem cells, but once distinct subsets of leukocytes are established, their dependence on replenishment from the bone marrow differs widely. The turnover of neutrophils is very rapid (i.e., more than 10^8 neutrophils enter and leave the circulation in a normal adult daily), so there is dependence on new formation in the bone marrow. In contrast, macrophages are long-lived and have little dependence on new formation of precursor cells. The adaptive immune system, comprising antigen-specific T and B lymphocytes, is almost completely established at around puberty and therefore essentially consists of independent bone marrow in the adult.

As a consequence of their high proliferation rate, stem cells in the bone marrow are likely to be extremely vulnerable to cytostatic drugs and chemicals like CWAs. Lineages like neutrophils with rapid turnover will be most vulnerable and will be affected first by such treatments and exposures. After prolonged exposure, macrophages and T or B cells of the adaptive immune system are also suppressed.

Effects on Maturation of Lymphocytes

T lymphocytes mature in the thymus by a very complex selection process that takes place under the influence of the thymic microenvironment and ultimately generates an antigen-specific, host-tolerant population of mature T cells. This process involves cellular proliferation, gene rearrangement, apoptotic cell death, receptor up- and down-regulation, and antigen-presentation processes, and is very vulnerable to a number of chemicals. Drugs may target different stages of T-cell differentiation like naive T cells, proliferating and differentiating thymocytes, antigen-presenting thymic epithelial cells and dendritic cells, and cell death processes (Vos et al., 1999). In general, immunosuppressive drugs may cause a depletion of peripheral T cells, particularly after prolonged treatment and during early stages of life, when thymus activity is high and important in establishing a mature T-cell population. In addition, suppression of T cells may result in suppression of the adaptive immune system by affecting the maturation of B cells, and thus antibody level.

Effects on the Initiation of Immune Responses

The innate and adaptive immune systems act together to eliminate invading pathogens. Ideally, T cells tailor the responses to neutralize invaders with minimal damage to the host. The recognition of autoantigens is maintained by two distinct signals that govern lymphocyte activation. One is the specific recognition of antigens via clonally distributed antigen receptors, and the other is antigen-nonspecific co-stimulation or help, which involves interactions of various adhesive and signaling molecules expressed in response to tissue damage, linking initiation of immune responses to situations of acute danger for the host (Vos et al., 1999). This helps to aim immune responses at potentially dangerous microorganisms (nonself), while minimizing deleterious reactions to the host (self). Xenobiotics, however, can interfere with the initiation of immune responses if they act as antigens, by forming haptens or by releasing previously hidden self-antigens. They may also trigger an inflammatory response or disturb the cooperation between T and B cells.

CWAs with large molecular weights can function as antigens and become targets of specific immune responses themselves. This is particularly relevant for foreign protein pharmaceuticals, as these can activate both T and B lymphocytes. The resulting immune responses may lead to formation of antibodies, and induce specific memory, which can lead to allergic responses to the drug. Immunotoxic effects may occur after repeated treatment with the same CWA. However, low-molecular-weight CWAs cannot function as antigens because they are too small to be detected by T cells. Reactive chemicals that bind to proteins, however, can function as haptens and become immunogenic if epitopes are derived from prime T cells, which in turn provide co-stimulation for hapten-specific B cells. This effect is responsible for allergic responses to many new epitopes formed by chemical haptens.

Modification of autoantigens can also lead to autoimmune responses to unmodified self-epitopes. Haptenated autoantigens can be recognized and internalized by antigen-presenting cells. These cells subsequently present a mixture of neo- and self-epitopes complexed to distinct class II major histocompatibility (MHC-II) molecules on their surface and neospecific T cells. T helper (Th) cells provide signals for B cells. This leads to production of either anti-hapten or

anti-self antibodies, depending on the exact specificity of the B cell. Moreover, once these B cells are activated, they can stimulate autoreactive Th cells recognizing unmodified self-epitopes. This process is called *epitope (determinant) spreading* and causes the diversification of adaptive immune responses. For example, injection of mercury salts initially induces a response directed only to unidentified chemically created neoepitopes, but after 3–4 weeks, it includes reactivity to unmodified self-epitopes. Thus, the allergic response may gradually culminate as autoimmune responses reflecting the relative antigenicity of the neo- and self-epitopes involved (Lu and Kacew, 2002).

Induction of Inflammation and Noncognate T–B Cooperation

Cytotoxic chemicals or their reactive metabolites can induce tissue damage, which causes the release of proinflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, and attracts inflammatory cells like granulocytes and macrophages. Cytokines produced during this inflammatory response activate antigen-presenting cells and accumulation of tissue debris. The epitopes of antigens on debris provide co-stimulation for Th cells, which lead to the initiation of an adaptive immune response. Reactive xenobiotics may also stimulate adaptive immune responses by disturbing the normal cooperation of Th and B cells. Normally, B cells receive stimulation from Th cells that recognize epitopes of the same antigen. However, when Th cells respond to nonself-epitopes on B cells, such B cells may be noncognately stimulated by the Th cells. This occurs during graft-versus-host responses following bone marrow transplantation, when Th cells of the host recognize nonself-epitopes on B cells of the graft and vice versa. This leads to T- and B-cell activation and results in production of autoantibodies to distinct autoantigens like deoxyribonucleic acid (DNA), nucleoli, nuclear proteins, erythrocytes, and basal membranes. Drug/chemical-related lupus is characterized by a similar spectrum of autoantibodies, and noncognate—graft-versus-host-like—T–B cooperation, therefore, is suggested to be one of the underlying mechanisms.

EXPOSITION OF AUTOANTIGENS AND INTERFERENCE WITH CO-STIMULATORY SIGNALS

Self-tolerance involves specific recognition of autoantigen leading to selective inactivation of autoreactive lymphocytes at birth, but tolerance is not established for epitopes of autoantigens that are normally not available for immune recognition. Pharmaceuticals can expose such sequestered epitopes by disrupting barriers between the antigen and the immune system [i.e., blood–brain barrier (BBB), blood–testis barrier, and cell membranes]. Tissue damage, cell death, and protein denaturation induced by chemicals can largely increase the chances of such autoantigens for immune recognition. Antigen recognition followed by co-stimulation of signaling molecules leads to the activation of lymphocytes and initiation of immune responses. Many xenobiotics have the inherent capacity to induce or inhibit this co-stimulation due to their intrinsic adjuvant activity.

REGULATION OF THE IMMUNE RESPONSE

The type of immune response elicited in response to a foreign pathogen or allergen is the result of a complex interplay of cytokines produced by macrophages, dendritic cells, mast cells, granulocytes, and lymphocytes. Immunotoxic chemicals that somehow influence the immune system can lead to either immunosuppression or immune exaggeration; i.e., hypersensitivity and autoimmunity. Hypersensitivity is an immune response that damages the body's own tissues. Hypersensitivity reactions require a presensitized (immune) state of the host. They are divided into four classes (Type I–IV) based on the mechanisms involved and the time course of the hypersensitive reaction. Type I hypersensitivity is an immediate or anaphylactic reaction, often associated with allergy. Symptoms can range from mild discomfort to death. Type I hypersensitivity is mediated by IgE released from mast cells and basophils. Type II hypersensitivity occurs when antibodies bind to antigens on the patient's own cells, marking them for destruction. This is also called *antibody-dependent (or cytotoxic) hypersensitivity*, and is mediated by IgG and IgM antibodies. Immune complexes (aggregations of antigens, complement proteins, and IgG and IgM antibodies) deposited in various tissues trigger Type III hypersensitivity reactions. Type IV hypersensitivity (also known as *cell-mediated* or *delayed type hypersensitivity*) usually takes between 2 and 3 days to develop. Type IV reactions are involved in many autoimmune and infectious diseases, but they may also involve contact dermatitis (poison ivy). These reactions are mediated by T cells, monocytes, and macrophages. The actual development of clinical symptoms is influenced by the route and duration of exposure, the dosage of the pharmaceutical, and by immunogenetic (MHC haplotype, Th1-type versus Th2-type responders, etc.) and pharmacogenetic (acetylator phenotype, sulfoxidizer, Ah receptor, etc.) predisposition of the exposed individual. Moreover, atopic individuals that tend to mount Th2 immune responses are more susceptible to anaphylaxis triggered by an IgE response to

chemical haptens than typical Th1 responders. Genetic variation in metabolism of pharmaceuticals is important as it determines the formation and clearance of immunotoxic metabolites. The slow-acetylating phenotype, for instance, predisposes for drug-related lupus because reactive intermediates of phase I metabolism have an increased opportunity to bind proteins as they are only slowly conjugated.

Immune dysregulation can also be in the form of immune suppression and both innate and adaptive arms of the immune systems play crucial roles. A wide variety of physiological, pharmacological, and environmental factors can exert a negative influence on the immune system and sometimes result in immunotoxicity. Recent experimental data have shown that emotional and environmental stressors influence the functioning of the immune system, and this is reflected in the various markers of specific immunity (Ray et al., 1991; Koner et al., 1998). Such experimental stressors consistently suppressed both humoral and CMI responses in experimental animals. Both antibody-forming cell counts and antibody titer were lowered, and a neuroendocrine–immune axis concept was proposed. Similar attenuations in CMI responses were also seen after such stressors and delayed type hypersensitivity (DTH) responses, leukocyte/macrophage migration indices and also cytokine profiles (both Th1- and Th2-dependent). Further analysis of the mechanisms involved indicated that central nervous system (CNS)–mediated changes could have contributed to this immunotoxicity. Depletion or antagonism of brain dopamine aggravated emotional stress-induced immune suppression, whereas psychoactive drugs like benzodiazepines and opioids prevented this response (Ray et al., 1992; Puri et al., 1994). In another set of experiments, rats exposed to several environmental pollutants like dichlorodiphenyltrichloroethane (DDT) showed graded degrees of immune suppression and immunotoxicity when the exposure lasted for a reasonably long period of time. Gradual accumulation in the various body tissues resulted in a variety of untoward effects in the immune system, which was particularly susceptible to such xenobiotic-induced damage (Banerjee et al., 1996; Koner et al., 1998). Both humoral and CMI response were affected, depending on the quantum and duration of exposure to these xenobiotics. Further, a combination of emotional stress and xenobiotic exposure had additive effects on the immunotoxicity parameters studied (Banerjee et al., 1997). Recent studies revealed that such emotional stress and xenobiotic-induced immunotoxicity was accompanied by derangements in oxidative stress parameters, such as enhancements in 3,4-methylenedioxyamphetamine (MDA) levels and lowering of glutathione (GSH) and superoxide dismutase (SOD) levels in the blood (Koner et al., 1997; Gulati et al., 2007; Ray and Gulati, 2007).

IMMUNOTOXICITY OF CWAS

Very few studies have been conducted to explore the immunomodulation and immunotoxic potential of CWAs, and there is little evidence that these drugs are associated with such undesirable, immunologically significant effects. The reason may be due to confounding factors such as stress, nutritional status, lifestyle, co-medication, and genetics (Vos et al., 1999). Few conventional compounds have been shown to induce unexpected enhancement of immune competence. Xenobiotic-induced hypersensitivity reactions and autoimmune disorders are a major concern, whereas some of these chemicals result in immunosuppression. In particular, impaired activity of the first line of defense of the natural immune system can have disastrous consequences. These are generally not influenced by the genetic predisposition of the exposed individual, but on actual outbreak of infections and the general immune status prior to exposition. This explains why immunosuppressive xenobiotics are most likely to have clinical consequences in immunocompromised individuals such as young children or the elderly, and can be aggravated further by stressful situations.

The exposure to CWA, depending on the type of agent and duration of exposure, can result in immunodepressed conditions or allergic and autoimmune diseases. CWAs can be classified in many different ways. They may take the form of solids, liquids, or gases. Volatile substances mainly contaminate the air, and persistent substances, which are nonvolatile, mainly cover surfaces. Some agents (e.g., sulfur mustard) may appear as solids in northern European winter conditions (freezing point 14.4°C), as a liquid at a wide range of temperatures (boiling point 219°C) or as a vapor evaporating from the liquid phase. CW agents may also be encountered as mixtures or solutions of one agent in another, or of an agent in a solvent. The mixing of lewisite with sulfur mustard has been undertaken to lower the vapor pressure and freezing point of the mustard, and hence to increase its persistence, without reducing the effective CW payload of weapon systems.

CWAs mainly used against people may also be divided into lethal and incapacitating categories. A substance is classified as an incapacitating agent if less than 1/100 of the lethal dose causes incapacitation (e.g., through nausea or visual problems). The limit between lethal and incapacitating substances is not absolute but refers to a statistical average. CWAs are generally classified according to their principal target organs, as follows:

1. **Nerve agents**. These agents are extremely toxic compounds that work by interfering with the nervous system. They include soman, sarin, cyclosarin, tabun, and VX.

2. **Blister agents/vesicants**. These compounds severely blister the eyes, respiratory tract, and skin on exposure. They include nitrogen mustard, sulfur mustard, and lewisite.
3. **Choking agents**. These agents cause severe irritation primarily affecting the respiratory tract. They include phosgene, ammonia, methyl bromide, and methyl isocyanate.
4. **Blood agents**. These agents are absorbed into the blood and interfere with the oxygen-carrying capacity (e.g., arsine, cyanides, and carbon monoxide).

Nerve Agents

Nerve agents are highly toxic organophosphorus compounds (OPs) that represent potential threats to both military and civilian populations, as evidenced in recent terrorist attacks in Japan (Ohtomi et al., 1996). Commonly known as *nerve agents* or *nerve gases*, these are the deadliest of CWAs. These agents have both chemical names and two-letter NATO codes. These are categorized as G agents: GA (tabun), GB (sarin), GD (soman), GF (cyclosarin), and V agents: VE, VG, VM, and VX. (The letter *G* represents Germany, the country of origin; and the letter *V* denotes "venomous"). Their initial effects occur within 1–10 min of exposure followed by death within 15–30 min for sarin, soman, and VX, and within 30–60 min for tabun. The ease and low cost of production make sarin gas a weapon of mass destruction in the hands of terrorist groups and dictators. While people in the immediate vicinity of a sarin attack may receive neurotoxic doses, people remote from the vicinity are likely to receive subclinical exposures.

Short- and long-term health effects from exposure to OP nerve agents and insecticide nerve agents are compiled on the basis of scientific literature published on health effects in humans and animal studies. Four distinct health effects are identified: acute cholinergic toxicity, OP-induced delayed neuropathy (OPIDN), subtle long-term neuropsychological and neurophysiological effects, and a reversible muscular weakness called *intermediate syndrome.* Each effect has data suggesting threshold exposure levels below which it is unlikely to be clinically detectable. High-level exposure results in definitive cholinergic poisoning; intermediate-level cholinergic effects include miosis, rhinorrhea, or clinically measurable depression of cholinesterase; and low-level exposure results in no immediate clinical signs or symptoms. Threshold exposure levels for the known long-term effects from OP nerve agents are at or above intermediate-level exposures (Brown and Brix, 1998). However, subclinical doses of sarin cause subtle changes in the brain, and subclinical exposure to sarin has been proposed as an etiology of Gulf War syndrome. For further details of Gulf War Syndrome, see chapter 6.

The wide use of cholinesterase inhibitors in various spheres of human activities and the risk of acute and chronic intoxications associated with this process prompted investigation of the role of acetylcholinesterase (AChE) and nonspecific esterases in the immunotropic effects of these chemicals. They irreversibly bind to AChE that normally catalyzes the hydrolysis of acetylcholine (ACh) at the cholinergic synapses and neuromuscular junctions (NMJs). The inhibition of degradation results in accumulation of ACh in the cholinergic synapses, causes the overstimulation of peripheral and central cholinergic nervous systems, and is clinically manifested as acute cholinergic crisis (featuring convulsions, respiratory failure, and sometimes death) (Marrs, 1993; Taylor, 2006).

Immunotoxicity of Nerve Agents

Kalra et al. (2002) suggested that low doses of sarin are highly immunosuppressive, and suppress glucocorticoid production. The effects of sarin exposure on the immune system are attenuated by ganglionic blockers and decreased glucocorticoid level may be a biomarker for cholinergic toxicity. In addition, nerve agents cause the activation of multiple noncholinergic neurotransmitter systems in the CNS, resulting in mutagenic, stressogenic, immunotoxic, hepatotoxic, membrane, and hematotoxic effects (Bajgar, 1992). The CNS and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. The ability of OP compounds to induce an alteration of the immune system was primarily demonstrated in animals or humans exposed to OP insecticides (OPIs). The results provide evidence that a number of functions are altered in animals or humans exposed to OP compounds, especially neutrophil function, natural killer (NK) cells, cytotoxic T-cell and humoral immune functions, and spontaneous as well as mitogen-induced lymphocyte proliferations (Casale et al., 1984; Hermanowitz and Kossman, 1984; Li et al., 2002; Newcombe and Esa, 1992; Sharma, 2006). In addition, a decreased number of cells in the spleen and thymus (Ladics et al., 1994), an inhibition of chemotaxis in neutrophils (Ward, 1968), inhibition of monocyte accessory functions, and inhibition of interleukin-2 production (Pruett and Chambers, 1988; Casale et al., 1993) were reported following the exposure to OPs, at relatively high toxic doses.

Lee et al. (1979) were the first to draw attention to the possible effects of OPs on human leukocyte function. They demonstrated that lymphocyte proliferation to phytohemaglutinin *in vitro* was decreased in the presence of OPs. Although most of the studies described the results of OPI exposure, there are studies about the immunotoxic effects of highly toxic nerve agents and their by-products. Marked impairment in neutrophil chemotaxis and neutrophil adhesion, and a reduction in the NK cell and cytotoxic

T-cell function were observed in workers exposed to OPIs and by-products of sarin (Hermanowitz and Kossman, 1984; Newcombe and Esa, 1992; Li et al., 2002). Kant et al. (1991) documented a decrease in the weight of the thymus, an important immune organ in severely affected soman survivors, but other tests of immune function did not show differences between control and soman-exposed rats. Samnaliev et al. (1996) described a decrease in the number of plaque-forming cells (PFCs) in soman-exposed rats after the administration of sheep red blood cells (SRBCs) as an antigen. However, Johnson et al. (2002) demonstrated that OP-induced modulation of immune functions can involve not only their suppression, but also their activation. Similar activation of some immune functions involving "acute phase response," such as increase in the synthesis of acute phase proteins, increase in release of histamine from basophile leukocytes, and activation of macrophages, was observed following exposure to soman (Newball et al., 1986; Sevaljevic et al., 1992). Although most of the studies dealt with exposure to high doses, Kassa et al. (2004) confirmed that not only symptomatic, but also asymptomatic, doses of sarin were able to modify various immune functions. The proportion of T lymphocytes was found to be decreased, while the B-cell levels were raised. However, sarin significantly suppressed nonspecific, *in vitro*, stimulated proliferation of both T and B cells, which suggests that it can also block normal immune response to infection. While the lymphocyte-mediated immunity is suppressed, the peritoneal and alveolar macrophages and NK cells were activated after exposure to both levels of sarin, which was determined to be the result of compensatory reactions of immune functions rather than the result of direct effects of inhalation.

Immunosuppression may result from direct action of acetylcholine upon the immune system, or it may be secondary to the toxic chemical stress associated with cholinergic poisoning (Pruett et al., 1992). Further, immunomodulation at low levels seems to be very complex, and it is suggested that other protein targets probably are very sensitive to some anticholinesterases (anti-AChEs), including nerve agents. However, the function of these protein targets is not yet known (Ray, 1998). Some immune functions are probably stimulated due to the development of "acute phase response" generally characterized by the inflammatory reaction of OP-exposed organisms (Sevaljevic et al., 1989, 1992). Other immune functions are suppressed due to immunotoxicity of OP compounds. Although these findings are difficult to extrapolate directly to low-level exposures to nerve agents, they indicate that subtle alteration of the immune system could also occur in humans at exposure levels that do not cause any clinical manifestation. Post-intoxication immunodeficiency can promote infectious complications and diseases.

It has been shown that T lymphocytes have AChE located on the plasma membrane, while B cells are esterase-negative (Szelenyi et al., 1982). Thus, AChE inhibition by toxic agents in sublethal doses may play an important role in immunodeficiency following exposure to nerve gases. Zabrodskii et al. (2003) showed inhibition of AChE in T cells and a decrease in the number of esterase-positive T lymphocytes (and, to a certain extent, monocytes and macrophages) directly correlated with suppression of T-cell-dependent antibody production and with the degree of DTH reduction, upon exposure to dimethyl dichlorovinyl phosphate, sarin, VX, lewisite, tetraethyl lead, and dichloroethane. This presumably involves the loss of some functions by T lymphocytes (e.g., by Th1 cells), which leads to the attenuation of T-dependent immune reactions. This can be explained by excessive ACh stimulation of the muscarinic and nicotinic receptors present on T lymphocytes. As a result, the optimal ratio of cyclic adenosine monophosphate (cAMP) to cyclic guanosine monophosphate (cGMP) in immunocytes, which is essential for their proliferation and differentiation, is distorted (Richman and Arnason, 1979). Thus, the anti-AChE effect of lewisite, TEL, and DCE may be one of the important mechanisms in the formation of T-cell-mediated immunodeficiency.

A study showed that malathion (an OPI) in acute noncholinergic doses enhanced the humoral immune response to SRBCs and macrophage function, and also caused mast cell degranulation. When effects of acute administration of malathion were observed in mast cell-deficient mice, the humoral activation was not observed, suggesting that the mast cells contribute to the increases in macrophage function and humoral immunity observed in normal mice (Rodgers et al., 1996).

The effects of subchronic doses of malathion exposure on humoral and CMI responses were studied in male albino mice, rats, and rabbits using SRBCs, tetanus toxoid, and ovalbumin as antigens. The humoral immune response was assessed by estimating serum immunoglobulin (IgM and IgG) concentrations, antibody titer against antigens, and splenic-PFCs. The CMI response was studied by using the leukocyte migration inhibition (LMI) and macrophage migration inhibition (MMI) tests. In general, there were the following responses: (i) attenuation in the antigen-induced antibody response, (ii) suppression of PFCs, and (iii) marked inhibition of LMI and MMI factors. Subchronic malathion exposure-induced differential degrees of humoral and CMI suppression in these experimental animals. However, both cellular and humoral immune responses were decreased in a dose-time-dependent pattern and a consistent trend was observed. The threshold level of the malathion for inducing immune suppression varies on the basis of species of animals, type of antigen used, and method of immunological assay (Banerjee et al., 1998).

In another recent study, rats were administered with malathion alone and in combination with bradykinin potentiating factor (BPF), and the effects were compared to the vehicle group on immune parameters. The results showed that the concentration of total globulin, total immunoglobulins, IgG, IgM, circulatory immune complexes, total number of RBCs and platelets, and hemoglobin concentrations decreased significantly in malathion-exposed animals. The number of total leukocytes and lymphocytes increased. Histopathological changes of bone marrow and spleen after malathion exposure were consistent with these findings. Administration of double and triple injections of BPF resulted in the recovery of bone marrow and splenic changes, normalization of peripheral blood elements, reduction of elevated proinflammatory markers (IL-2, IL-4, and TNF-α), total plasma peroxide, and oxidative stress index (OSI), while the total antioxidant capacity (TAC) increased. Our results suggest that exposure to malathion has negative effects on immune function that is mediated through alteration of cytokines, antioxidants, and direct damage of bone marrow (BM). Also, BPF can ameliorate both physiological and morphological changes (Ahmed, 2012).

In another study involving malathion-poisoned human subjects, significant levels of malathion residue of 503–702 mg/L were seen, and this was associated with significant enhancements of IL-2, IL-4, and TNF-α levels in blood, whereas no significant changes in immunoglobulin levels were seen. This study thus showed altered levels of cytokines in the blood of malathione-exposed subjects (Seth et al., 2008). Kassa et al. (2001) showed that rats exposed once or repeatedly to three various low concentrations (0.8, 1.25 and 2.5 µg/L) of sarin for 60 min in an inhalation chamber induced immunotoxicity. Nonconvulsive concentrations of sarin caused subtle suppression of spontaneous, as well as lipopolysaccharides-stimulated proliferation of spleen lymphocytes and bactericidal activity of peritoneal macrophages.

Blister or Vesicant Agents

Blister agents act on skin and other epithelial tissues and severely damage the eyes, respiratory tract, and internal organs and destroy different substances within cells of living tissue. The symptoms vary depending upon the compound and the sensitivity of the individual. Acute mortality is low; however, they can incapacitate the enemy and overload the already burdened health care services during wartime. Some of these agents are sulfur mustard (HD), nitrogen mustard (HN), lewisite (L), and phosgene oximine (CX).

HD was the most widely used CWA in the Iran–Iraq war (1980–1988), resulting in over 100,000 chemical casualties. It acts as an alkylating agent with long-term toxic effects on several body organs, mainly the skin, eyes, and respiratory system (Willems, 1989). The extent of tissue injury depends on the duration and intensity of exposure. When absorbed in large amounts, SM can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system (Willems, 1989).

Immunotoxicity

Evidence that HD causes immunosuppression in humans has emerged from several areas of investigation. The earliest evidence came from clinical observations of humans directly exposed to HD during World War I, who showed significant changes (quantitative and qualitative) in the circulating elements of the immune system. Stewart (1918) studied 10 fatal cases of HD poisoning and observed striking depression of the bone marrow production of WBCs. Among the HD casualties during the Iran–Iraq conflict, leukopenia was the most common finding, accompanied by total bone marrow aplasia and extensive losses of myeloid stem cells (Balali-Mood, 1984; Eisenmenger et al., 1991). These findings provide further evidence of an association between suppression of immunologic functions and an increased incidence of infectious disease.

HD was widely used during the Iran–Iraq conflict, and there are many reports of influence of HD on the respiratory, gastrointestinal, and endocrine systems, as well as the immune system (Balali-Mood, 1984; Budiansky, 1984; Balali- Mood and Farhoodi, 1990; Sasser et al., 1996; Emad and Razaian, 1997). The influence of HD on the immune system has been the subject of many researchers since 1919 (Krumbhaar and Krumbhaar, 1919; Hektoen and Corper, 1921). Early investigations on HD casualties during the Iran–Iraq war showed decreased immunoresponsiveness, expressed as leukopenia, lymphopenia, and neutropenia, as well as hypoplasia and atrophy of bone marrow (Willems, 1989; Tabarestani et al., 1990; Balali-Mood et al., 1991). Chronic exposure to HD has been associated with the impairment of NK cells among workers in poison gas factories in Japan (Yokogama, 1993). Similarly, CMI was found to be suppressed following mustard gas exposure (Zandieh et al., 1990).

Leukopenia has been the first manifestation to appear within the first days of postexposure. Thrombocytopenia and anemia followed later if the patients survived (WBCs of some patients dropped to less than 1,000 per cm^3). Although most of these patients suffered skin burns, clinicians reported cases that had minor skin lesions and yet developed leukopenia. Bone marrow biopsies revealed hypocellular marrow and cellular atrophy involving all elements (Willems, 1989). Studies on the status of immunocompetent cells in the blood of patients exposed to HD showed that T cell

and monocyte counts dropped in 54% and 65% of the patients, respectively, from day 1 and up to the 7th week postexposure (Hassan and Ebtekar, 2002). Eosinophil counts dropped in 35% and neutrophil numbers in 60% of the patients. B lymphocyte counts were normal up to the 7th week (Manesh, 1986). The majority of the patients showed increased levels of IgG and IgM during the 1st week, but the percentage decreased over the next 6 months. The percentage of patients with increased levels of C3, C4, and CH50 was somewhat higher than that of healthy controls during the 1st week and up to the 6th month (Tabarestani et al., 1990), and it remained higher 3 years postexposure, especially in the severely affected group. And 8 years after exposure, there was a significant increase in the number of atypical leukocytes, such as myelocytes. The severely affected group presented with significantly lower CD56 NKs, as well as CD4 and CD8 counts compared with healthy controls (Yokogama, 1993). Hassan and Ebtekar (2002) reported that there was no major difference between the severely affected patients and healthy controls concerning CD19 B cells, CD14 monocytes, and CD15 granulocytes. The moderately and mildly affected patients did not significantly differ in their leukocyte subset counts from the control group 8 years after exposure (Mahmoudi et al., 2005). Follow-up studies on the clinical conditions of exposed Iranian victims still show that they suffer from three major problems: recurrent infection, septicemia and death, respiratory difficulties, and lung fibrosis, as well as a high incidence of malignancies, septicemia, and death.

Hassan and Ebtekar (2002) suggested that patients with moderate clinical manifestations may be experiencing a shift from Th1 to Th2 cytokine patterns since leukocyte cultures from this patient group showed a decrease in IFN-γ levels. When absorbed in large amounts, HD can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system (Sasser et al., 1996). Moreover, this alkylating agent has been reported to produce short- and long-term suppression of antibody production in both animals and humans. It also affects complemented system factors C3 and C4. Incidences of acute myelocytic and lymphocytic leukemia are reported to be 18 and 12 times higher in patients exposed to HD, in comparison with the normal group (Zakeripanah, 1991). Willems (1989) reported that exposure to HD could result in the impairment of human immune function, especially in the number of B and T lymphocytes. Hence, SM is still a potential threat to the world and effective therapeutic measures must be taken for the relief of the victims of this incapacitating agent. Ghotbi and Hassan (2002) showed that the percentage of NK cells, which play an important role in cellular immunity, was significantly lower in severely affected patients than in the control group.

Studies on animal models have shown that alkylating agents such as HD mainly affect B cells, which is why hypogammaglobulinemia is one of the main features in animal models, whereas studies of human cases, following a treatment with cytotoxic drugs, suggest that low-dose exposure to alkylating agents impairs cellular immunity, and high-dose exposure to such agents impairs both cellular and humoral responses (Marzban, 1989; Malaekeh et al., 1991). There are reports suggesting that HD can produce toxicity through the formation of reactive electrophobic intermediates, which in turn covalently modify nucleophilic groups in biomolecules such as DNA, ribonucleic acid (RNA), and proteins (Malaekeh et al., 1991), resulting in disruption of cell function, especially cell division (Crathorn and Robert, 1966). As a result, these agents are particularly toxic to rapidly proliferating cells, including neoplastic, lymphoid, and bone marrow cells. Mahmoudi et al. (2005) reported higher IgM levels after 16–20 years of exposure to HD compared to the control group. A significant decrease in the number of NK cells in severely affected patients is probably due to the destructive effect of this alkylating agent on NK cell precursors in bone marrow. However, the activity of NK cells was found to be noticeably above normal, which possibly compensates for the reduction in the number of these cells.

Korkmaz et al. (2006) explained the toxicodynamics of HD in three steps: (i) binding to cell surface receptors; (ii) activation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to peroxynitrite ($OONO^-$) production, and (iii) $OONO^-$-induced damage to lipids, proteins, and DNA, leading to poly ADP ribose polymerase (PARP) activation. This could provide a lead for devising strategies for protection against and treatment of mustard toxicity.

A study was conducted to evaluate the incidence of immunocompetence among survivors of the chemical bombardment of Halabja in the Kurdistan region of Iraq. In the research, 40 exposed and 40 unexposed controls were studied to determine their immune system status 12 years after bombardment. Skin reactivity to tuberculin, DPT (diphtheria, pertussis, tetanus) vaccine, TD (tetanus, diphtheria) toxoid and measles vaccine was negative in 62.5% of the exposed cases, compared to unexposed persons, none of whom showed any negative reactions. The total leukocyte count was normal among 70% of the exposed cases, whereas the total lymphocyte count was within subnormal ranges in 80% of the exposed cases. All the subjects displaying negative skin reactions had subnormal lymphocyte counts, which reflected impaired CMI. The immunoglobulin assay for exposed cases revealed subnormal values for IgG and IgA, while IgM levels were above the normal range in 22.5% of cases when compared to that of controls, which showed no abnormal values. This result revealed that

there was a deficiency in antibody-mediated immunity. There were significant differences between the exposed and the control samples with respect to total leukocytes, neutrophil count, lymphocyte count, IgG, and IgA. The immunological reactions were more closely related to the effects of mustard gas, which appeared to be long lasting. They concluded that long-term effects were produced by CWAs on victims who have survived in Halabja and, in particular, on their immune system at both antibody and cell-mediated levels. This study confirmed the immunosuppressive properties of mustard compounds and indicated increased vulnerability of the exposed individuals to secondary opportunistic or pyogenic bacterial infections due to injuries that occurred frequently among chemical survivors in Halabja (Hama et al., 2008).

In conclusion, the results suggest that exposure to HD causes a higher risk of opportunistic infections, septicemia, and death following severe suppression of the immune system, especially in the case of lesions and blisters produced by these agents. As alkylating agents, they form covalent linkages with biologically important molecules, resulting in disruption of cell function, especially cell division. As a result, these agents are particularly toxic to rapidly proliferating cells including neoplastic, lymphoid, and bone marrow cells. However, there is still a paucity of information regarding the long-term immunosuppressive properties of HD in the setting of battlefield exposure to this agent.

Choking Agents

Choking agents act on the pulmonary system causing severe irritation and swelling of the nose, throat, and lungs; e.g., phosgene (CG), diphosgene (DP), chlorine, and chloropicrin (PS). These inhalational agents damage the respiratory tract and cause severe pulmonary edema in about 4 h, leading to death. The effects are variable, rapid, or delayed, depending on the specific agent (Gift et al., 2008).

Phosgene was first used as a chemical weapon in World War I by Germany, and later by French, American, and British forces. In this conflict, phosgene was often combined with chlorine in liquid-filled shells, so it was difficult to state the number of casualties and deaths attributable solely to phosgene. In military publications, it has been referred to as a "choking agent," "pulmonary agent," or "irritant gas." Since World War I, phosgene has rarely been used by traditional militaries, but the extremist cult Aum Shinrikyo used this agent in an attack against the Japanese journalist Shouko Egawa in 1994. Nowadays, phosgene is primarily used in the polyurethane industry for the production of polymeric isocyanates (USEPA, 1986). Phosgene is also used in the polycarbonate industry and in the manufacture of carbamates and related pesticides, dyes, pharmaceuticals, and isocyanates.

As mentioned earlier, the primary exposure route for phosgene is by inhalation. Suspected sources of atmospheric phosgene are fugitive emissions, thermal decomposition of chlorinated hydrocarbons, and photooxidation of chloroethylenes. Individuals are most likely to be exposed to phosgene in the workplace during its manufacture, handling, and use (USEPA, 1986). Phosgene is extremely toxic by acute inhalation exposure. Severe respiratory effects, including pulmonary edema, pulmonary emphysema, and death have been reported in humans. Severe ocular irritation and dermal burns may result following eye or skin exposure. Chronic inhalation exposure to phosgene has been shown to result in some tolerance to the acute effects noted in humans, but it may also cause irreversible pulmonary changes of emphysema and fibrosis (U.S. Department of Health and Human Services, 1993).

Primarily because of phosgene's early use as a war weapon, many exposure studies have been performed over the past 100 years to examine the effects and mode of action of phosgene following a single, acute (less than 24 h) exposure. Many studies have examined the effects of acute phosgene exposure in animals, but the human data are limited to case studies following accidental exposures.

Most studies were performed in rodents and dogs, with exposure concentrations ranging between 0.5 and 40 ppm (2–160 mg/m^3) and duration intervals ranging from 5 min to 8 h. Acute exposure studies in animals suggest that rodent species may be more susceptible to the edematous effects of phosgene acute exposure than larger species with lower respiratory volumes per body weight such as dogs and humans (Pauluhn, 2006; Pauluhn et al., 2007).

Pauluhn et al. (2007) reported that acute phosgene exposure results in increased lung lavage protein, phospholipid content, enzyme levels, number of inflammatory cells, and lethality (LC_{50}). Rats seem to be able to survive approximately threefold higher levels of lung edema than humans (hundredfold versus thirtyfold), thus rat responses in short- and long-term assays may still be relevant to humans even if it is ultimately shown that rats produce higher levels of edema following acute phosgene exposure.

Immunotoxicity

Acute exposure to phosgene has been shown to result in immunosuppression in animals, as evidenced by an increased susceptibility to *in vivo* bacterial and tumor cell infections (Selgrade et al., 1989) and viral infection (Ehrlich and Burleson, 1991), as well as a decreased *in vitro* virus-killing and T-cell response (Burleson and Keyes, 1989). Selgrade et al. (1989) reported that a single 4-h exposure to phosgene concentrations as low as 0.025 ppm significantly enhanced mortality due to

streptococcal infection in mice. Furthermore, when the exposure time was increased from 4 to 8 h, a significant increase in susceptibility to *streptococcus* was seen at an exposure concentration of 0.01 ppm.

Selgrade et al. (1995) administered *Streptococcus zooepidemicus* bacteria via an aerosol spray to the lungs of male Fischer-344 rats immediately after phosgene exposure and measured the subsequent clearance of bacteria. They also evaluated the immune response, as measured by an increase in the percentage of polymorphonuclear (PMN) leukocytes in lung lavage fluid of uninfected rats similarly exposed to phosgene. This experiment showed that all phosgene concentrations from 0.1 to 0.5 ppm impaired resistance to bacterial infection and that the immune response is stimulated by phosgene exposure. After 4 weeks following exposure, bacterial resistance and immune response returned to normal.

Yang et al. (1995) also reported a decrease in bacterial clearance in the lungs at 24 h after infection following a single 6-h exposure to phosgene concentrations of 0.1 and 0.2 ppm. In comparison with single exposures, the multiple daily exposures extending to 4 and 12 weeks in the Selgrade et al. (1995) report showed a slight enhancement of effect in the 0.1 ppm group at 24 h postinfection, but no adaptation, or lessening of the effect. Yang et al. (1995) found that if the bacteria are administered 18 h after single phosgene exposures rather than immediately, the clearance is normal, which indicates that recovery from the toxic effect of phosgene is rapid.

When inhaled, phosgene is either rapidly hydrolyzed to hydrogen chloride (HCl) and carbon dioxide (CO_2) and exhaled (Diller, 1985; Schneider and Diller, 1989) or penetrates deep into the lungs and is eliminated by rapid reactions with nucleophilic constituents of the alveolar region (Pauluhn et al., 2007). As phosgene is electrophilic, it reacts with a wide variety of nucleophiles, including primary and secondary amines, hydroxy groups, and thiols. In addition, it also reacts with macromolecules, such as enzymes, proteins, or other polar phospholipids, resulting in a marked depletion of GSH (Sciuto et al., 1996) and forms covalent adducts that can interfere with molecular functions. Phosgene interacts with biological molecules through two primary reactions: hydrolysis to hydrochloric acid and acylation reactions. Although the hydrolysis reaction does not contribute much to its clinical effects, the acylation reaction is mainly responsible for the irritant effects on mucous membranes. The acylation reactions occur between highly electrophilic carbon molecules in phosgene and amino, hydroxyl, and sulfhydryl groups on biological molecules. These reactions can result in membrane structural changes, protein denaturation, and depletion of lung GSH. Acylation reactions with phosphatidylcholine are particularly important, as it is a major constituent of pulmonary surfactant and lung tissue membranes. Exposure to phosgene has been shown to increases the alveolar leukotrienes, which are thought to be important mediators of phosgene toxicity to the alveolar–capillary interface. Phosgene exposure also increases lipid peroxidation and free radical formation. These processes may lead to increased arachidonic acid release and leukotriene production. Proinflammatory cytokines, such as interleukin-6, are also found to be substantially higher 4–8 h after phosgene exposure. In addition, studies have shown that postexposure phosphodiesterase activity increases, leading to decreased levels of cAMP. Normal cAMP levels are believed to be important for maintenance of tight junctions between pulmonary endothelial cells, and thus for prevention of vascular leakage into the interstitium. Oxygenation and ventilation both suffer, and breathing is dramatically increased.

Diller (1985) and Schneider and Diller (1989) reported that inhalation of phosgene at high concentrations results in a sequence of events, including an initial bioprotective phase, a symptom-free latent period, and a terminal phase characterized by pulmonary edema. The first is an immediate irritant reaction likely caused by the hydrolysis of phosgene to hydrochloric acid on mucous membranes, which results in conjunctivitis, lacrimation, and oropharyngeal burning sensations. This symptom complex occurs only in the presence of high-concentration (>3–4 ppm) exposures but does not have any prognostic value for the timing and severity of later respiratory symptoms. The most important finding to identify during this stage is a laryngeal irritant reaction causing laryngospasm, which may lead to sudden death. The irritant symptoms last only a few minutes and then resolve so long as further exposure to phosgene ceases.

The second phase, when clinical signs and symptoms are generally lacking, may last for several hours after phosgene exposure. The duration of the latent phase is an extremely important prognostic factor for the severity of the ensuing pulmonary edema. Patients with a latent phase of less than 4 h have a poor prognosis. Increased physical activity may shorten the duration of the latent phase and worsen the overall clinical course. Unfortunately, there are no reliable physical examination findings during the latent phase to predict its duration. However, histologic examination reveals the beginnings of an edematous swelling, with exudation of blood plasma into the pulmonary interstitium and alveoli. This may result in damage to the alveolar type I cells and a rise in hematocrit. The length of this phase varies inversely with the inhaled dose. The third clinical phase peaks approximately 24 h after an acute exposure and, if lethality does not occur, recedes over the next 3–5 days. In the third clinical phase of phosgene toxicity, the accumulating fluid in the lungs results in edema. Oxygenation and ventilation both suffer, and breathing is dramatically increased. Often, positive end expiratory

pressure (PEEP) is required to stent open alveoli that would otherwise collapse and result in significant ventilation/perfusion mismatch. This hyperventilation causes the protein-rich fluid to take on a frothy consistency. A severe edema may result in an increased concentration of hemoglobin in the blood and congestion of the alveolar capillaries.

Increased levels of protein in bronchoalveolar lavage have been shown to be among the most sensitive endpoints characterizing the early, acute effects of phosgene exposure, and they are rapidly reduced after the cessation of exposure (Sciuto, 1998; Sciuto et al., 2003). With continuous, chronic, low-level phosgene exposure, there may be transition of edema to persistent cellular inflammation, leading to the synthesis of abnormal Type I collagen and pulmonary fibrosis. An increased synthesis of Type I relative to Type III collagen can lead to chronic fibrosis (Pauluhn et al., 2007). Surfactant lipids are important for maintaining alveolar stability and for preventing pulmonary edema. Pauluhn et al. (2007) reported that the induction of surfactant abnormalities following phosgene exposures is a key pathophysiological event leading to pulmonary edema and chronic cellular inflammation, which caused the stimulation of fibroblasts and the synthesis of abnormal collagen in pulmonary fibrosis. As discussed earlier, a breach in the chemical layer of defense followed by pulmonary edema may lead to a cascade of other immunological responses and reactions. There are limited studies, in both humans and experimental animals, to evaluate the immunotoxicity of chronic low-level environmental exposures to phosgene. The lack of studies examining the effects in humans or laboratory animals from chronic exposure to phosgene is a concern and the sequelae of effects leading to phosgene-induced pulmonary fibrosis is not well understood.

Animal infectivity models have been important in the demonstration of enhanced susceptibility to viral and bacterial infection as a result of low-level toxicant exposure. Ehrlich and Burleson (1991) demonstrated an enhanced and prolonged viral infection using an influenza virus infectivity model in the rat following inhalation of the toxicant gas phosgene. Fischer-344 rats were exposed to either air or a sublethal concentration of phosgene, and they demonstrated peak pulmonary influenza virus titers 1 day after infection. Virus titers in rats exposed to air declined rapidly, falling below detectable levels by the 4th day of infection. However, a significantly enhanced and prolonged pulmonary influenza virus infection was observed on days 3 and 4 after infection in the rats exposed to phosgene. Virus was cleared below detectable limits on day 5 after infection in the animals exposed to phosgene. Thus, inhalation of sublethal concentrations of phosgene resulted in an increased severity of pulmonary influenza virus infection. This study provides a demonstration of the prolongation of rat viral infectivity by exposure to phosgene.

Phosgene, also known as carbonyl chloride, carbon oxychloride, and chloroformyl chloride, is a toxic air pollutant and a potential occupational health hazard. Studies were initiated to evaluate the measurement of pulmonary NK activity as a method to assess pulmonary immunocompetence, and to determine whether exposure to phosgene resulted in local pulmonary or systemic immune dysfunction. Fischer-344 male rats were exposed either to filtered air or to 1.0 ppm of phosgene gas for 4 h. The effect of phosgene on lung NK activity was quantified at different times after acute phosgene exposure. Pulmonary NK activity was measured by mincing lung tissue into small pieces prior to incubation with collagenase. Whole-lung homogenate was assayed for NK activity. Acute phosgene exposure resulted in suppressed pulmonary NK activity on days 1, 2, and 4 after exposure; however, normal levels of biological activity were observed 7 days after exposure. The suppressed NK activity was not restored after adherent cells were removed from the lung homogenate, thus indicating that the effect of phosgene on NK activity was not due to immunosuppression via mobilization of suppressor alveolar macrophages. Pulmonary immunotoxicity was also observed after exposure at 0.5 ppm, while no adverse effects were observed at 0.1 ppm of phosgene. Systemic immunotoxic effects were observed for NK activity in the spleen, but not in the peripheral blood, making it important to evaluate systemic immune functions since secondary effects—distant to the original interaction—may occur, with potentially serious consequences (Burleson and Keyes, 1989).

Blood Agents

Agents like SA (arsine), cyanide, and carbon monoxide are absorbed into the blood and affect its oxygen-carrying capacity and are thus termed *blood agents.* They are highly volatile and rapid-acting, and they produce seizures, respiratory failure, and cardiac arrest. Hydrogen cyanide has been known as a potent toxicant for over 200 years. It was used as a CWA during World War I by France. Although it is highly volatile (and was later considered militarily useless because of its volatility), no deaths from its military use during World War I were ever reported. After World War II, the importance of hydrogen cyanide as a CWA diminished rapidly, primarily as a result of the rise of nerve agents. Although reduced in importance, there are some reports of hydrogen cyanide being used as a war gas by Vietnamese forces in Thailand territories and during the Iran–Iraq war in the 1980s (Sidell, 1992).

Hydrogen cyanide can be detoxified rapidly by humans. It is very volatile, and massive amounts of the gas are needed for it to be effective as a CWA. Cyanide is primarily an environmental contaminant of industrial processes. It is used in the metal-processing industry for electroplating, heat treating, and metal polishing, and it can be found in wastewater from many mining operations that use cyanide compounds in the extraction of metal, such as gold and silver, from ore.

The acute toxicity of cyanide has been well documented in humans and experimental animals. Symptoms of toxicity in humans include headache, breathlessness, weakness, palpitations, nausea, giddiness, and tremors (Gupta et al., 1979). Depending on the degree of intoxication, symptoms may include metallic taste, anxiety, confusion, headache, vertigo, and hyperpnea, followed by dyspnea, convulsions, cyanosis, respiratory arrest, bradycardia, and cardiac arrest. Death results from respiratory arrest (Berlin, 1977), and onset is usually rapid. Effects on inhalation of lethal amounts may be observed within 15 s, with death occurring in less than 10 min. Hydrogen cyanide should be suspected in terrorist incidents involving prompt fatalities, especially when the characteristic symptoms of nerve agent intoxication are absent. Chronic exposure to low-level cyanide can result in neuropathies, goiter, and diabetes. Cyanide and derivatives prevent the cells of the body from using oxygen. Cyanide acts by binding to mitochondrial cytochrome oxidase, blocking electron transport and thus inhibiting enzymes in the cytochrome oxidase chain, and in turn blocking oxygen use in metabolizing cells and preventing the use of oxygen in cellular metabolism. These chemicals are highly toxic to cells and, in high doses, may result in death. Cyanide is more harmful to the heart and brain, as these organs require a large amount of oxygen.

Immunotoxicity

There are very few reports about the immunotoxicity of the hydrogen cyanide; however, acrylonitrile [also known as vinyl cyanide (VCN)], an environmental pollutant that is metabolized to cyanide, has been shown to be an animal and human carcinogen, particularly in the gastrointestinal tract (Mostafa et al., 1999; National Toxicology Program Technical Report Series, 2001). Hamada et al. (1998) evaluated the systemic and local immunotoxic potential of VCN and demonstrated that VCN induces immunosuppression, as evident by a decrease in the PFC response to SRBCs, a marked depletion of spleen lymphocyte subsets, and bacterial translocation of the normal flora, leading to brachial lymph node abscess. These results suggested that VCN has a profound immunosuppressive effect, which could be a contributing factor in its gastrointestinal tract carcinogenicity.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The immune system of humans and animals is extremely vulnerable to the action of xenobiotics for several reasons. The immune response is associated with rapidly multiplying cells and synthesis of regulatory/effector molecules, and the immune system works as an amplifier for this integrated information network. Immunologic tissue damage can result from the activation of the cellular and biochemical systems of the host. The interactions of an antigen with a specific antibody or with effector lymphocytes trigger a sequence of humoral and cellular events that produce the pathophysiologic effects that lead to tissue injury or disease. Stem cells often appear to be sensitive targets for therapeutic and environmental toxicants, most likely because of their rapid proliferation. Xenobiotics or various drugs that are toxic to the myelocytes of the bone marrow can cause profound immunosuppression due to loss of stem cells.

Humans are now under a sustained and increasing pressure of xenobiotic exposure. Xenobiotics can stimulate the immune system as antigens by provoking a substantial immune response. Even mild disturbances of this network could result in detrimental health effects. The influence of the xenobiotics on the immune system is either suppressive or enhancing. The former leads into immunosuppression, with consequent increased susceptibility to infection and cancer. The latter is associated with the development of autoimmune reactivity, such as delayed hypersensitivity, atopy, systemic or organ-specific immunopathology, and granuloma formation. It is likely that overall immunosuppressive effects of xenobiotics are caused by interference with cellular proliferation and differentiation, down-regulation of cytokine signaling, and enhanced apoptosis of immune cells. In contrast, autoimmune reactions are induced by abnormal activation of immune cells followed by dysregulated production of cytokines, resulting in harmful inflammatory response.

The field of immunotoxicology is developing rapidly. Attempts must be made to conduct basic research into the cellular and molecular mechanisms of the immunomodulatory actions of various xenobiotics. Newly emerging technologies, such as genomics, proteomics, and bioinformatics, certainly will be helpful to investigate the interactions between the immune system and xenobiotics in their full complexities. Toxic compounds may be antigenic or act as haptens and can evoke an antibody response. If these antibodies bind to the determinant on the parent molecule, which is responsible for causing toxicity, that development can lead to the biological inactivation of the parent molecule, thereby preventing toxicity. This may constitute an immunological antidote

that could neutralize the toxicity of certain compounds. Thus, passive administration of these antibodies may be used to prevent the toxic effects of the specific compound, and this approach may be useful in biological or chemical warfare to protect against the toxicity of known chemicals or toxins. The antibodies can also be used to protect industrial workers against the toxic effects of known chemicals or gases during accidental exposure. Although this assumption seems logical, it will involve elaborate and time-consuming research to identify the site of the parent molecule responsible for causing toxicity, to chemically link the molecule with a large protein molecule that should be immunogenic but not toxic, and to screen various antibodies raised for their capacity to prevent the toxicity of the compound.

CWAs have been widely condemned since their first use on a massive scale during World War I. In 1993, the United States signed the Chemical Weapons Treaty, which required the destruction of all chemical weapon agents, dispersal systems, and production facilities by April 2012. The United States destroyed 45% of its stockpile of chemical weapons by 2007. As of 2012, stockpiles have been eliminated at 7 of the 9 chemical weapons depots, and 89.75% of the 1997 stockpile has been destroyed by the treaty deadline of April 2012. The rest are scheduled to be destroyed by 2017. The most recent arms control agreement in international law, the Convention of the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and Their Destruction, known for short as the CWC, outlaws the production, collection, and use of chemical weapons. It is administered by the Organization for the Prohibition of Chemical Weapons (OPCW), an intergovernmental organization based in The Hague. However, in early 2007, multiple terrorist bombings had been reported in Iraq using chlorine gas. These attacks wounded or sickened more than 350 people. Thus, in view of the current global scenario, it appears that the use of CWAs may continue in different types of warfare, as these agents are not only inexpensive, but easy to disseminate with the help of unsophisticated devices. Hence, the medical profession should assemble on a common platform through globally recognized organizations like the World Health Organization (WHO) and make efforts to monitor, research, and study the scientific and medical aspects of CWAs in the interest of humanity. The guidelines on the prevention and management of CWA-induced insults should be updated to reduce morbidity and mortality. Nations worldwide should ensure that adequate supplies of antidotes, protective equipment, and decontamination devices are available in adequate quantities and at all times.

The impact of the immune system is enormous in the health of human beings. CWA-exposed individuals are prone to immune system-mediated diseases. Immunological diseases are growing at epidemic proportions that require aggressive and innovative approaches to develop new treatments. Developing vaccines by recognizing the molecular patterns of some of the xenobiotics can be another approach to tackle the issue and is the most critical challenge for the research community. Furthermore, one of the great therapeutic opportunities for the survivors of war is organ transplantation. However, immune system-mediated graft rejection remains the single greatest barrier to widespread use of this technology. Thus, the most pressing requirement is a multisectorial approach involving health, defense, agriculture, and environmental specialists, with clearly defined roles for each, to establish and maintain effective, robust, and sustainable strategies to countermeasure this threatening situation.

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CHAPTER

45

Alternative Animal Toxicity Testing of Chemical Warfare Agents

Gopala Krishna and Saryu Goel

INTRODUCTION

Chemical warfare agents have been in existence for centuries. They are substances with toxic properties that are used to kill, injure, or incapacitate humans. Broadly, these are categorized into two groups: chemical or biological.

1. Chemical warfare agents use poisons that kill, injure, or incapacitate. These can be gases or liquids and are more commonly dispersed as aerosols.
2. Biological warfare agents use living organisms such as bacteria (e.g., *Bacillus anthracis*, the causative agent of anthrax) or viruses (e.g., Variolae, the virus that causes smallpox).

Further, these warfare agents have been subdivided into multiple classes in the public domain. These include choking gases and lung irritants, blister agents (vesicants), blood agents, nerve agents, incapacitants and psychoactive chemicals, harassing or riot-control agents, vomiting agents, herbicides, Napalm, and obscurant smoke and masking agents. The main routes of entry of these include inhalation, ingestion, injection, and absorption.

The US Food and Drug Administration (FDA), in collaboration with the US Department of Defense (DOD), continues to support the US military and the nation's counterterrorism efforts. The FDA has helped make critical medical products available for combat readiness so that armed forces are better-equipped for combat. It has helped US Special Forces obtain medical products for airborne hospitals used in evacuating battlefield casualties. The FDA has provided consultation and review to help make investigational and licensed medical products such as vaccines and drugs available to combat forces. In developing such warfare agents for counterterrorism, extensive testing of their efficacy and safety is required, especially in animals and humans. A schematic diagram showing the effects of warfare agents and use of 3Rs concept is shown in Figure 45.1. The agreement on Mutual Acceptance of Data (MAD; www.oecd.org/chemicalsafety/testing/mutualacceptanceofdatamad.htm) eliminated the need for duplication of tests in each country before approval of or ban on a substance within Organization for Economic Cooperation and Development (OECD) countries, thus reducing animal use.

It is noted that animal testing is highly controversial and has become an emotional issue in recent years (Krishna, 2010; SOT, 2013a,b). Keeping the focus of this chapter in mind, testing of chemical warfare agents in humans is not practical, especially at toxic doses, and is considered unethical. To address this issue, it is important to understand and recognize the growing impact of alternatives to animal and/or human testing. These methods include *in vitro* toxicity, metabolism, and efficacy/potency testing in a variety of scenarios.

Anadon et al. (2013) highlighted the value of *in vitro* testing in science. They stated that there is a correlation of toxicological data from *in vivo* animal studies to *in vitro* assays. They propose an integrated *in vitro* testing strategy by taking into consideration the following: exposure modeling of chemical agents for *in vitro* testing; data gathering, sharing, and read-across for testing a class of chemical; a battery of tests to assemble a broad spectrum of data on different mechanisms of action to predict toxic effects; applicability of the test and the integrated *in vitro* testing strategies; and flexibility to adjust the integrated *in vitro* testing strategies to test substances (Goldberg and Frazier, 1989; Hartung,

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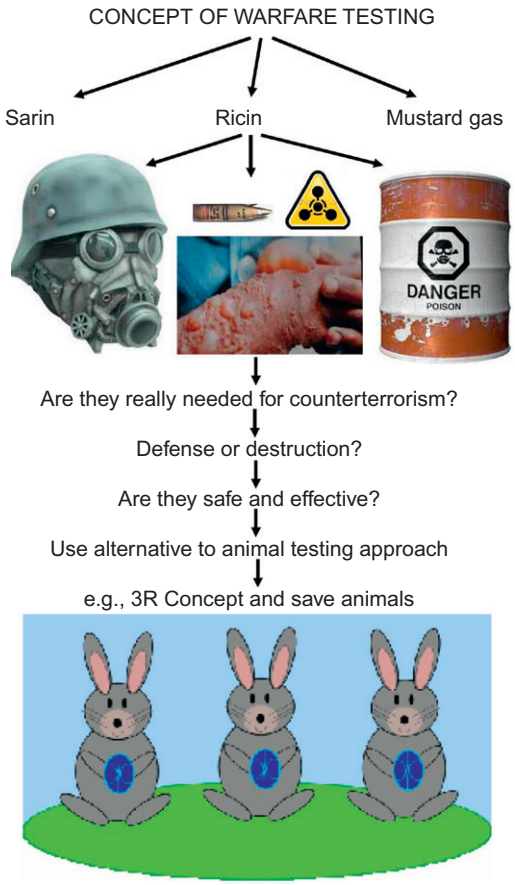

FIGURE 45.1 Alternative concept of warfare testing.

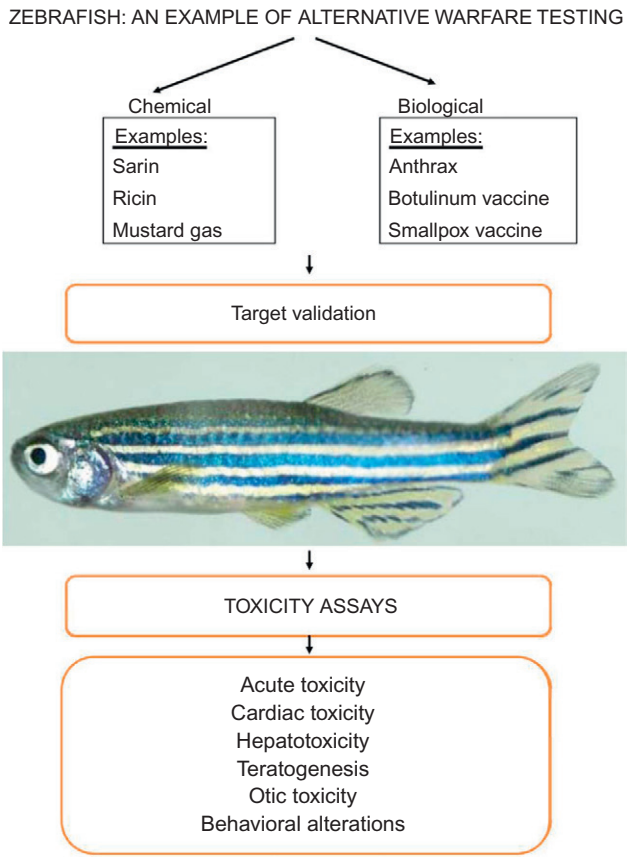

FIGURE 45.2 Zebrafish as an alternative animal model in warfare testing.

2011; Hartung et al., 2013). However, they emphasize validating *in vitro* systems to ensure reliability and suitability for humans; thus, the data can be invaluable and effective. Schechtman (2002) reviewed the implementation of the 3Rs (refinement, reduction, and replacement) validation and regulatory acceptance considerations for alternative toxicological test methods and suggested pros and cons of *in vitro* testing.

Animal testing is an essential part of basic research, especially in inventing and developing effective chemical warfare. Animal testing is defined as the use of nonhumans, such as vertebrate and nonvertebrate animals or organisms, in scientific experimentation in search of answers for the good of humankind and the pursuit of basic knowledge (Krishna et al., 2014). Vertebrate animals ranging from zebrafish to nonhuman primates are commonly used worldwide. However, their use is heavily regulated. An example of zebrafish testing strategy is shown in Figure 45.2. More recently, the zebrafish has been identified as an important vertebrate model for studying the development of embryos and pathogenesis of human diseases (Lieschke and Currie, 2007). When compared with mammalian models, experimental results show that zebrafish embryos exhibit similar responses to test agents (Hill et al., 2005). This suggests that the zebrafish model can be used as a bridge between the *in vitro* and the *in vivo* model in the toxicity screening process.

Similarly, invertebrates such as fruit flies (*Drosophila*) and earthworms are also commonly used in research, when applicable, but their use is excluded from regulations. Around the globe, animal testing is conducted under the strict regulatory guidelines that set fundamental standards for the humane use of animals for training, experimentation, biological testing, or for other related purposes. These regulations and guidelines require minimizing harm to animals through 3Rs, reduction, refinement, and replacement, as initially described by Russell and Burch (1959) searching for alternatives including consideration for hierarchical use of species and *in vitro* methods.

In this chapter, a brief history of chemical warfare use in humans, their classification, use of alternative methods of animal testing, particularly *in vitro* toxicology tests, structure activity relationships, and the use of the 3Rs including "Animal Rule" (FDA, 2009, 2010) and "human-on-a-chip" to possibly test warfare agents are described.

BRIEF HISTORY OF CHEMICAL WARFARE USE

- Poisonous agents have been used as tools of war for thousands of years, even as early as 600 BC; some examples include poisoned arrows, boiling tar, arsenic smoke, and noxious fumes (www.opcw.org/about-chemical-weapons/history-of-cw-use/).
- In the 1900s, modern chemical warfare began on the battlefields of World War I. Chlorine and phosgene gases were released from canisters on the battlefield and dispersed by the wind. The first large-scale attack with chlorine gas occurred on April 22, 1915, at Ieper in Belgium.
- The use of several different types of chemical weapons, including mustard gas (yperite), resulted in 90,000 deaths and more than one million casualties during the war. Those injured in chemical warfare suffered from the effects for the rest of their lives; thus, the events at Ieper during World War I scarred a generation. By the end of World War I, 124,000 metric tons of chemical agents had been expended. The means of delivery for chemical agents evolved over the first half of the twentieth century, increasing these weapons' already frightening capacity to kill and maim through the development of chemical munitions in the form of artillery shells, mortar projectiles, aerial bombs, spray tanks, and landmines.
- After witnessing the effects of such weapons in World War I, it appeared that few countries wanted to be the first to introduce even deadlier chemical weapons on the World War II battlefields. However, preparations were made by many countries to retaliate in kind should chemical weapons be used in warfare. Chemical weapons were deployed on a large scale in almost all stages during World War I and World War II, leaving behind a legacy of old and abandoned chemical weapons that still presents a problem for many countries.
- During the Cold War, the United States and the Soviet Union both maintained enormous stockpiles of chemical weapons, amounting to tens of thousands of metric tons. The amount of chemical weapons held by these two countries was enough to destroy much of the human and animal life on Earth.
- Iraq used chemical weapons in Iran during the war in the 1980s, and Iraq also used mustard gas and nerve agents against Kurdish residents of Halabja, in Northern Iraq, in 1988.
- The two most recent examples of the use of chemical weapons are the sarin poisoning incident in Matsumoto, a Japanese residential community, in 1994, and the sarin attack on the Tokyo subway in 1995, both perpetrated by the Aum Shinrikyo doomsday cult. These two attacks re-focused international attention on the potential use of chemical weapons by terrorists and on the dangers posed by these chemical weapons.
- The devastating impact chemical weapons have had in the past and the potential for the use of modern—even more deadly—chemical agents not only by States at war, but also by non-State actors in other violent conflicts, provide the imperative for an international effort to uphold the ban on such weapons and to work toward the complete global elimination of chemical weapons.

TOP FIVE CHEMICAL WARFARE AGENTS

It has been reported that sarin, ricin, mustard gas, agent 15, and chlorine gas are the top chemical warfare agents (www.livescience.com/39332-5-chemical-warfare-agents.html).

1. *Sarin* is a deadly toxic organophosphate compound with no color, taste, or odor. Although it is produced as a liquid, its low evaporation point allows it to turn into a gas quickly when exposed to the environment. Sarin, also known as GB by military personnel, was originally developed as a pesticide in Germany in 1938; since then, it has been classified by many national governments as a chemical nerve agent. Nerve agents are the most toxic and fast-acting chemical warfare agents in the world. People exposed to large amounts of sarin quickly lose control over their bodily functions and, if not treated immediately, can fall into a coma or succumb to respiratory failure.
2. *Ricin* is derived from a common plant, the castor bean (*Ricinus communis*), and is native to the Mediterranean and Middle East and cultivated elsewhere as an ornamental plant. It is also the source of castor oil, which has many uses in medicine, food, and industry. Ricin is also a highly potent toxin that can kill a person in amounts as small as a few grains of sand.
3. *Mustard gas*, or sulfur mustard $(Cl\text{-}CH_2CH_2)2S$, is a chemical agent that causes severe burning of the skin, eyes, and respiratory tract. It can be absorbed into the body through inhalation, ingestion, or by contact with the skin or eyes. First used during World War I, the gas is effective at incapacitating its victims en masse. Sulfur mustard is generally colorless in its gaseous state, although it may have a faint yellow or green tint. It is easily recognized by its trademark "mustardy" odor, although some compare its smell with that of garlic, horseradish, or sulfur.

4. *Agent 15* is also called compound 3-quinuclidinyl benzilate, BZ, or "Buzz." It is a powerful chemical warfare agent. As one of the most potent psychoactive chemical agents, only a small amount of BZ is needed to produce complete incapacitation. When used as an aerosol, BZ is absorbed through the respiratory system (it has no odor). It can also be absorbed through the skin or the digestive system. It takes approximately 1 h for BZ to take effect, and the symptoms of exposure include confusion, tremors, stupor, hallucinations, and coma that can last for more than 2 days. Use of BZ was suspected in the Bosnian conflict in 1995. Similarly, in January 2013, Syrian government troops may have used Agent 15 on rebels.
5. *Chlorine gas* is another chemical agent with a history of use going back almost 100 years. During World War I, chlorine gas, sometimes known as bertholite, was used by the German army during the Second Battle of Ypres in Belgium. Because chlorine can be pressurized and cooled into a liquid, it can be easily shipped and stored in tanks. When released as a gas, chlorine stays close to the ground and spreads quickly, making it an ideal agent for warfare or terrorism. Although it is used less often today (because more lethal agents exist), chlorine is easy to manufacture and disguise because it has many other civilian uses such as water sanitation. In 2007, chlorine gas bombs were used to kill dozens of people during the fighting in Iraq.

Toxicology of these warfare agents and many more have been elegantly described elsewhere in this book. Table 45.1 lists a variety of warfare agents. The focus of this chapter is alternatives of animal testing of such warfare agents. The tests mentioned and the strategies described might have to be optimized to suit such test-setting, depending on the type of warfare agent whether it is liquid, solid, aerosol, or even a biological agent.

TABLE 45.1 Listing of Multiple Categories of Chemical Warfare Agents

Name	CAS Number	Chemical Name
BLOOD AGENTS		
Arsine	7784-42-1	
Cyanogen chloride	506-77-4	
Hydrogen cyanide	74-90-8	
VOMITING AGENTS		
Adamsite	578-94-9	(10-Chloro-5,10-dihydrophenarsazine)
Diphenylchloroarsine	712-48-1	
Diphenylcyanoarsine	23525-22-6	
NERVE AGENTS		
Cyclohexyl sarin	329-99-7	Cyclohexylmethylphosphonofluoridate
GE	1189-87-3	Phosphonofluoridic acid, ethyl-, 1-methylethyl ester
Sarin	107-44-8	Phosphonofluoridic acid, methyl-, 1-methylethyl ester
Soman	96-64-0	Phosphonofluoridic acid, methyl-, 1,2,2-trimethylpropyl ester
Tabun	77-81-6	Phosphoramidocyanidic acid, dimethyl-, ethyl ester
VE	1189-87-3	Phosphonofluoridic acid, ethyl-, 1-methylethyl ester
Amiton	78-53-5	Phosphorothioic acid, *S*-[2-(diethylamino)ethyl] *O*,*O*-diethyl ester
GV (also GP)	141102-74-1	Phosphoramidofluoridic acid, dimethyl-, 2-(dimethylamino)ethyl ester
VM	21770-86-5	Phosphonothioic acid, methyl-, *S*-[2-(diethylamino)ethyl] *O*-ethyl ester
VX	50782-69-9	Phosphonothioic acid, methyl-, *S*-[2-[bis(1-methylethyl)amino]ethyl] *O*-ethyl ester

(*Continued*)

Name	CAS Number	Chemical Name
BLISTER AGENTS		
Mustard gas	505-60-2	Bis(2-chloroethyl) sulfide
Nitrogen mustard	0538-07-08	Bis(2-chloroethyl)ethylamine
Nitrogen mustard	51-75-2	Bis(2-chloroethyl)methylamine
Nitrogen mustard	555-77-1	Tris(2-chloroethyl)amine
Phosgene oxime	1794-86-1	
2-Chloroethyl ethyl sulfide	0693-07-02	
Sesqui mustard	3563-36-8	1,2-Bis(2-chloroethylthio)ethane
Ethyldichloroarsine	598-14-1	
Lewisite	541-25-3	2-Chlorovinyldichloroarsine
Lewisite-2	40334-69-8	Bis(2-chlorovinyl)chloroarsine
Lewisite-3	40334-70-1	Arsine, tris(2-chloroethenyl)-
Methyldichloroarsine	593-89-5	Arsonous dichloride, methyl-
Mustard/Lewisite		
Phenyldichloroarsine	696-28-6	Arsonous dichloride, phenyl-
RIOT CONTROL/TEAR AGENTS		
Bromobenzylcyanide	16532-79-9	4-Bromophenylacetonitrile
Chloroacetophenone	532-27-4	
Chloropicrin	1976-06-02	
CNB		10% CN, 45% benzene, 45% carbon tetrachloride
Fentanyl	437-38-7	Propanamide, *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]-
CNC		30% CN in chloroform
CNS		23% CN, 38% chloropicrin, 38.4% chloroform
Dibenz-(*b,f*)-1,4-oxazepine	0257-07-08	
O-chlorobenzylidenemalononitrile	2698-41-1	CS1, CS2, CSX all have CS as agent
PULMONARY (CHOKING) AGENTS		
Ammonia	7664-41-7	
Chlorine	7782-50-5	
Hydrogen chloride	7647-01-0	
Phosgene	75-44-5	
Diphosgene	503-38-8	Trichloroacetyl chloride
Titanium tetrachloride	7550-45-0	
Nitric oxide	10102-43-9	
Perflurorisobutylene	382-21-8	1,1,3,3,3-Pentafluoro-2-(trifluoromethyl)-1-propene
Red phosphorous	7723-14-0	
Sulfur trioxide-chlorosulfonic acid		(Smoke mixture)
Sulfur trioxide	7446-11-9	
Chlorosulfonic acid	7790-94-5	
Zinc oxide	1314-13-2	
Agent 15 (similar to BZ)		
3-Quinuclidinyl benzilate	6581-06-2	3-Quinuclidinyl benzilate
Delta-9-THC	33086-25-8	Delta-9-Tetrahydrocannabinol
China White	79704-88-4	Alpha-methylfentanyl
Fentanyl	437-38-7	Propanamide, *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]-
LSD	50-37-3	D-Lysergic acid *N,N*-diethylamide
Phenothiazine	92-84-2	Phenothiazine
Thorazine	50-53-3	Chlorpromazine

THE CONCEPT OF 3RS

The concept of 3Rs is further explained by the National Center for the Replacement, Reduction, and Refinement of animals in research (NC3Rs; http://eslav-eclam.org/other-associations/nc3rs). This is a widely accepted ethical framework for conducting scientific experiments using animals humanely. This includes:

- *Replacement*: Use methods that avoid or replace the use of animals defined as "protected" under the Animals (Scientific Procedures) Act 1986, amended 2012 (ASPA), in an area where they would otherwise have been used. "Protected" animals are all living vertebrates (other than humans), including some immature forms, and cephalopods (e.g., octopus, squid, cuttlefish). These can be:
  - Absolute replacements—those that do not involve animals at any point.
  - Relative replacements—those that avoid or replace the use of "protected" animals.
  - Examples include:
    - Computer modeling (such as Lhasa, 2014) (Figure 45.3).
    - Human volunteers (e.g., for noninvasive imaging studies).
    - Invertebrates, such as *Drosophila* (fruit fly) and nematode worms.
    - Immature forms of vertebrates—mammal, bird, and reptile embryos up to the last third of their gestation or incubation period, larval forms of amphibians and fish until the stage where they become capable of independent feeding, and cephalopods until the point at which they hatch.
    - *In vitro* methodologies, utilizing established human or animal cell lines, animal cells, tissues, and organs from animals killed by a humane method, and abattoir material from the meat industry (Figures 45.4 and 45.5).
- *Reduction*: Use methods that minimize animal use and enable researchers to obtain comparable levels of information from fewer animals or to obtain more information from the same number of animals, thereby further reducing future use of animals.
  - Examples include:
    - Sharing data and resources, such as MAD agreement.
    - Improved experimental design and statistical analysis.
    - Integrated genotoxicity testing using fewer animals and collecting maximum information. The concept of integrating toxicity assessment, which uses fewer animals but a variety of relevant toxicity data can be collected and

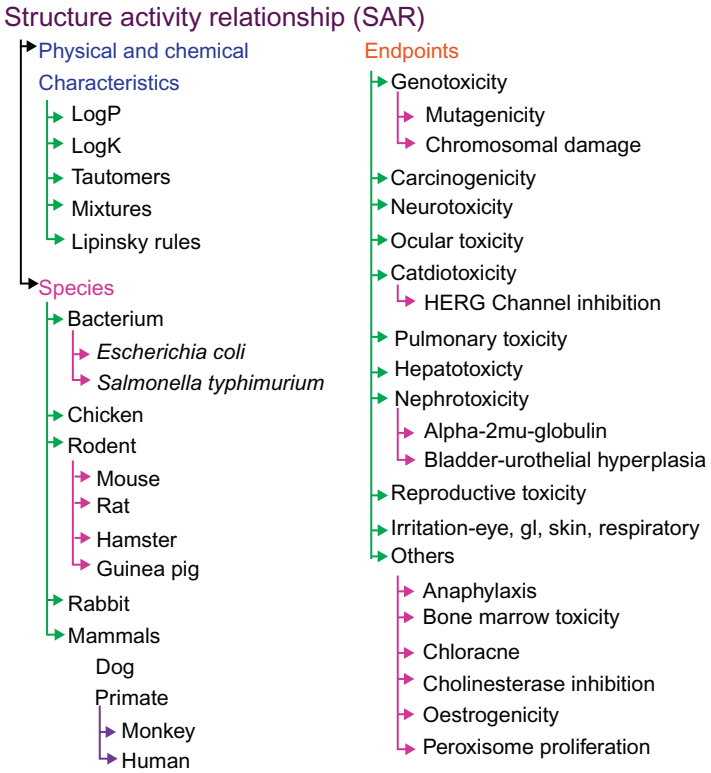

FIGURE 45.3 *In silico* endpoints commonly used to achieve 3Rs.

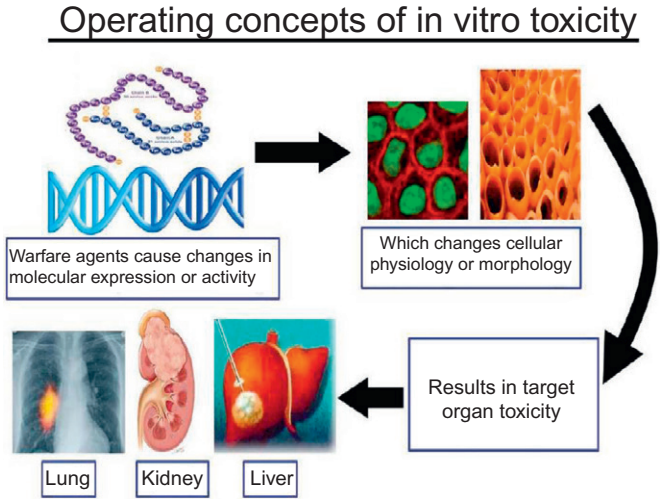

FIGURE 45.4 Warfare agents cause changes at the molecular level (DNA or protein) that are expressed at the cellular level, which in turn result in target organ toxicity (e.g., liver, kidney) and can be studied and extrapolated to whole animal or humans based on *in vitro* studies.

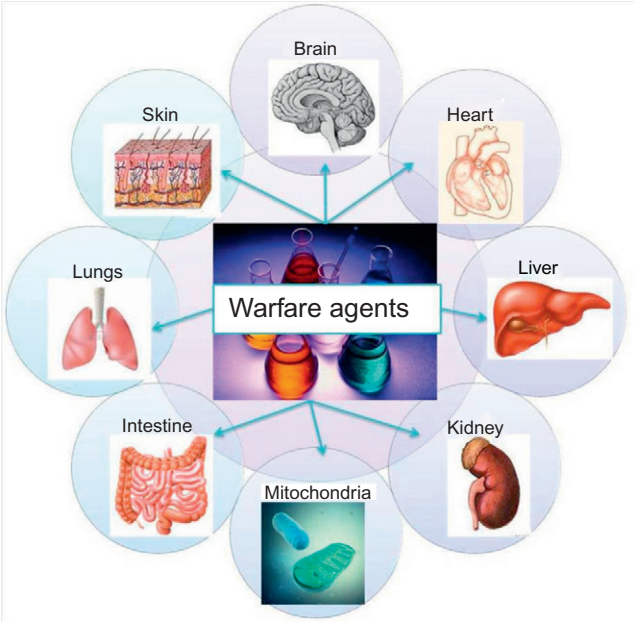

FIGURE 45.5 Warfare agents interact with various cells, organelles, and organ systems to produce their toxicological effects. These effects can now be studied using various *in silico* or *in vitro* tools minimizing the use of animal testing.

evaluated (Krishna et al., 1994, 1995a,b, 1998, 2000; Figure 45.6). As an example, an integrated *in vivo* genotoxicity testing philosophy and a practical approach, as applied to pharmaceuticals, is currently practiced and is recommended by the International Conference on Harmonization (ICH) guidance (ICH, 2006). In this case, a rodent (primarily rat) micronucleus assay is integrated with routine 2-week to 4-week repeat dose toxicity and toxicokinetic studies. This approach has several advantages. First, it utilizes the general principles of toxicology that govern the overall toxicity profile of a test substance. Second, factors such as the dose and/or route of test agent administration, metabolism, principles of toxicokinetics, and saturation of defense mechanisms are considered in evaluating genotoxicity. Third, it uses the concept of administering multiple tolerable doses aiding in achieving steady-state plasma test agent levels, which is more relevant for risk assessment compared with high acute doses. Fourth, it helps minimize the amount of test agent, number of animals used, and other resources. This integration approach can be extended to other toxicology studies and other relevant genotoxicity endpoints may be assessed. Based on experience reported in the literature, integrating micronucleus assessment in routine toxicology testing is promising and should be utilized when practical. A number of genotoxicity endpoints can be easily evaluated in peripheral blood lymphocytes of exposed subjects by genetic monitoring.

- Modern imaging techniques
  - Noninvasive, whole-body imaging of small animals using techniques such as X-ray, CT, SPECT, PET, and MRI is helping to reduce the number of animals used in basic research and testing of chemical warfare agents. Figure 45.7 illustrates such an example. The same animal can be imaged multiple times to monitor visually, often in real time, the progression or regression of infection or disease. This avoids the need to sequentially sacrifice animals at different time points, allowing significant reductions in the number of animals used per study.

- *Refinement*: Use improvements to scientific procedures and husbandry that minimize actual or potential pain, suffering, distress, or lasting harm and/or improve animal welfare in situations in which the use of animals is unavoidable. Refinement applies to the lifetime experience of the animal. There is evidence that refinement not only benefits animals, but also can improve the quality of research findings. Figure 45.8 shows a unique refinement of housing mice.
  - Examples include:
    - Noninvasive techniques.

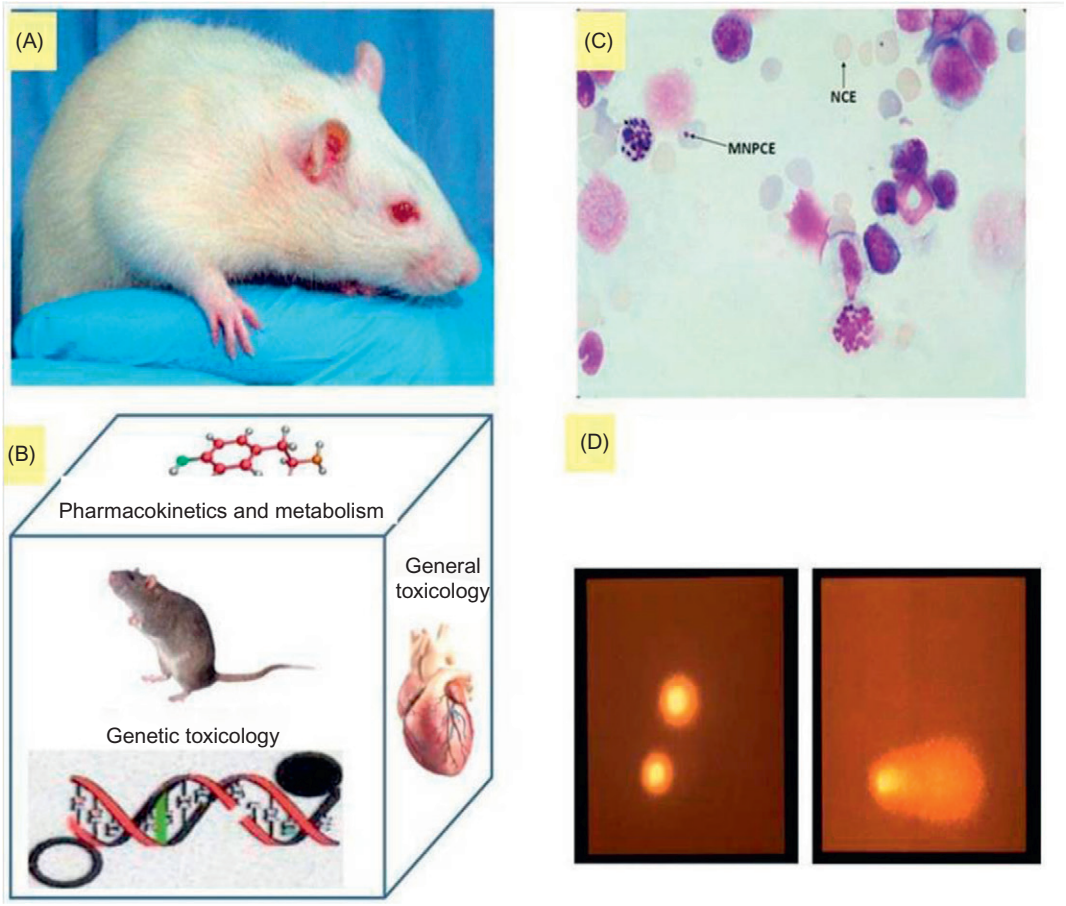

FIGURE 45.6 Integrated *in vivo* genotoxicity of warfare agents can be evaluated using samples obtained during the general toxicity study. (A) Samples are obtained from the animal (e.g., rat). (B) The test agent is exposed to *in vivo* biochemical processes, including metabolism and opportunity to interact with the DNA, thereby providing intact animal with homeostatic assessment of the genotoxic potential of test agent. (C) The blood sample/bone marrow is obtained and stained to detect presence of micronuclei in polychromatic erythrocytes; if positive, then the test agent is considered as clastogenic. (D) Cells, particularly from the liver, are processed and tested for DNA strand breaks in the COMET assay. The breaks in the DNA are observed as a comet-shaped migration of DNA from the nucleus of the cell after gel electrophoresis in treated compared with intact round nucleus, suggesting unaffected DNA (control).

- Appropriate anesthetic and analgesic regimes for pain relief.
- Training animals to voluntarily cooperate with procedures (e.g., blood sampling) so that they have greater control over the procedure and are less stressed.
- Accommodation and environmental enrichment that meet the animals' physical and behavioral needs (e.g., providing opportunities for nesting for rodents).

INTERNATIONAL COOPERATION ON ALTERNATIVE TEST METHODS

In 2009, the United States, Canada, Japan, and European Union signed a memorandum of cooperation that could reduce the number of animals required for consumer product safety testing worldwide (International Cooperation on Alternative Test Methods (ICATM); http://ntp.niehs.nih.gov/?objectid=62A650A4-DD4B-D0A8-C26C7AE0A57F82E8). A similar approach can be used for evaluating chemical warfare agents. The agreement is hoped to yield globally coordinated scientific recommendations for alternative toxicity testing methods that should speed their adoption in each of these countries, thus reducing the number of animals needed for safety testing. A flowchart of this is shown in Figure 45.9.

ICH: The ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use.

OECD: The OECD has a Test Guideline program that deals with chemicals.

ICCR: The International Cooperation on Cosmetics Regulation (ICCR) is an international group of cosmetic regulatory authorities from the United States (FDA), Japan (Ministry of Health, Labor, and Welfare),

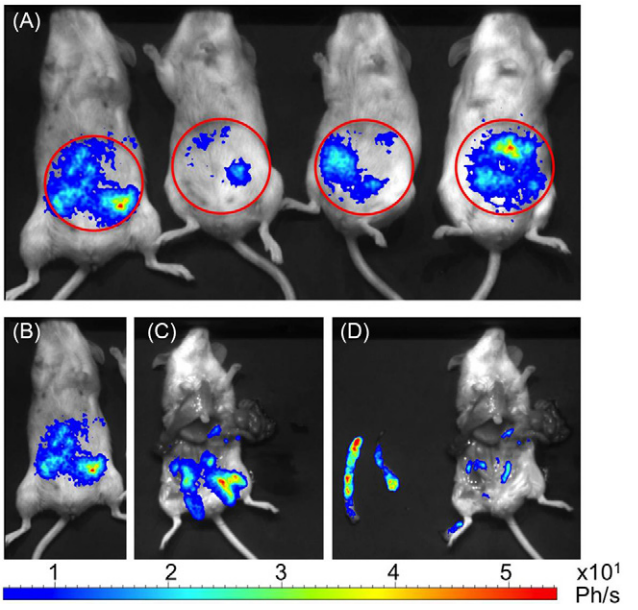

FIGURE 45.7 Localization of bioluminescent parasites in intra-abdominal mesenteric fat in infected mice. Infection was monitored by bioluminescence imaging (BI) technique. On day 40 after infection (A), mice showed intraperitoneal parasites localized by BI. One representative mouse (B) was dissected and the adipose tissue localization was verified *in situ* by BI (C) and confirmed after removing the adipose tissue and re-imaging (D).

FIGURE 45.8 The "mouse house" is a refinement using a transparent red plastic house that enables the mice to perform natural behaviors such as nesting, hiding, and climbing, which are important for their welfare. The house appears dark to the mice, yet the transparent walls allow the scientist to make observations without disturbance (www.scanbur.com).

the European Union (EC, DG Enterprise), and Canada (Health Canada). This multilateral framework maintains the highest level of global consumer protection while minimizing barriers to international trade.

The alternative toxicology methods listed and suggested may be used for chemical warfare agents, as applicable. Nonanimal methods for toxicity testing currently being used are listed in Table 45.2. This was initially compiled by an organization called AltTox (2013), but was modified to serve the purposes of this chapter.

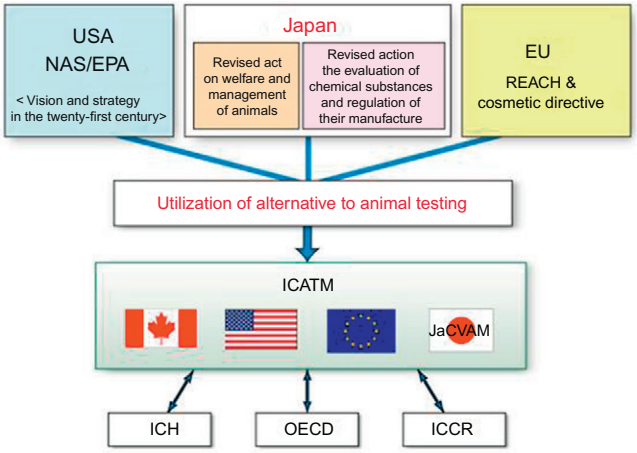

FIGURE 45.9 International Cooperation on Alternative Test Methods.

These methods are also used by organizations such as Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH, 2013).

ALTERNATIVES TO ANIMAL TESTING OF CHEMICAL WARFARE AGENTS

Greenfield et al. (2014) report that the US Army Medical Research Institute of Chemical Defense (USAMRICD) has been commissioned with a mission to develop medical countermeasures against exposure to chemical warfare agents as well as against agents of biological origin. Over the years, this organization has reduced the number of animals used in their research protocols by 92%. Based on a report by the US House of Representatives Committee on the Armed Services regarding the use of animals in DOD military experiments, initiatives to promote alternatives to reduce, refine, and replace (3Rs) were undertaken in 1992. During this time, while adopting the 3Rs, the DOD also added a fourth R, responsibility. This organization developed a unique concept called the management by objective program based on science and technology objectives. One such major objective was to develop reduction, refinement, and replacement strategies for the use of animals in research. The goal was to develop technologies that would incrementally reduce reliance on animal and human subject research and improve the experimental conditions using animals. This further requires introducing a minimum of one improvement per year in experimental protocols using animals.

The scientists at the USAMRICD have taken advantage of emerging computer and biological technologies in the design of molecular modeling software and in the development and maintenance of cell culture models to

TABLE 45.2 Alternative Test Methods and Testing Strategies for Chemical Warfare Agents

Endpoint	Method Name	Test Type
Acute mammalian toxicity (oral)	Acute toxic class method	*In vivo*
	Fixed dose procedure	*In vivo*
	Up-and-down procedure	*In vivo*
	Normal human keratinocyte neutral red uptake (NHK NRU) assay	*In vitro*
	Balb/c 3T3 neutral red uptake assay	*In vitro*
Acute mammalian toxicity (hematotoxicity)	Colony Forming Unit-Granulocyte/Macrophage Assay for acute neutropenia in humans	*In vitro*
Acute mammalian toxicity (inhalation)	Acute toxic class method	*In vivo*
Acute toxicity testing of pesticides	Guidance for waiving or bridging of mammalian acute toxicity tests for pesticides (acute oral, dermal, inhalation; primary eye and dermal; dermal sensitization)	*In vivo*
Biologics and vaccines	Enzyme Linked Immuno-Sorbent Assay (ELISA) for swine erysipelas vaccines batch potency testing	*In vitro*
	ELISA for human tetanus vaccines batch potency testing	*In vitro*
	Toxin binding inhibition test for human tetanus vaccines batch potency testing	*In vitro*
	Batch potency testing of erythropoietin concentrated solution	*In vivo*
	Deletion of target-animal safety test for batch safety testing of veterinary vaccines after consistency in 10 consecutive batches	*NA*
	ELISA for *in vitro* batch potency testing of *Leptospira* veterinary vaccines	*In vitro*
	Use of humane endpoints in animal testing of veterinary biologics, including rabies vaccines	*In vivo*
	Veterinary vaccine potency assays: exemptions from Standard Requirements tests; master reference qualification and requalification	*NA*
	Cell-based assay for stability and potency of botulinum neurotoxin type A products	*In vitro*
	Alternative test procedure for tuberculin, Purified Protein Derivative (PPD) Bovis, intradermic	*In vivo*
Carcinogenicity	Three cell transformation assays (CTA): (Syrian Hamster Embryo (SHE) CTA performed at pH 6.7, SHE CTA performed at pH 7.0, and BALB/c 3T3 CTA)	*In vitro*
Chronic toxicity	*Ending* 1-year dog studies of pesticides	*In vivo*
Dermal absorption/ penetration	*In vitro* skin absorption methods	*In vitro*
Ecotoxicity	Acute aquatic toxicity: upper threshold concentration step-down approach	*In vivo*
	Acute avian toxicity (oral): Sequential testing procedure to minimize numbers of birds used	*In vivo*
	Fish embryo toxicity	*In vivo*
Endocrine active substances	Androgen receptor binding assay (rat prostate cytosol)	*Ex vivo*
	Aromatase inhibition assay (human recombinant)	*In vitro*
	Stably transfected trans-activation *in vitro* assays to detect estrogen receptor agonists	*In vitro*
	Estrogen receptor binding assay rat uterine cytosol (ER-RUC)	*Ex vivo*
	H295R steroidogenesis assay	*In vitro*
	US EPA Tier 1 Screening Battery	*In vitro/In vivo*
	BG1Luc ER TA test method for estrogen agonists and antagonists	*In vitro*
Eye corrosion	Bovine corneal opacity permeability (BCOP) test	*Ex vivo*
	Cytosensor Microphysiometer modified	*In vitro*
	Fluorescein Leakage	*In vitro*
	Hen's egg test-chorioallantoic membrane (HET-CAM)	*In vitro*
	Isolated chicken eye (ICE) test	*Ex vivo*
	Isolated rabbit eye test	*Ex vivo*
	Routine use of topical anesthetics, systemic analgesics, and humane endpoints	*In vivo*
	Sequential testing strategy for eye irritation and corrosion	*In vitro/Ex vivo/In vivo*

(*Continued*)

Endpoint	Method Name	Test Type
Eye irritation	Cytosensor Microphysiometer modified	*In vitro*
	Rabbit low-volume eye test (LVET)	*In vivo*
	Routine use of topical anesthetics, systemic analgesics, and humane endpoints	*In vivo*
	Sequential testing strategy for eye irritation and corrosion	*In vitro/Ex vivo/In vivo*
Genotoxicity	Bacterial reverse mutation (Ames) test	*In vitro*
	In vitro cell gene mutation test	*In vitro*
	In vitro chromosomal aberration test	*In vitro*
	In vitro mammalian cell micronucleus test	*In vitro*
	In vitro sister chromatid exchange test	*In vitro*
	In vitro unscheduled DNA synthesis test	*In vitro*
	Saccharomyces cerevisiae gene mutation assay	*In vitro*
	S. cerevisiae mitotic recombination assay	*In vitro*
Immunotoxicity/skin sensitization	Local lymph node assay (LLNA)	*In vivo*
	Reduced LLNA: rLLNA	*In vivo*
	Nonradiolabelled LLNA: DA	*In vivo*
	Nonradiolabelled LLNA: BrdU-ELISA	*In vivo*
	LLNA for Potency Categorization of Skin Sensitizers	*In vivo*
Phototoxicity	3T3 Neutral Red Uptake Phototoxicity Test	*In vitro*
	3T3 NRU Phototoxicity Test: Application to UV filter chemicals	*In vitro*
Preclinical and nonclinical safety studies for drug development	Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2) (harmonized guidance can reduce use of animals)	*In vivo*
	Preclinical safety evaluation of biotechnology-derived pharmaceuticals, ICH S6(R1)	*In vivo*
Pyrogenicity	Human whole blood IL-1	*In vitro*
	Human whole blood IL-6	*In vitro*
	Human cryopreserved whole blood IL-1	*In vitro*
	PBMC IL-6	*In vitro*
	MM6 IL-6	*In vitro*
	Limulus amebocyte lysate (LAL) test	*In vivo/In vitro*
Reproductive and developmental toxicity	Embryonic stem cell test for embryotoxicity	*In vitro*
	Micromass embryotoxicity assay	*Ex vivo*
	Whole rat embryotoxicity assay	*Ex vivo*
	Extended one-generation reproductive toxicity study	*In vivo*
Skin corrosion	EST-1000 human reconstructed epidermis	*In vitro*
	Membrane Barrier Corrosivity Test Method (Corrositex®)	*In vitro*
	EpiSkin® human skin model	*In vitro*
	EpiDerm™ human skin model	*In vitro*
	Rat skin transcutaneous electrical resistance (TER) assay	*Ex vivo*
	SkinEthic™ human skin model	*In vitro*
	Vitrolife-Skin™ human reconstructed epidermis	*In vitro*
Skin irritation	EpiSkin® skin irritation test (with MTT reduction)	*In vitro*
	EpiDerm™ skin irritation test (with MTT reduction)	*In vitro*
	EpiDerm™ SIT model (EPI-200)	*In vitro*
	SkinEthic™ RHE model	*In vitro*

meet the animal reduction goals. In addition, scientists, when possible, have adopted the use of less sentient animal species.

As an example, computer modeling of the molecular structure of nerve agents, physiological enzymes, and neurotransmitters are used to predict and eventually determine how these chemical compounds interact at the molecular level. A test compound that inhibits acetylcholinesterase (AChE) aging has also been studied via computer modeling to determine the specific molecular events that occur at the peripheral anionic site of AChE, which subsequently prevents a potent AChE inhibitor, such as the nerve agent sarin, from irreversibly binding to the AChE molecule (Khan et al., 2000). With this knowledge, the most likely candidates for chemical intervention in the prevention or treatment of nerve agent exposure can be determined. To this end, multiple cell lines or cell cultures are used in research and have led to a reduction of animal usage in research programs for blistering chemical warfare agents. In an effort to evaluate the effects of blister agents, or vesicants, particularly sulfur mustard, and to develop medical countermeasures against such agents, alternatives to animals have proven particularly useful. Several cell line or culture systems, such as peripheral blood lymphocytes, human epidermal keratinocytes, and the HeLa, a human epithelial tumor line, have been adapted and developed by scientists into valuable models for studying vesicant injury. In addition, studies have used a commercial human skin equivalent model and skin biopsy specimens from the Cooperative Human Tissue Network. The USAMRICD also has developed the technology to process and generate a human epidermal model, which possesses typical structural components of human epidermis *in vivo* to include hemidesmosomes, anchoring filaments, and elements of a true basement membrane. The use of these alternative models has especially led to a decrease in the number of rodents required for these studies.

The USAMRICD also promotes the use of less sentient or less regulated animal species, for example, the use the *Aplysia californica*, a large naked marine mollusk or gastropod with anterior sensory tentacles, commonly referred to as the sea hare or sea slug. These slugs possess large and discrete neural ganglia and unique neural anatomy that makes these invertebrate animals an excellent and widely used model for neural transmission and neurotoxin research. Murphy and Glanzman (1997) have described "classical conditioning" based on neurological studies in Aplysia. It appears to be mediated, in part, by long-term potentiation due to activation of *N*-methyl-D-aspartate-related receptors. In certain sulfur mustard experimental protocols, the USAMRICD has used SKH-1 hairless mice as an appropriate substitute for the hairless guinea pig. The main histological feature of skin lesions produced in a SKH-1 hairless mouse after exposure to sulfur mustard is the formation of microblisters at the dermal–epidermal junction. Similar microblisters also occur in sulfur mustard lesions of hairless guinea pigs. Although laboratory mice are not subject to the Animal Welfare Act, provisions for their care and use are contained in the *Guide for the Care and Use of Laboratory Animals*.

ANIMAL EFFICACY RULE

The animal efficacy pathway was formed soon after the terror attacks of 2001, which included a harrowing series of incidents in which spores of anthrax were sent through the US Postal Service, killing several people and sickening many more (www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=314&showFR=1&subpartNode=21:5.0.1.1.4.9).

In response to the attacks, legislators and regulators determined that a regulatory process needed to be established to test the safety and effectiveness of so-called medical countermeasures while taking into account the extremely dangerous nature of the pathogens against which they would need to protect their subjects. The problem, at its most basic level, was testing a drug product in humans that could potentially kill them. However, testing for efficacy in humans is not performed. Instead, the FDA ultimately established the animal efficacy rule, which is an approval pathway through which manufacturers can apply for conditional approval of a medical countermeasure based on efficacy testing in human analogues such as pigs or chimpanzees or other appropriately relevant models. Safety testing is still conducted in humans.

Pyridostigmine bromide is the first drug approved under the "animal efficacy rule" that allows use of animal data for evidence of the drug's effectiveness for certain conditions when the drug cannot be ethically or feasibly tested in humans.

The "animal efficacy rule," which became effective on June 30, 2002, is an important component of the FDA's efforts to make medical countermeasures available to treat or prevent the effects of biological and chemical agents. The "animal efficacy rule" enabled the FDA to approve pyridostigmine bromide to increase survival after soman poisoning despite the impossibility of ethically conducting human studies on the effectiveness of the drug.

The nerve agent soman causes loss of muscle control and death from respiratory failure. Evidence of the effectiveness of pyridostigmine bromide as a pretreatment for exposure to soman was obtained primarily from studies in monkeys and guinea pigs. This evidence shows that administration of the drug before exposure to soman, together with atropine and pralidoxime administered after exposure, increases survival. The FDA believes

that, based on the animal evidence of effectiveness, pyridostigmine bromide is likely to benefit humans exposed to soman.

The agency's safety assessment is based on long-term use of pyridostigmine bromide, which was first approved by the FDA in 1955, to treat a neuromuscular disease called myasthenia gravis. The Department of the Army has submitted data from multiple controlled trials and uncontrolled clinical experience demonstrating pyridostigmine bromide is well-tolerated at the doses intended for military use. The dose used for myasthenia gravis is higher than the dose used for pretreatment to protect against soman.

To use this potentially life-saving drug correctly, military personnel must carefully follow instructions and use the drug only under specific circumstances. For example, if US troops faced the threat of exposure to soman, then they would be given instructions to take pyridostigmine bromide every 8 h before the anticipated exposure. Soldiers would be warned that the drug is not effective and should not be taken at the time of, or after, exposure to soman.

The troops are to use the drug in conjunction with other protective measures, including chemical protective masks and battle dress garments. Furthermore, effectiveness depends on the rapid use of the antidotes atropine and pralidoxime and discontinuation of pyridostigmine bromide at the first indication of nerve gas exposure. The Department of Defense plans to provide all military personnel with extensive training, before deployment, regarding the proper use of pyridostigmine bromide, as well as other methods used in the prevention and treatment of nerve agent poisoning.

A leaflet that explains the drug's use, benefits, and side effects will be provided to military personnel when the drug is distributed. The leaflet advises that pyridostigmine bromide should not be used by persons who have a history of bowel or bladder obstruction, or sensitivity to certain medicines used during surgery (like physostigmine). Side effects that may occur include stomach cramps, diarrhea, nausea, frequent urination, headaches, dizziness, shortness of breath, worsening of peptic ulcer, blurred vision, and watery eyes.

The approved dose of pyridostigmine bromide for soman pretreatment is one 30 mg tablet every 8 h. The leaflet states that pyridostigmine should be started at least several hours before exposure to soman and emphasizes that it must be discontinued on exposure to nerve gas, at which point the antidotes atropine and pralidoxime are administered.

During the Gulf War, the FDA had allowed distribution of pyridostigmine bromide under its Investigational New Drug provisions because pretreatment with this drug had the potential to help save lives if nerve agents were used.

Johnson & Johnson's Levaquin (levofloxacin), an antibiotic intended to treat pneumonic plague, became the first product to receive FDA (2012a) approval via the Animal Rule. The product received approval based on testing in African green monkeys infected with pneumonic plague. None of the placebo control group survived contact with the virus, whereas 94% of the Levaquin group survived.

The FDA's (2012b) approval of raxibacumab marks just the second approval under the Animal Rule, and it is also the first monoclonal antibody. The drug is also notable in that it is intended to treat inhalation anthrax—the pathogen perhaps most responsible for the formation of the animal efficacy rule.

Raxibacumab was tested in one study involving monkeys and three studies involving rabbits. As with Levaquin, none of the animals in the control groups survived being infected with inhalation anthrax, whereas 64% of monkeys and 44% of rabbits in one study treated with raxibacumab survived. The FDA also said GSK's animal studies showed the drug was an improvement over existing antibiotic therapies, according to the animal studies. It is said that the drug is specifically aimed at defending against another bioterrorism-type event. Although antibiotics are approved to prevent and treat anthrax infection, raxibacumab is the first approved agent that acts by neutralizing the toxins produced by *B. anthracis*.

- *Improvements to Animal Rule*
  Both approvals come as the FDA and other government entities are looking into ways to refine the Animal Rule process and the protections it affords to the human subjects it is intended to protect.

  Members of the Alliance for Biosecurity (Gronvall et al., 2007) have made three recommendations for effective implementation of Animal Rule.
- First, the Department of Health and Human Services, the FDA, and other US agencies provide strategic direction about how countermeasures may be used. This will aid development and testing.
- Second, the FDA, along with the National Institute of Allergy and Infectious Diseases, should actively develop scientific consensus on animal models for specific disease threats.
- Third, the FDA should develop a consistent interpretation of the Rule within the agency.
- Aebersold (2012) reviewed the FDA experience with medical countermeasures under the Animal Rule. The author concludes that even though only a few drugs or biologicals have been approved since the Animal Rule became effective, several investigational drugs have been placed in the National Strategic Stockpile for use as medical countermeasures, if needed.

TABLE 45.3 Agents Approved Using "Animal Rule"

Compound	Company	Purpose
Abthrax (Raxibacumab)	Glaxo Smith-Kline (GSK)	Anthrax vaccine
Cynokit	Orphan Medical, Inc/Merck Sante	Cyanide poisoning
Ciprofloxacin	Bayer	Anthrax
Levofloxacin	Janssen Pharmaceuticals	Plague (*Yersinia pestis*)
Pyridostigmine bromide (PB)	US Army	Soman

- The FDA has also clarified suggested issues, one of which is an animal model qualification program. The qualification process is limited to animal models used for product approval under the Animal Rule (www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284078.htm). A qualified model may be used for efficacy testing in development programs for multiple investigational drugs for the same targeted disease or condition. Such animal models are considered to be product-independent (i.e., not linked to a specific drug).

The Animal Rule states that the FDA can rely on the evidence from animal studies to provide substantial evidence of the effectiveness of a drug only when all of the following four criteria are met:

a. There is a reasonably well-understood pathophysiological mechanism of the toxicity of the substance and its prevention or substantial reduction by the product.
b. The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans.
c. The animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity.
d. The data or information regarding the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allow selection of an effective dose in humans.

The FDA (2003) approved pyridostigmine bromide for combat use by US military personnel to protect them from the lethal effects of the nerve gas soman (www.fda.gov/Drugs/EmergencyPreparedness/BioterrorismandDrugPreparedness/ucm130342.htm).

Sullivan et al. (2009) have reviewed the usefulness of the Animal Rule in the development and regulatory approval process of the Ebola virus. Ebola virus infection is a highly lethal disease for which there are no effective therapeutic or preventive treatments. Several vaccines have provided immune protection in laboratory animals, but because outbreaks occur unpredictably and sporadically, vaccine efficacy cannot be proven in human trials, which are required for traditional regulatory approval. The FDA has introduced the Animal Rule to allow laboratory animal data to be used to show efficacy when human trials are not logistically feasible. In this review, the authors describe immune correlates of vaccine protection against Ebola virus in animals. This research provides a basis for bridging the gap from basic research to human vaccine responses in support of the licensing of vaccines through the Animal Rule. Table 45.3 lists a few agents approved using the Animal Rule.

HUMAN ON A CHIP

Hartung and Zurlo (2012) have discussed various alternative approaches for medical countermeasures to biological and chemical terrorism and warfare. One such approach focuses on the development of a human-on-a-chip shown in Figure 45.10. This involves the combination of different three-dimensional (stem) cell-based organ equivalents combined with microfluidics. The prospects of such approaches, their impact on the field of alternative approaches, and necessary complementary activities are discussed. They emphasize the need to adapt quality assurance measures and experiences from validation while executing such newer approaches.

Similarly, scientists at the Edgewood Chemical Biological Center (ECBC) and academic collaborators are performing research on organoids (small swatches of human tissue) on microchips (www.kurzweilai.net/human-on-a-chip). This research focuses on *in vitro* human organ constructs, such as for the heart, liver, lung, and the circulatory system, in communication with each other. The goal is to assess effectiveness and toxicity of drugs in a way that is relevant to humans and their ability to process these drugs. It is said that "the screening models will be used to assess the efficacy and safety

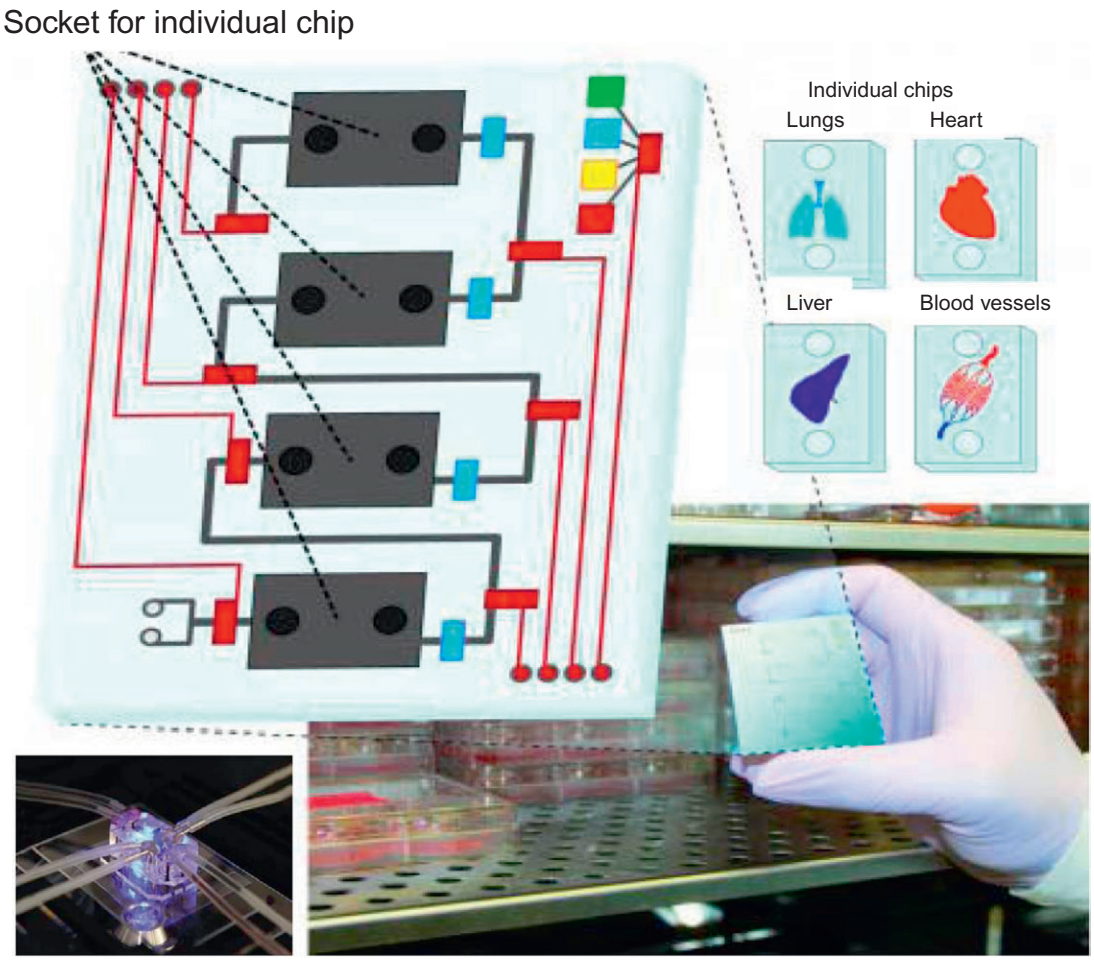

FIGURE 45.10 "Human on a chip" *in vitro* human organ constructs (for heart, liver, lung, and the circulatory system) in communication with each other used to assess effectiveness and toxicity of warfare agents that are relevant to humans (Human on a Chip, 2014).

of medical mitigation procedures and countermeasures for the soldier and the nation as a whole."

Each organ on a chip is approximately the size of a thumb drive and is an "organoid" (a structure that resembles an organ in appearance or function) designed to mimic the properties of an actual human organ. The organoids are created by induced pluripotent stem cells made from adult skin cells. They comprise multiple layers of cells growing on a membrane and connected to each other by microfluidics (tiny microchannels) that copy the function of blood vessels. Their primary purpose is to take the place of animal research. It is proposed that animal tests do not always give the results seen in people. Because of the species-specific differences by which compounds are metabolized, a drug tested on a laboratory rat does not always translate well to a human. In some cases, such as with asthma, no animal testing can mimic the human response. Because human on a chip is made from human cells, it is the next best thing. Human tissue reacts like human tissue.

New Predictive Models of Toxicity

The researchers anticipate that new predictive models of toxicity will result from the more accurate human on a chip testing, saving time and money. This technology is hoped to result in fewer test failures than animal studies. The ECBC center houses the only laboratories in the United States that the Chemical Weapons Convention permits to produce chemical warfare agents for testing purposes. It is said ECBC will test the human on a chip against chemical warfare agents to learn more about how the body will respond to agent exposure and explore various treatment options for exposures. For the military, human on a chip research is hoped to save lives.

Labant (2014) points out using *in vitro* ADME-Tox assays and getting more biological data from each experiment. The use of specialized instrumentation, such as single-stage accelerator mass spectrometer (SSAMS), is suggested so trace amounts of metabolites can be

FIGURE 45.11 SSAMS is a robust and ultrasensitive bioanalytical tool. AMS technology can provide a compound-independent and matrix-independent platform that enables innovative *in vitro* and *in vivo* study designs.

identified and quantitated (Figure 45.11). Similarly, use of flow cytometry in toxicological testing has truly enhanced the collection of a large amount of data from each experimental unit either *in vivo* or *in vitro* while reducing animal use (Krishna et al., 1993; Criswell et al., 1998a,b, 2003; Darzynkiewicz et al., 2011; Dertinger et al., 2011; Willjam et al., 1991).

Examples of alternative studies conducted using a variety of warfare agents are briefly listed here. Worek et al. (2007) described the use of highly toxic organophosphorus (OP) warfare nerve agents and underlined the necessity for effective medical treatment. Acute OP toxicity is primarily caused by inhibition of AChE. Reactivators (oximes) of inhibited AChE are a mainstay of treatment. *In vitro* studies with human tissue enabled the evaluation of oxime efficacy without animal experiments and with no need for interspecies extrapolation. Dorandeu et al. (2007) emphasized the use of swine, especially minipigs, based on similarities in a number of interesting biological and physiological characteristics (skin, cardiovascular), as an alternative to other commonly used species such as dogs, macaque, and marmoset. Szinicz et al. (2007) described development of antidotes against chemical warfare agents as "orphan drugs" given that these poisonings are rare. Computer models are being established to estimate the therapeutic effect of an antidote in various human poisoning scenarios. This approach compensates for the lack of human clinical pharmacodynamic studies given the obvious ethical issues preventing human volunteer studies with these agents. Krishnan et al. (2009) and Reddy et al. (2011a,b) tested a variety of warfare agents in multiple alternative methods, including short-term genotoxicity and physiologically based pharmacokinetic modeling of warfare agents, for example, cyclotrimethylenetrinitramine (RDX), ethylenediaminedinitrate, diethylenetriaminetrinitrate, and 3-nitro-1,2,4-triazol-5-one.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Alternatives in animal testing have come a long way in thought and action. Over the years, scientists have continuously improved such methods so that the goals of the 3Rs are achieved. For example, the roadmap for the development of alternative (nonanimal) methods for systemic toxicity testing by Basketter et al. (2012) and challenges in developmental neurotoxicity testing in the twenty-first century versus *in vitro* opportunities by Smirnova et al. (2014) are noteworthy. In recent years, the application of alternatives in testing warfare agents has been much appreciated in light of the threat and/or actual terrorist attacks. A variety of such methods either has been validated or are being validated. Continued global efforts in innovation with reliable assays, instrumentation, and strategies are hoped to produce much anticipated results so these methods can be routinely used in warfare testing and counterterrorism. Regulatory agencies around the globe are working in collaboration with academia and industry, so the stakeholders, thoughts, talk, and action are on the same page. Meaningful cooperation is sought, as needed, to achieve a common goal of reliable and quality data for our efforts. This kind of collective and collaborative effort and efficient use of resources with funding from multiple bodies, especially the government, will continue to provide impetus in fully achieving the ultimate goal of reducing, refining, and replacing animal use, as applicable, in toxicity and efficacy testing and finding viable alternatives, especially for warfare testing. Going forward, it is believed that with continued innovations in technologies, the instrumentation, ideas, and vision, such as "organ or organs-on-a-chip" and "human-on-a-chip" as proposed by Lee et al. (2013) and others, would become a reality. With these advancements, the concerns of excessive use of animals in testing would continue to decrease, but with quality data still generated while using these remarkable alternatives.

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SECTION IV

SPECIAL TOPICS

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CHAPTER

46

Genomics and Proteomics in Brain Complexity in Relation to Chemically Induced PTSD

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INTRODUCTION

Posttraumatic stress disorder (PTSD) is an anxiety disorder rooted in an extremely traumatic experience, usually one that threatens severe injury or death. PTSD is characterized by symptoms in three primary domains: (i) reminders of the exposure (flashbacks, intrusive thoughts, and nightmares); (ii) activation (hyperarousal, insomnia, agitation, irritability, impulsivity, and anger); and (iii) deactivation (numbing, avoidance, withdrawal, confusion, derealization, dissociation, and depression). Signs and symptoms can last beyond 1 month (even though such periods can occur long after the original traumatic exposure) and therefore reflect a persistent, abnormal adaptation of neurobiological systems to the stress of witnessed trauma. According to the World Health Organization, mental disorders account for 4 out of 10 of the leading causes of disability in developed countries, and epidemiological data suggests that 7.8% of people will experience PTSD in their lifetimes (Kessler et al., 1995; Roth et al., 2011). Clinical studies have shown a high prevalence of PTSD in war veterans, especially in those who suffered from chemical warfare. Based on symptom severity and persistence, PTSD is generally categorized into three types: acute, chronic, and delayed onset. In acute PTSD, symptoms normally last less than 3 months. In chronic PTSD, symptoms may last 3 months or more. In delayed onset of PTSD, symptoms first appear at least 6 months after the traumatic event. The estimated lifetime prevalence of PTSD is about 8% in the US population, with women (10.4%) more than twice as likely as men (5%) to experience PTSD. According to the same report, the prevalence ranges from 3% to 58% in high-risk groups, such as combat veterans and victims of violent crimes such as rape, sexual molestation, physical attack, and violence.

Current diagnostics for PTSD rely on subjective measures and patient recall, making it difficult to accurately diagnose the condition or differentiate its symptoms from those of depression or anxiety. The exact causes of PTSD and the long-term changes in the body, especially the brain, caused by it are still unknown. Identifying specific areas of the brain whose key functions are altered in response to a specific disorder is key to understanding the fundamental causes and sustaining factors of disease pathology. Recent advances in brain imaging technologies have allowed scientists to analyze the brains of patients afflicted with a variety of neurological disorders. Whole-brain imaging studies offered valuable information about the permanent changes and long-term effects of PTSD in the brain (Hull, 2002; Brown et al., 2014). However, some of these key brain areas are also involved in a number of closely associated brain disorders, such as anxiety and depression (Kroes et al., 2011); and distinguishing subtle morphological changes between these closely related neurological disorders through brain imaging is extremely challenging. Additionally, whole-brain imaging studies can provide only a snapshot of the current morphological status, requiring the development of advanced methodologies that can predict the predisposition of an individual, mainly the altercations in genetic makeup, which serve as molecular signatures of PTSD. High-throughput genomics and proteomics approaches have been widely applied for identifying

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biomarkers for many diseases, as well as characterizing the molecular toxicology of chemical agents.

There are many questions that are relevant to address while trying to characterize the molecular fingerprint of a complex disease like PTSD. Determine whether there are changes in gene expression—that is, can the activity or output of particular genes associated with the onset of PTSD after exposure to traumatic events, or some environmental factors such as toxic chemical exposure, affect the way our genes behave and the RNA they make, ultimately resulting in the alteration of the body's production of various proteins and hormones? Do those changes contribute to the development of PTSD? Can we identify specific variants in the genetic architecture that are involved in alterations in gene expression and associated with PTSD? Answers to these questions will help researchers understand what happens with our genes and the proteins they make and related basic biology as we are exposed to trauma and toxic environment. This, in turn, could educate us concerning new intervention efforts, including medication that offsets biological factors leading to PTSD's development. In this chapter, we briefly discuss the outcomes of current research in the anatomical changes in the brain, genomic factors leading to PTSD development, and recent advances in genomics, transcriptomics, and proteomics to understand PTSD at the molecular level and develop early diagnostic tools and specific pharmacotherapy.

THE EFFECT OF PTSD ON DIFFERENT REGIONS OF THE BRAIN

Earlier brain imaging studies have identified a few key regions in which patients with PTSD appear to have altered stress response structure and function. The neurobiological systems regulating stress response pathways and the network of brain regions known to regulate fear and emotive behavior also seem to play a role in PTSD. Investigations to identify neurobiological markers for PTSD originally presumed that abnormalities were acquired as a consequence of traumatic experience. However, certain abnormalities in a patient with PTSD could simply represent preexisting conditions or functionally dormant pathology, which arise through trauma exposure and hence are detected upon investigation. Patients with PTSD showed drastic changes in the hippocampus and amygdala, as well as cortical regions including the anterior cingulate, insula, and orbitofrontal region (Shin et al., 2006; Bremner et al., 2008). All these areas form a neural circuit mediating, among other functions, adaptation to stress and fear conditioning, and changes in these circuits are considered directly related to the development of PTSD (Rauch et al., 2006).

THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The "headquarters" of the mammalian neuroendocrine stress response system is the hypothalamic-pituitary-adrenal (HPA) axis; as such, it has been the main target for investigation in PTSD patients. Briefly, the HPA axis is made of endocrine hypothalamic components, including the anterior pituitary and the adrenal glands as effector organs. Upon exposure to stress, neurons in the hypothalamic paraventricular nucleus secrete corticotropin-releasing hormone from nerve terminals in the median eminence into the hypothalamohypophyseal portal circulation, which stimulates the production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH in turn stimulates the release of glucocorticoids from the adrenal cortex. Prolonged glucocorticoid exposure has adversarial effects on hippocampal neurons, involving reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis (Fuchs and Gould, 2000).

Cortisol, secreted in the adrenal cortex in response to stress, is an informative biomarker that can be used to differentiate PTSD from normal patients. Outcome from a simulation study supported the hypothesis proposed by Yehuda and colleagues that high stress intensity and strong negative feedback loops may cause a hypersensitive neuroendocrine axis that results in hypocortisolemia in PTSD (Yehuda et al., 1993; Sriram et al., 2012). Also, low-dose dexamethasone suppression testing suggests that hypocortisolemia in PTSD occurs due to increased negative feedback sensitivity of the HPA axis (Wingenfeld et al., 2007). In summary, these studies suggest that decreased availability of cortisol, due to irregular regulation of the HPA axis, may lead to abnormal stress reactivity and fear processing.

HIPPOCAMPUS

Reduced hippocampal volume is a well-known hallmark of PTSD. Initial magnetic resonance imaging studies determined smaller hippocampal volumes in Vietnam veterans with PTSD and patients with abuse-related PTSD compared with controls (Bremner et al., 1997). Therefore, severity of trauma and memory impairments were related to the decrease in hippocampal volumes. However, reduced hippocampal volume has not been observed in children with PTSD (De Bellis et al., 1999). Additionally, proton magnetic resonance spectroscopy investigations further demonstrated a reduction of the levels of *N*-acetyl aspartate (NAA), a marker of neuronal integrity, in the hippocampus of adult patients with PTSD (Villarreal et al., 2002). Hippocampal volume reduction in PTSD likely reflects a neurotoxic effect

of repeated exposure to stress-increased glucocorticoid levels or glucocorticoid sensitivity, although decreased hippocampal volumes might also be a preexisting vulnerability factor for developing PTSD (van der Werff et al., 2013).

Convincing evidence suggested a smaller hippocampal volume in trauma-exposed persons diagnosed with more severe, unremitting PTSD (Gilbertson et al., 2002). In fact, early life stressors can affect hippocampal volume and predispose for the disorder (Yehuda et al., 2010). However, at this stage, the way that trauma affects hippocampal volumetric (as well as other histopathological) measures is not well understood. Interestingly, functional neuroimaging studies have also shown deficits in hippocampal activation during verbal declarative memory tasks in PTSD patients (Bremner et al., 2003). Both hippocampal atrophy and functional deficits select serotonin reuptake inhibitors (SSRIs) most likely due to an increase of neurotrophic factors and neurogenesis (Nestler et al., 2002).

AMYGDALA

Investigators have suggested the idea that aberrant stress response and an enhanced amygdala-induced augmentation of emotional memories in PTSD subjects may be underpinned by abnormal amygdala functioning (Elzinga and Bremner, 2002), since its functional role is in mediating both stress responses and emotional learning. However, no clear evidences for structural alterations of the amygdala have been confirmed in PTSD patients. Functional imaging studies have revealed amygdala hyperresponsiveness in PTSD subjects when stressful scripts (Hendler et al., 2003; Shin et al., 2004), cues, and trauma reminders were presented (Shin et al., 2006). On the other hand, PTSD patients showed increased amygdala responses even to general emotional stimuli that were not trauma-associated (Shin et al., 2006) or subliminally threatening cues (Hendler et al., 2003; Bryant et al., 2008). Amygdala hyperactivity seems to be related to specific genetic traits (Hariri et al., 2002; Kilpatrick et al., 2007); hence, it may likely play a role in developing PTSD. Actually, several investigations have found a positive relationship between activation of the amygdala and PTSD symptom severity (Shin et al., 2004).

PTSD patients show a hyperactive amygdala as a consequence of trauma and throughout the course of the disorder; hence, reducing the activity of the amygdala could potentially prevent onset of the disorder, or it could be therapeutic for individuals who have already developed psychopathology. These findings led to the hypothesis that noradrenergic activity could promote amygdala-dependent fear memories. Indeed, researchers have speculated that adrenergic receptor antagonists could potentially reduce the severity of PTSD symptoms; adrenergic receptor modulators, such as prazosin (α1 receptor antagonist; (Raskind et al., 2007), clonidine (α2 receptor agonist; (Boehnlein and Kinzie, 2007; Strawn and Geracioti, 2008) and propranolol (β receptor antagonist; (Brunet et al., 2011; Pitman, 2011), have shown promising results as elements of therapy for PTSD.

CORTEX

The prefrontal cortex (PFC), and particularly the medial PFC, are highly involved in the extinction of fear memories (Sherin and Nemeroff, 2011) and, in general, in the inhibitory control of inappropriate cognitive and emotional responses that are mediated in part by the amygdala (Elzinga and Bremner, 2002). Given this large inhibitory role of the PFC over the amygdala, researchers have speculated that impaired PFC functioning may trigger amygdala hyperactivity and hence exacerbate emotional responsiveness. Actually, individuals with PTSD exhibit smaller volumes of PFC, its major subregions, and specifically the anterior cingulate cortex (ACC); (Yamasue et al., 2003; Woodward et al., 2006). Therefore, PTSD symptom severity could likely be related to these volumetric reductions (Zoladz and Diamond, 2013). Moreover, shape abnormalities (Corbo et al., 2005), as well as a decrease in NAA levels in the ACC (De Bellis et al., 2000) have also been reported.

However, recent evidence has suggested that volume loss in the PFC and ACC could also be a consequence of PTSD rather than a preexisting risk factor (Kasai et al., 2008). Functional imaging studies have demonstrated a reduced activity of the medial PFC in PTSD patients in response to stimuli, such as trauma scripts (Shin et al., 2004; Britton et al., 2005), combat pictures and sounds (Bremner et al., 1999), trauma-unrelated negative narratives (Lanius et al., 2003), fearful faces (Shin et al., 2005), and impaired performance on PFC-dependent tasks (Koenen et al., 2001). Finally, PTSD patients show impaired abilities to extinguish fear (Peri et al., 2000) and reduced activities of PFC regions during extinction trials (Bremner et al., 2005).

These individuals exhibited a reduced activity of PFC or a complete failure to activate PFC brain regions during the presentation of trauma-associated stimuli (Shin et al., 2004; Britton et al., 2005); hence, it is likely that a reduced activation of PFC, along with amygdala hyperactivity, could lead to the development of the intrusive emotional thoughts and memories often experienced by PTSD patients. Actually, reduced PFC activity could lead to greater governance of behavior by more primitive brain areas, such as the amygdala, impairing brain processes involved in adaptation, behavioral flexibility, and cognition. As for the volume decrease, it is still unclear

whether the reduced PFC inhibition of lower brain areas, such as the amygdala, is a result of the disorder or a preexisting condition for developing PTSD. Investigations of twin brothers who were discordant for PTSD (Gilbertson et al., 2006) supported the hypothesis that reduced PFC functioning is both a preexisting condition and a risk factor for the development of PTSD. Moreover, animal and clinical studies mainly suggest a complex interaction among early life stress (Karssen et al., 2007; Lyons et al., 2010), gene-environment influences (Garrido, 2011), and the well-described adverse effects of stress on PFC functioning in adulthood (Arnsten, 2009) which could interact to influence PTSD susceptibility and posttrauma expression. SSRI treatment has been shown to restore medial PFC activation patterns (Shin et al., 2006).

Although these findings using brain imaging studies advances our understanding of PTSD pathology, it falls short of identifying predispositions to the disease. The significant progress in genetics and genomics has generated approaches that screen the full genome and genetic network rather than individual genes. The recent move towards the "omics" fields, including genomics and proteomics, offer enormous opportunities to analyze genes, RNA transcripts, and protein expression patterns in diseased and normal tissues. Such approaches have equipped researchers with many advantages and given access to thousands of new molecular targets for disease screening and drug discovery. In the rest of this chapter, we discuss the genomic and proteomic methods that are currently used to understand the molecular level players in PTSD.

UNDERSTANDING PTSD: GENOMICS AND PROTEOMICS

The central dogma of biology coined by Francis Crick (1958) proposed that an organism's genetic information encoded in its DNA molecules is transcribed into RNA molecules (transcriptome), which are then translated to proteins (proteomes) that facilitate particular biological functions. In addition to the transfer of information from genome to proteome, proteome functions are regulated by epigenetic mechanisms such as posttranslational modifications and regulation via noncoding RNA (ncRNA). The development of advanced techniques over the past two decades have allowed the scientific community to take a closer look at the genome, transcriptome and proteome of any tissue or organ, and these tools have proved to be invaluable in studying complex tissues such as the brain. Monitoring the specific activity of a genome by measuring messenger RNA (mRNA) expression levels of large gene sets can identify molecular profiles correlated to disease states, which may then be developed as diagnostic tools.

The human brain is one of the most complex biological structures. It consists of approximately 100 billion neurons and a nearly equal number of nonneuronal cells (Azevedo et al., 2009). Neurons that perform a specific function are assembled into circuits, and the neurons within each circuit talk to each other through unique connections called *synapses.* Anomalies that affect communication within neural circuits lead to neurological disorders and affect regular brain functions (Geula, 1998). For over a century, researchers have tried to understand human brain functions using simple animal models such as worms, flies, snails, mice, and rats. Over the past two decades, molecular genetics studies have enabled a common conceptual framework for the development and basic function of the nervous system. However, because many debilitating human disorders are genetically complex and phenotypic screens are difficult to perform, large-scale genomic approaches to discover genes that are uniquely expressed in brain circuits and regions that control complex behaviors are becoming highly significant.

A comprehensive picture of the genomic factors underlying an individual's susceptibility to complex diseases is essential to understanding a disease like PTSD, where it is often difficult to make an absolute clinical diagnosis. One such study showed that a single-nucleotide polymorphism (SNP) of the gene Oprl1 is associated with PTSD symptoms in humans, and an altered expression of this gene in the amygdala of mouse models led to alterations in fear processing that may lead to PTSD development (Andero et al., 2013). Another study of adult civilians with PTSD has shown that individuals with a history of childhood abuse have very discrete and profound changes in gene activity patterns compared to adults with PTSD but no history of child abuse (Mehta et al., 2013). The outcome of both these studies provide evidence that susceptibility to developing PTSD may be coded in one's DNA; and the exposure to traumatic events may act as the trigger for PTSD onset. In addition, scores of recent studies on epigenetic mechanisms state that DNA is not the only element that carries all the information for a specific phenotypic outcome, and specific alterations in epigenetic signatures such as DNA methylation were more frequent in the PTSD group that suffered child abuse (Mehta et al., 2013). This study also found that the subjects with PTSD and a history of child abuse demonstrated more changes in genes linked with nervous system development and regulation of the immune system, while the PTSD patient group with no child abuse history showed more changes in genes linked with apoptosis (cell death) and regulation of growth rate. Although the symptoms in both the PTSD groups were the same, these findings showed that the specific affected biological pathways might lead to different mechanisms of PTSD symptom formation within

the brain. These studies provide ample evidence that PTSD is associated with distinct molecular fingerprints, and identifying the key genetic factors for PTSD etiology will not only improve understanding of the underlying pathophysiology, but may also lead to new avenues for preventing and treating this distressing disease in the early stages.

APPLICATIONS OF GENOMIC AND TRANSCRIPTOMICS METHODS

Transcriptomics and gene expression profiling methods analyze the expression of thousands of genes simultaneously in a single biological sample by quantifying the levels of individual mRNA transcripts. Some of the earlier methods used for the identification of genes in different parts of the brain include differential display, representational difference analysis, serial analysis of gene expression, and massively parallel signature sequencing. These techniques are relatively expensive and laborious, which led to the search for high-throughput screening techniques that can capture dynamic gene expression changes and perform large-scale gene expression profiling in complex neuronal systems (Kadakkuzha and Puthanveettil, 2013).

Microarrays hold the promise of becoming a revolutionary tool for large-scale analyses of genome sequence and gene expression (Young, 2000; Noordewier and Warren, 2001). The most commonly used formats are the oligonucleotide microarray (Fodor et al., 1993) and the complementary DNA (cDNA) microarray (Schena et al., 1995). Over the years, microarray technology has advanced to support maximum coverage of the transcriptome. The successful applications of microarray technology offer a robust and unbiased approach to acquire global gene expression patterns in the whole brain, specific tissue, or single neuron. cDNA arrays are often used in RNA expression analyses, while oligonucleotide arrays are used for sequence analyses. Oligonucleotide arrays offer a number of advantages over cDNA microarrays, such as increased specificity of hybridization, which is key to SNPs (LaForge et al., 2000), mutational analysis (Hacia, 1999), identification of splice forms, and, alternatively, polyadenylated transcripts (Hu et al., 2001). Microarrays have been extensively used to study expression profiles of complex neuropsychiatric disorders using both animal models (Lin et al., 2012) and RNA from postmortem human brain tissue (Lehrmann et al., 2003). Thus, microarrays can be effectively applied toward robust screening of a large number of genes, which are altered with PTSD onset.

Validation of microarray results is often performed by another sensitive technique such as qualitative real-time polymerase chain reaction (qRT-PCR), which detects the PCR product by combining a thermal cycler with a fluorescent spectrophotometer (Higuchi et al., 1993). The qRT-PCR reaction can be performed in two different formats. In the first, the double-stranded DNA generated is detected by the binding of a fluorescent dye such as SYBR green I that intercalates only to the double-stranded DNA (Ponchel et al., 2003). The second uses a fluorescent reporter molecule that is released due to the exonuclease activity of the DNA polymerase enzyme. qRT-PCR is widely used as a robust method for quantitative gene expression analysis (Gibson et al., 1996). Apart from qRT-PCR, another widely used approach to validate gene expression in transcriptomics is *in situ* hybridization. Fluorescence *in situ* hybridization (FISH) was developed in the 1980s, and it rapidly became a powerful technique (Langer-Safer et al., 1982). In the FISH method, a small RNA fragment of the mRNA transcript to be tested is fluorescently labeled and used for hybridization in fixed samples. The probe binds to the complementary sequences within the sense transcript, which can be visualized by fluorescence microscopy. FISH can identify whole chromosomes, centromeres, telomeres, specific regions or genes, or aberrations in interphase tumor nuclei, and it can be effectively used to identify novel mRNA transcripts, the levels of specific genes expressed, and their cellular localization. Using this technique, a genomewide, three-dimensional map of the entire human brain is constructed that details where each gene is "turned on or off."

Very often, however, the molecular characterization of clinical samples is complicated and limited by the available amount of samples. Strategies to overcome this problem include amplification of the starting material or of the signal to be detected, or miniaturization of the method. Novel means are also required to measure gene expression with allele-specific and splice-variant-specific profiles. Such technologies promise to be a further big step from bench to bedside. Although microarrays are still widely used, an increasing number of studies now use direct sequencing of transcripts by high-throughput sequencing technologies such as RNA Sequencing (RNA-Seq), also known as "next-generation" or "deep" sequencing (Wang et al., 2009). Unlike microarrays, RNA-Seq is not limited to detecting transcripts that correspond to existing genomic sequences and has significantly low, if any, background signaling. In addition, RNA-Seq does not have an upper limit for quantification, offering a large dynamic range of expression levels over which transcripts can be detected. The most commonly used techniques to analyze genetic and genomic various are shown in Figure 46.1.

SNP genotyping is an advanced genotyping method that measures SNPs between genomes. In an SNP, a single base pair is mutated at a specific locus, leading to the malfunction of the specific gene product and a disease

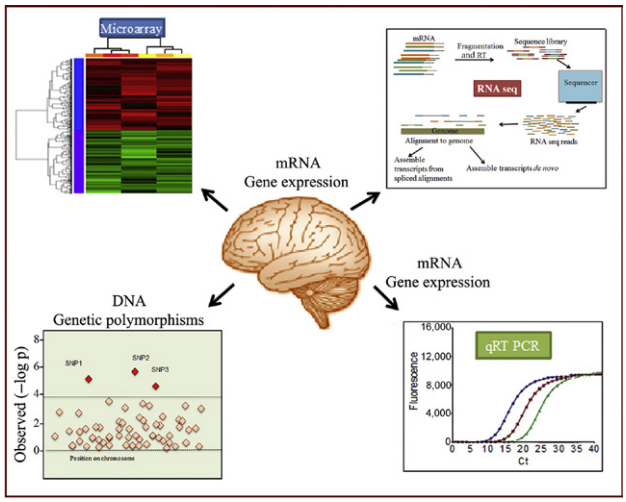

FIGURE 46.1 The most commonly used techniques to identify the genomic and transcriptomic changes associated with specific diseases. In microarray, RNA-Seq, and qRT-PCR, mRNA isolated from samples is characterized to observe any changes in gene expression. In SNP, DNA is used to identify a single base pair mutation at a specific locus on the chromosome.

phenotype. Over 1 million SNPs are currently reported to be present within the human genome (Sachidanandam et al., 2001). Even though very small numbers of SNPs cause changes in gene function or expression, it is critical to identify SNPs, as they are implicated in a number of diseases. There are multiple platforms to perform SNP genotyping, and multiple studies have shown that specific SNPs are associated with PTSD. A genomewide SNP association analysis in 1,578 European Americans, 300 of whom suffered from PTSD, and 2,766 African Americans, 444 of whom had PTSD, using an Illumina Omni1-Quad microarray, found novel common risk alleles for PTSD (Xie et al., 2013). This study yielded close to 90,000 SNPs and found a new susceptibility gene for PTSD called the *Tolloid-Like 1 gene (TLL1).* A similar genomewide association study (GWAS) from a cohort of veterans reported an SNP (rs8042149) located in the retinoid-related orphan receptor alpha (*RORA*) gene associated with PTSD susceptibility (Logue et al., 2013). RORA has been implicated in prior GWAS studies of psychiatric disorders and is known to have an important role in neuroprotection and other behaviorally relevant processes. This study represents an important step toward identifying the genetic underpinnings of PTSD.

Another well-studied example is the FK506 binding protein 5 (*FKBP5*), a glucocorticoid receptor co-chaperone regulator (Mehta et al., 2011) that has reduced expression levels in PTSD (Yehuda et al., 2009). In a cross-sectional study on childhood abuse, four specific SNPs of the *FKBP5* gene were found to be predictors of adult PTSD symptoms (Binder et al., 2008). These four SNPs were identified as rs3800373, rs9296158, rs1360780, and rs9470080 in a study done on European Americans and African Americans who were originally screened for lifetime PTSD (Xie et al., 2010). All four of these SNPs showed a similar linking pattern (Shinozaki et al., 2011), which supported a genetic as well as an environmental basis for childhood abuse and the subsequent onset of PTSD in adulthood.

In another study, the peripheral expression levels of *FKBP5* gene expression and volumes of specific brain structures such as the hippocampus, amygdala, and medial orbitofrontal cortex in 39 patients with PTSD were compared before and after cognitive behavioral therapy. Microarray and qRT-PCR analysis found that there was a significant increase in FKBP5 expression and hippocampal volume in patients with PTSD. Another similar study on pituitary adenylate cyclase-activating polypeptide (PACAP)–PAC1 receptor found that these were involved in abnormal stress responses underlying PTSD in a sex-specific manner in heavily traumatized individuals (Ressler et al., 2011). Analyzing the levels of PACAP in the blood and extensive SNP genotyping of *PACAP* and *PAC1* genes showed a single SNP within the *PAC1* gene (rs2267735) that predicts PTSD diagnosis and symptoms in females only. The study also found that methylation of the PAC gene in peripheral blood is associated with PTSD.

THE ROLE OF ncRNA AND EPIGENETICS IN PTSD

Earlier in this chapter, SNPs associated with a number of genes were described as genomic indicators of PTSD vulnerability. Recent studies provide evidence that epigenetic mechanisms and ncRNA are also involved in the development of PTSD, increasing the molecular complexity further. An initial animal model study examining DNA methylation of the brain-derived neurotrophic factor (*BDNF*) gene in rat models of PTSD found a significant increase in *BDNF* DNA methylation in the dorsal hippocampus, the highest increase in the dorsal CA1 subregion, and a significant methylation decrease in the ventral hippocampus (CA3) following the stress regimen (Roth et al., 2011). However, this study has found no change in *BDNF* DNA methylation in the medial PFC or basolateral amygdala. Interestingly, the mRNA levels of *BDNF* decreased in both the dorsal and ventral CA1, providing key evidence that traumatic stress can induce central nervous system (CNS) gene methylation and alter gene expression in key brain areas, leading to the pathophysiology of PTSD.

A recent study on African Americans involved in the Grady trauma project, which investigated the relative contribution of genetic and trauma-related risk factors for PTSD in a cross-sectional study of a highly

traumatized, low-socioeconomic-status, minority urban population, has found that DNA demethylation altered the transcription of *FKBP5*, leading to long-term dysregulation of the stress hormone system for stress regulation associated with PTSD (Klengel et al., 2013). It has been reported that PTSD patients who faced significant abuse in childhood displayed more changes in gene expression associated with CNS development and immune system regulation, whereas those without a history of childhood abuse displayed more changes in gene expression associated with cell death and growth rate regulation. Specific altercations in the DNA methylation profile were up to 12-fold higher in PTSD patients with a history of childhood abuse (Mehta et al., 2013). In a similar study, gene expression profiles in the dorsolateral prefrontal cortex Brodmann area (BA) of 46 postmortem patients with or without PTSD have been investigated using human mitochondria-focused cDNA microarrays (hMitChip3) (Su et al., 2008). A total of 119 differentially expressed genes were found between the control and PTSD patients, and the majority of the genes altered in the PTSD samples belong to the neuronal function-survival networks. Similarly, a recent study using bold sera from US military service members evaluated temporal changes in DNA methylation in select promoter regions of immune system–related genes between PTSD diagnosis, pre- and post-diagnosis, and in control patients. This study found reduced mC levels at the promoter regions of a long-ncRNA H19 and interleukin-18 (IL18) in those who did not develop PTSD after deployment, while those who did develop PTSD had increased levels of IL18 (Rusiecki et al., 2013).

MicroRNA (miRNA) has recently emerged as an epigenetic modulator of gene expression in psychiatric diseases like schizophrenia and depression (Miller and Wahlestedt, 2010). miRNAs are short, single-stranded RNA sequences that regulate gene expression by binding to the regulatory regions of mRNA and preventing translation, representing another mechanism of regulating gene expression besides up- or down-regulation of transcription (Fabian et al., 2010). In a recent study reporting the connection between miRNAs and PTSD, miRNA profiles of the PFCs from fluoxetine-treated and control wild-type C57BL/6N mice were dissected 74 days after they were subjected to either a single, traumatic electric shock to the foot or a mock treatment (Schmidt et al., 2013). Fluoxetine is an antidepressant effectively used both in PTSD patients and in mice suffering from a PTSD-like syndrome. Using miRBase 18.0 screening and qRT-PCR validation, the study found five miRNAs, including one (mmu-miR-1971) that showed significant reduction in fluoxetine-treated shocked mice.

The relevance of the noncoding genome to human disease has mainly been studied in the context of the miRNA expression and function. However, it is still a beginning of the recognition of the nature and extent of the involvement of other ncRNA in disease. A GWAS of PTSD was conducted using a group of primarily African-American women from the Detroit Neighborhood Health Study that included 94 PTSD cases and 319 controls exposed to at least one traumatic event, as well as an independent cohort of primarily European-American women from the Nurses' Health Study II, which was composed of 578 PTSD cases and 1,963 controls (Su et al., 2008). More than 700,000 markers were screened using Illumina HumanOmniExpress BeadChip. The results found a genomewide significant association of one marker mapping to a novel RNA gene, lincRNA AC068718.1. The study also performed pathway analysis to obtain a protein functional interaction network, and found pathways related to telomere maintenance and immune function. This study demonstrated the emerging evidence that noncoding RNAs may play a crucial role in shaping the landscape of gene regulation with putative pathological effects that lead to phenotypic differences.

TOXIC CHEMICAL EXPOSURE AND HUMAN DISEASES

Scores of studies point to the influence of an individual's genetic information and social conditions in the development of PTSD. However, it is also important to address the role of other external factors, such as brain cell damage and nervous system damage caused by chemical exposure. It has been reported that residents from regions of high-intensity warfare and chemical weapons during the Iran–Iraq war (1980–1988) had a significantly higher risk of meeting the criteria for lifetime and current PTSD compared with the residents of high-intensity warfare alone (Hashemian et al., 2006). Currently, PTSD development following trauma or exposure to chemical warfare agents (CWAs) is poorly understood. Although every individual with PTSD has been exposed to a traumatic event, studies show that the majority of people who experience trauma do not develop PTSD. It is intriguing why some people remain resilient, some experience short-term difficulties, and others develop a chronic problem such as PTSD.

According to the Research Advisory Committee report on "Scientific Progress in Understanding Gulf War Veterans' Illnesses: Report and Recommendations," almost 30% of the veterans from the Gulf War were disabled by chronic symptoms such as severe headaches, memory problems, confusion, dizziness, blurred vision, and tremors (http://www.va.gov/RAC-GWVI/Gulf_War_Illnesses_links.asp). Notably, Gulf War veterans have developed amytrophic lateral sclerosis at twice the rate of veterans who did not serve in the Gulf War. Based on the research data collected, the committee identified

the nerve gases sarin and pyridostigmine bromide, as well as multiple pesticides, as potential factors in the development of neurological disorders. More research is needed to better understand the veterans' vulnerability to PTSD after exposure to CWAs, especially to consider why only a small portion of the veterans who experienced childhood abuse developed the symptoms.

GENOMIC APPLICATIONS: UNDERSTANDING THE RELATIONSHIP BETWEEN PTSD AND CHEMICAL TOXICITY

The development of modern genomic analysis techniques offers an unparalleled opportunity to investigate neurological diseases such as PTSD due to exposure to CWAs and other toxic chemicals. CWAs like organophosphate (OP) insecticides and nerve gases are capable of changing neuronal activity, primarily via cholinergic pathways. These CWAs bind irreversibly to acetylcholinesterase (AChE), an important regulator of the neurotransmitter acetylcholine (ACh) that leads to lasting changes in neuronal activity. Studies in rodent brains have shown that the exposure could affect brain regions critical for attention, anxiety, and addiction. Nerve agents affect the CNS and the peripheral nervous system, as well as the cardiovascular, respiratory, gastrointestinal, and metabolic systems. Most importantly, they cause behavioral and psychological changes in humans that lead to memory loss and depression. One of the nerve agents used in previous wars, sarin, is suspected to be one of the key factors responsible for Gulf War syndrome.

Animal models have greatly aided our understanding of the effects of CWAs on the cellular functions, molecular pathways, organ functions, and gene expression changes that are observed in humans. Earlier studies on rats showed that sarin usually induced neurotoxicity by affecting the mRNA expression of alpha tubulin in the CNS. The study used northern blots to look at the differential expression of alpha tubulin mRNA in different regions of rat brain. Another similar study found a differential distribution of AChE mRNA expression following exposure to sarin (Damodaran, 2003). These studies clearly showed that exposure to sarin causes gene expression changes in the CNS that may lead to neurotoxicity, as well as behavioral and psychological changes. Another study looked at sarin-induced global gene expression pattern changes at different times after exposure by microarray (Damodaran et al., 2006). Analysis of gene expressions at an early point (15 min; 0.5 × LD_{50}) and a later point (3 months; 1 × LD_{50}) identified specific gene expression changes to each time and seven genes that were consistently altered in both time points (Ania-9, Arrb-1, CX-3C, Gabab-1d, Nos-2a, Nrxn-1b, and PDE2). Further genomewide study in the rat brain at another early point (2 h: 0.5 × LD_{50}) following sarin exposure showed that 46 genes were significantly altered compared to the control animals. Most of these 46 genes belong to ion channels, calcium channels, and binding proteins. The study also found that many genes are involved in the pathogenesis of sarin-induced pathology and OP-induced delayed neurotoxicity. These studies indicate that exposure to sarin can lead to neurodegeneration at a later time and result in neuropathological alterations. Pachiappan and colleagues applied single (3 and 24 h) or repeated (2 × 24 h) doses of sarin (5 μg/mL) on human neuronal cells (SH-SY5Y) to identify altered gene expressions (Pachiappan et al., 2009). Microarray analysis in this study identified over 200 genes that were significantly altered following sarin administration. The study also found that repeated doses over 48 h persistently down-regulated genes linked to neurodegenerative mechanisms, which indicates the adverse effects of sarin exposure for a prolonged time.

O-ethyl-*S*-2-diisopropylaminoethyl methylphosphonothiolate (VX), another member of the family of OP compounds used in chemical warfare, is a very potent nerve agent. A recent microarray study using cultured human neural cells (hN2) exposed to 0.1 or 10 μM of VX for 1 h has shown changes in gene expressions after 6, 24, and 72 h (Gao et al., 2013). The altered genes were subjected to functional pathway analysis, and many of them were found to be involved in pathways related to nervous system development and function. Advancement in genomics may also shed light on some other poorly understood toxic effects of nerve agents. It has been reported that civilians exposed to industrial OPs, such as pesticides, have a higher incidence of Parkinson's disease (Hatcher et al., 2008; Manthripragada et al., 2010), although it is not clear whether nerve agents lead to similar neurodegenerative effects. There are many relevant questions about neurological disorders and the use of CWAs. Some questions to be asked are as follows: Are long-term regional differences seen in the brain after nerve agent exposure also reflected in large-scale gene expression changes? Can changes in gene expression profiles be correlated with altered behavioral patterns? And does neuroinflammation, one of the acute effects of nerve agents (Svensson et al., 2001; Williams et al., 2003; Chapman et al., 2006), diminish across time? Genomics analysis of subjects exposed to CWAs may provide ample information to answer these questions and provide additional information in the efforts to understand the spectrum of chronic effects of nerve agent exposure.

Microarrays are still highly useful to characterize changes in gene expression due to a toxicant of interest, identify up- or down-regulated genes, map regulatory pathways modulated by the toxicant, and, in the case

of CWAs, identify potential therapeutic targets in these pathways (Thomas et al., 2001; Hamadeh et al., 2002). Nevertheless, RNA-Seq offers high technical reproducibility and large dynamic range compared to microarrays, and it can be used effectively to identify differential gene expression to understand complex disorders like PTSD. Recent efforts, such as the STRONG STAR Consortium to Alleviate PTSD (STRONG STAR-CAP), led by the University of Texas Health Science Center at San Antonio, is employing next-generation sequencing and mass spectrometry (MS) to research PTSD, including treatments. The enormous potential of RNA-Seq is offering an invaluable resource to investigate the transcriptomic profile of the brain in different layers. A similar recent study showed a transcriptomic analysis of distinct regions of a brain with Alzheimer's disease using Illumina RNA-Seq analysis to examine gene expression levels, splicing isoforms, and alternative transcript start sites from the total brain and the frontal and temporal lobes of healthy and Alzheimer's postmortem tissue. The findings reveal a significant representation of genes associated with neuronal cytological structure and synapse function.

PROTEOMICS

High-throughput genomic and transcriptomics approaches are indispensable to understanding the molecular map of complex diseases. However, their application is limited by the fact that they can assess only gene expression changes. Translation of mRNA into protein is a highly regulated and complex mechanism that is modulated at different levels. Effects due to changes in protein expression, modification, or function can lead to the development of diseases and only be inferred from the analysis of protein expression profiling. The proteome was initially defined as the complete complement of proteins that are expressed by a genome; subsequently, it was denoted as "the total set of expressed proteins by a cell, tissue, or organism at a given time under a determined condition" by Wilkins et al. (1996). To assess protein expression status, different proteomics techniques need to be utilized.

Proteomics is widely applied in biological sciences to study protein expression, posttranslational modification, protein-protein interactions, and protein–nucleic acid interactions. It gives a new platform for studying complex biological functions involving large numbers and networks of proteins (Husi and Grant, 2001; Martins-de-Souza et al., 2011). Since Patrick O'Farrell first presented two-dimensional gel electrophoresis (2DE) in 1975, proteomics has given rise to a new scientific approach for comparative global proteome analyses, especially when combined with MS for protein identification. As a result of the development of in-gel digestion protocols and related optimizations (Shevchenko et al., 1996), the resolution power of 2DE can lead to the separation of more than 2,000 protein spots on large-format gels. Thus far, 2DE followed by MS is the most used proteomic technique in studies of psychiatric disorders (Kromer et al., 2005; Ditzen et al., 2006). Typically, mass spectrometers have three main features: an ionization source (e.g., matrix-assisted laser desorption/ionization and electrospray ionization), a mass analyzer (e.g., time of flight and quadrupole), and a detector (Bayes and Grant, 2009), which measures the mass-to-charge (m/z) ratio of ionized particles. The 2DE-MS approach provides direct information on intact proteins and protein isoforms. Figure 46.2 depicts a major technique currently used in proteomics methodology for PTSD diagnosis.

Link et al. (1999) launched shotgun proteomics in 1999, which increased the capacity for proteome characterization. Shotgun-MS was originally designed as a nongel/MS-direct approach, which was regarded as a more sensitive and reproducible proteome representation compared to 2DE proteomics. Shotgun-MS includes shotgun-MS, liquid chromatography-MS (LC-MS/MS), and multidimensional protein identification technology.

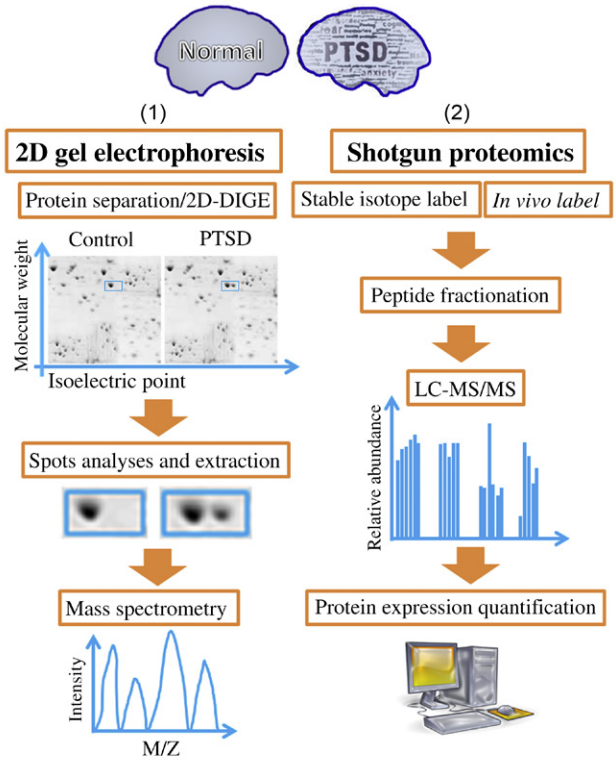

FIGURE 46.2 A major technique used in proteomics methodology for PTSD diagnosis. (1) Two-dimensional gel electrophoresis for proteomics; 2D-DIGE: proteomes mixed in gel; (2) shotgun proteomics methodology.

Basically, this approach involves digesting the whole proteome of interest using specific enzymes and subsequently identifying the resulting peptides by MS, while proteome quantification and sample comparison rely on a label-free MS methodology or stable isotope labeling. MS analysis is capable of identifying small modifications, like point mutations, phosphorylation, and glycosylation; it can also detect the primary structure of proteins and peptides (e.g., amino acid sequences (Davidsson et al., 2003)). Based on its applications, proteomics includes (i) expression proteomics, which studies protein expression profiles; (ii) comparative proteomics, which compares physiological and diseased states; (iii) structural proteomics for structure investigations; and (iv) functional proteomics, which studies the interaction between molecules. Due to its high levels of accuracy and ultrasensitivity, proteomics has become a preeminent tool in many ways, especially in neuroscience (Tyers and Mann, 2003).

NEUROPROTEOMICS: PROTEOMICS APPLICATIONS IN NEUROSCIENCE

Neuroproteomics is the study of the proteomes of the nervous system (Bayes and Grant, 2009), which is of great importance in functional studies. Bayés and Grant (2009) have reviewed four major categories of neuroproteomics, including (i) expression neuroproteomics, which refers to the qualitative and quantitative cataloguing or profiling of neuroproteomes; (ii) functional neuroproteomics, which addresses functional properties of individual proteins, as well as their organization into substructures, complexes, and networks; (iii) clinical neuroproteomics, which includes the identification of biomarkers and disease mechanisms for neurological, neurodegenerative, and psychiatric diseases which also benefit from drug discovery; and (iv) neuroproteomic informatics, which handles analyzing proteomic data sets using computational tools and databases. A typical example of a proteomic approach to study the effects of complex neurological diseases is the shotgun analysis of postmortem dorsolateral PFC brain tissue from major depression disorder (MDD) patients (Martins-de-Souza et al., 2012). Gel electrophoresis, followed by shotgun data-independent, label-free, LC-MS led to identification of distinct proteome fingerprints between MDD and control subjects. Another study tackled the proteomic profile of the anxiety disorder–applied quantitative proteomic approach, where metabolic labeling of the HAB/LAB mouse model with stable isotopes is used to identify a large number of proteins in a high-throughput manner (Zhang et al., 2011). Differentially expressed proteins were subjected to pathway discovery analyses that suggested several biological processes and pathways were affected in the genetic predisposition to extremes in trait anxiety. Although these studies drew no connection between the proteins and pathways connected with PTSD, it may encourage similar efforts in proteomic research.

PROTEOMICS APPROACHES TO UNDERSTANDING NATURAL AND CHEMICAL TOXICITY-INDUCED PTSD

Despite the enormous medical and economic consequences of traumatic injury to the CNS, relatively little study has been directed toward elucidating the proteomic profile of posttraumatic psychopathology, due to the complexity of the molecular mechanisms of the disease. Some of these proteins may serve as diagnostic and prognostic markers to assess the severity of tissue damage. Studies of proteomic biomarkers of PTSD risk have been in play for a long time; yet they have been limited by many technical issues, such as optimizing the sample collection time frame and the variation in the timing between the traumatic incident and the biological sample collection. In spite of these limitations, the initial research provided key insights into the biological mechanisms underlying PTSD vulnerability. A key observation from initial proteomics studies was the high concentrations of inflammatory proteins; together, these studies indicated increased concentrations of inflammatory cytokines related to the risk for PTSD onset (Gill et al., 2008, 2010). Proteomics makes it possible to identify molecular mechanisms and molecular targets for PTSD through a large-scale screen in patients and a validated animal model for protein expression profile analysis. In a recent study, a large-scale proteomic approach and MS with the use of human proteomic databases were employed to identify differential serum proteomic profile in combat-related PTSD and healthy controls (Kozarić-Kovăić et al., 2010). The study found more than 100 proteins expressed differently between individuals with PTSD and healthy controls. These results indicated the direction for a larger analysis of this type in people with PTSD. This pilot study offers a good basis for further proteomic research, which could help in better diagnosis and treatment of PTSD, as well as clarification of its etiology.

Sulfur mustard (SM, bis-(2-chloroethyl) sulfide), a potent alkylating agent and a chemical reagent used in chemical warfare, is indicated in PTSD development. A quantitative proteomic approach using stable isotope-labeling combined with immobilized metal affinity chromatography tested the effect of SM in a human keratinocyte cell culture model. The study found large-scale protein phosphorylation changes resulting from SM exposure and characterized more than 2,300 phosphorylation

sites, many of which showed altered levels in response to SM treatment. The study also found new proteins that are associated with SM toxicity (Everley and Dillman, 2010).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

It is clear that PTSD is not a monolithic disorder that can be characterized by unique and consistent mental and biological traits, as the development of this disorder results from complex interactions among numerous factors. Genetic background, as well as environmental factors, may contribute to whether one is sensitive to complex circumstances such as trauma exposure, leading to PTSD development. Recent research studies provided solid evidence that specific DNA functions can be modified by such exposure through epigenetic pathways, resulting in alterations in gene expression leading to a pathological phenotype. This chapter provided a comprehensive picture of the technological advances in genotyping, expression profiling, and proteomics, which offer exciting and promising approaches in understanding the basis of PTSD, as well as improved diagnosis and therapy. Additionally, short- and long-term exposure to toxic chemicals, such as OP nerve agents and insecticides, and other CWAs contribute toward PTSD onset. The combination of genomic and proteomic information will allow early and more accurate prediction of individuals' susceptibility to chemical exposure–related trauma, development of PTSD, and disease progression. Although significant discoveries have been made, there is obviously substantial promise and potential remaining to be fully realized through increasing use of and further development of these technologies.

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CHAPTER

47

Clinical and Cellular Aspects of Traumatic Brain Injury

Jason Pitt and Yiuka Leung

INTRODUCTION

Generally, traumatic brain injury (TBI) is a sporadic mechanical impact to the head that leads to acute or chronic impairments in neurological function. It is the most common cause of death in North America in individuals between ages 1 and 45 years (Rutland-Brown et al., 2006; Rosenfeld et al., 2012), accounting for 30.5% of all injury-related deaths. There are approximately 1.7 million cases of TBI annually in the United States, leading to 1.4 million emergency department (ED) visits, 275,000 hospitalizations, and 52,000 deaths. Costs of TBI, including direct medical expenses and indirect costs related to loss of productivity, amount to an estimated $76.5 billion each year. TBI occurs most commonly to children aged 0–4 years, adolescents aged 15–19 years, and adults older than 65 years. The leading causes of TBI-related deaths were injuries by firearms (34.8%), motor vehicle accidents (31.4%), and falls (16.7%). TBI-related death rates are highest in adults older than 75 years, and males are more likely to die from TBI than females across all age groups (Faul et al., 2010).

Our understanding of the pathophysiology of TBI has been advanced primarily through the use of several rodent models (Figure 47.1). This chapter summarizes our understanding of the cellular and molecular mechanisms that account for neurological abnormalities observed after TBI. The points of this work can be summarized as follows: TBI includes both primary and secondary injuries; primary injuries lead to necrosis; secondary injuries lead to apoptosis or alterations in synaptic function; and several pathological changes suggest that TBI is a risk factor for other neurological diseases.

A VARIETY OF MOUSE MODELS ARE USED TO STUDY TBI

Our understanding of the cellular and molecular changes that occur after TBI have been advanced through the use of animal models of TBI. In general, animal models of TBI deliver mechanical forces to the skull in a controlled manner. Many of the outcomes in animal models mimic those observed in human TBI, such as concussion, contusion/hemorrhage, and diffuse axonal injury (DAI). As with any experimental model, however, there is a trade-off between reproducibility and physiological relevance. A brief description of commonly used animal models of TBI follows. Readers interested in further details about different animal models of TBI should read the work by Xiong et al. (2013).

Highly reproducible models include fluid percussion injury and controlled cortical impact. In fluid percussion injury, a pressure pulse is sent through a fluid reservoir placed on the exposed dura. This pressure pulse displaces the brain to generate focal and diffuse injuries. Controlled cortical impact drives a rigid object directly onto the exposed dura. Both of these models require removal of a portion of the skull (craniotomy). This decreases variability of the injury but reduces the physiological relevance of the TBI.

More biomechanically relevant models include blast injuries, penetration injuries, weight-drop, and repeated mild injuries. Blast injuries deliver a pressurized gas wave to the head. Because this model uses a pressure wave that is difficult to focus, the animal's body must be protected using a Kevlar jacket. Blast injuries are excellent models of TBIs suffered as a result of improvised

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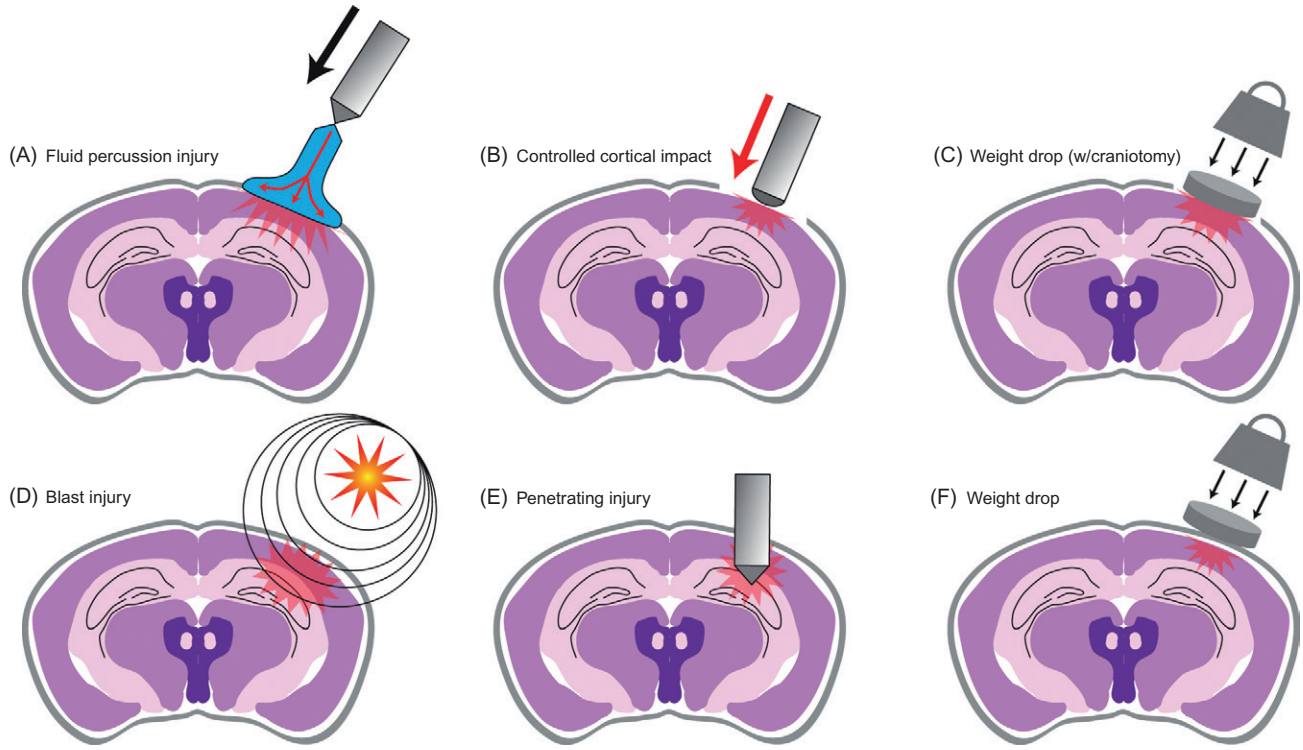

FIGURE 47.1 Mouse models of TBI.

explosive devices, which are the primary cause of head injuries in modern military conflicts. Penetrating injuries drive a projectile into the brain with high energy to mimic TBIs that occur from gunshot wounds. In weight-drop models, a free-falling weight is dropped on the exposed skull. In some weight-drop models, a craniotomy is performed to increase reproducibility.

Repeated mild injury models are of particular interest because they mimic injuries that occur in contact sports. Repeated injuries have been delivered using fluid percussion, blast, and weight-drop models. Although more characterization is needed, repeated mild injury models suggest that even mild TBIs can produce rapid, disastrous effects.

CLINICAL MANIFESTATION AND MANAGEMENT OF TBI

TBI Is Usually Classified Using the Glasgow Coma Scale

TBI is a heterogeneous entity. The severity of TBI can be classified according to various injury scoring systems, with each carrying different impacts on prognosis and treatment. The most commonly used scoring system is the Glasgow Coma Scale (GCS) at initial presentation (Vos et al., 2002; Moppett, 2007) (Table 47.1). A GCS score

TABLE 47.1 Using the GCS to Assess Head Injury Severity

Behavior	Response	Score
Eye opening	Open and blinking spontaneously	4
	Open to verbal stimuli	3
	Open to painful stimuli	2
	No response	1
Verbal	Aware of self, time and location	5
	Slight confusion	4
	Inappropriate replies	3
	Incomprehensible replies	2
	No reply	1
Motor	Follows commands	6
	Appropriate purposeful withdrawal from pain	5
	Normal flexion in response to pain	4
	Abnormal flexion in response to pain	3
	Extension in response to pain	2
	No response	1

Classification:
Coma—3
Severe head injury—4–8
Moderate head injury—9–12
Mild head injury—13–15.

of 13–15 is considered mild injury, 9–12 is moderate, and 8 or less represents severe TBI. The initial severity of TBI has prognostic value regarding the long-term outcome of the injury (Thornhill et al., 2000). Although the majority of TBI cases are mild (>75%) (Faul et al., 2010), the minority of patients presenting with moderate or severe TBI have a very poor prognosis, with mortality rate as high as 55%. Of all patients with a post-TBI GCS of 12 or less, 2% die within the first hour and 8% die within the first 6 h (Peek-Asa et al., 2001).

Although the GCS excels as a universal tool for TBI classification because of its simplicity, reproducibility, and prognostic power, its use may sometimes be limited by clinical confounders, such as anesthesia, sedation, paralysis, and coexisting intoxication. These confounding factors are especially prominent in patients with a low GCS score (Stocchetti et al., 2004). Hence, CT-based scoring systems are sometimes used by neurotrauma centers for a more objective classification on TBI. These include the Marshall scale, which has been shown to be accurate in predicting risks of increased intracranial pressure (ICP) but lacks reproducibility in cases of multiple brain injuries (Marshall et al., 1992), and the Rotterdam scale, which was developed more recently to overcome the limitations of the Marshall scale but still lacks validation in large populations (Maas et al., 2005). The details of these scales are beyond the scope of this chapter.

Primary Brain Injury and Secondary Insults Are Two Distinctive Phases of TBI

TBI is often divided into two distinct but related phases: primary brain injury and secondary insults (Werner and Engelhard, 2007; Greve and Zink, 2009). Clinically, primary brain injury often requires surgical attention, whereas secondary injury is managed in the intensive care setting, where the prevention and treatment of these secondary insults become the major focus of neurotrauma intensivists. Primary brain injury happens at the time of the initial mechanical impact to the skull.

The transfer of external mechanical forces to intracranial contents can lead to intra-axial damage and/or extra-axial injuries. Intra-axial damage involves the brain parenchyma. Focal cerebral contusions occur most commonly after TBI. Injuries involving abrupt acceleration/deceleration result in direct resonant impact of the brain with the base of the skull. Hence, the basal, frontal, and temporal areas are especially susceptible to contusion due to their anatomical distribution in relation to the bony areas of the cranial vault. In addition, shearing forces during mechanical impact can cause DAI. Patients with DAI often present with profound coma without elevated ICP, and often have a very poor prognosis. The cellular and molecular outcomes of intra-axial damages are described in greater details in other sections of this chapter.

Extra-axial damage involves nonbrain cranial structures. Penetrating trauma, blast waves, or direct impact can result in skull fracture and rupture of intracranial vessels. Depending on the location of the impacting force, different types of hematomas may result.

1. Epidural hematoma (EDH). Direct impact to the lateral surfaces of the head may cause rupture of the middle meningeal arteries embedded within the dura, forming an EDH. EDHs usually appear as a well-demarcated, biconvex lens-shaped density on CT scan. Bleeding in EDH is usually from a high-pressure arterial source but is initially tamponaded by the tightly tethered dura. However, as the hematoma expands and strips the dura from the skull, it gradually creates an intense headache. Classically, patients with EDH are described as having a lucid period right after injury, when bleeding is still contained and tamponaded by the dura. This lucid period is followed by sudden loss of consciousness, when expansion of hematoma becomes large enough to cause significant increase in ICP and compression or herniation of brain tissue.
2. Subdural hematoma (SDH). Impact to the frontal regions, as well as shearing forces from linear or rotational acceleration/deceleration, can tear the subdural bridging veins and result in SDH. On CT, SDHs appear as poorly demarcated crescent-shaped densities. Unlike EDH, SDH occurs between the dura and the arachnoid layers. Hence, SDH lacks the additional layer of "protection" offered by the rigid dura. Clinically, patients with SDH present with gradually increasing confusion and headaches. Acute SDH has a high mortality rate and often requires prompt surgical evacuation.
3. Subarachnoid hemorrhage (SAH). SAH refers to bleeding that occurs in the subarachnoid space, which is between the arachnoid and pia mater and is normally filled with cerebrospinal fluid (CSF). The majority (>85%) of SAHs occur in patients with a preexisting intracerebral vascular lesion, most commonly an aneurysm (van Gijn and Rinkel, 2001), which serves as a functional weak point that is susceptible to rupture when intravascular pressure is elevated. However, in the setting of trauma, sufficient external forces could lead to rupture of intracerebral vessels without preexisting vascular abnormalities. Radiologic clues for traumatic SAH include localized bleeding in the superficial sulci, adjacent skull fracture, cerebral contusion, as well as external evidence of traumatic injury (Rinkel et al., 1993). Clinical suspicion for SAH should be high for patients reporting sudden, severe headaches (often

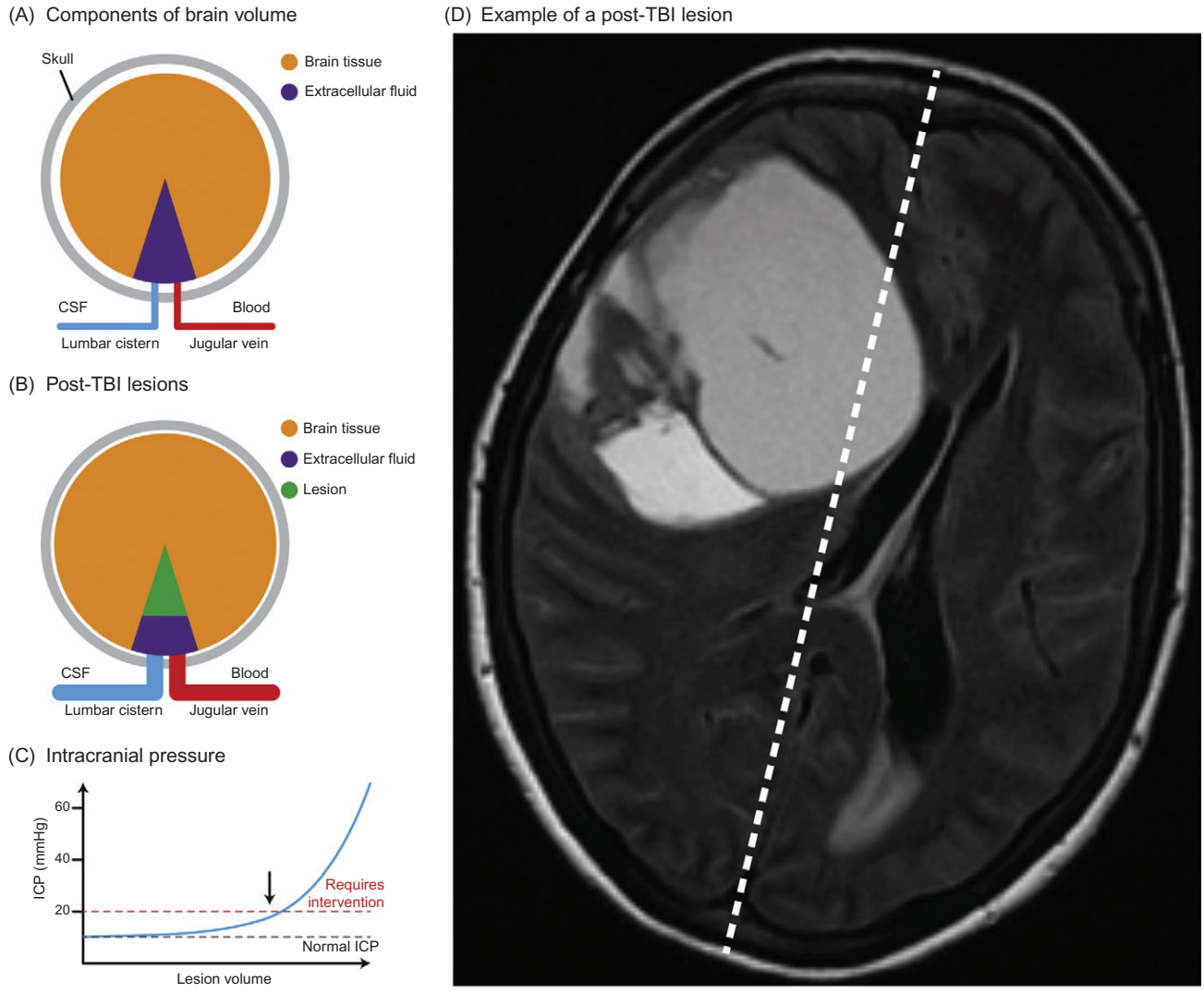

FIGURE 47.2 Regulation of brain volume is critical for maintaining safe ICP. (A) Brain tissue and the extracellular fluid (comprising blood and CSF) contribute to normal brain volume. (B) Brain lesions that occur after TBI increase intracranial volume. (C) An initial increase in intracranial volume only leads to minimal increase in ICP because of a compensatory outward flow of CSF and blood. Once this protective mechanism has been exhausted (arrow), ICP begins to increase with a steep slope. (D) Magnetic Resonance Imaging (MRI) showing midline shift of brain contents secondary to decompensated increase in intracranial volume (in this case by a growing tumor). Loss of sulci/gyri prominence and asymmetric compression of ventricles are seen. White line depicts the expected midline of the brain.

described as "thunderclap" headache or "the worst headache of my life"). In these cases, emergent CT of the head should be obtained. If CT fails to identify intracranial bleeding but clinical suspicion for SAH remains high, then a lumbar puncture must be performed to look for blood in the CSF.

Intracranial bleeding after TBI can be further exacerbated by coagulopathies, which develop in up to one-third of TBI patients (Zehtabchi et al., 2008; Allard et al., 2009; Wafaisade et al., 2010). Coagulopathies may result either from underlying anticoagulation medications (e.g., warfarin, clopidogrel) or from TBI-induced platelet dysfunction, release of systemic tissue factor, and activation of endogenous anticoagulation pathways (Maegele, 2013). All hematomas, intracranial bleeding from penetration injuries or skull fracture, entrainment of air, or foreign objects can all result in increases in ICP. In the acute setting, rapid increases in ICP can cause brain tissue to herniate within the skull, which can lead to compression of cranial nerves, leading to various neurological findings. The most dreaded complication is brain stem compression, which leads to suppression of many neurological functions important for survival, such as regulation of respiration and circulation (Figure 47.2).

Secondary insults of TBI result from cellular perturbations caused by primary injury. Secondary injury can happen at any later time point after the initial impact.

Although the primary injury of TBI is a major predictor of outcome, secondary insults can contribute to further worsening of prognosis (McHugh et al., 2007).

The mechanisms of secondary injury include neurotransmitter-mediated excitotoxicity, free-radical injury to cell membranes, electrolyte imbalances, mitochondrial dysfunction, inflammatory responses, apoptosis, secondary ischemia from vasospasm, focal microvascular occlusion, and vascular injury (Werner and Engelhard, 2007). These events can lead to cerebral edema and further increases in ICP. The detailed molecular mechanisms of these events are described later in this chapter.

A critical aspect in post-TBI intensive care management is the avoidance of these secondary brain insults, which would otherwise be well-tolerated but can exacerbate neuronal injury in cells made vulnerable by the initial TBI. Of particular importance is the prevention of hypotension and hypoxia (which decrease substrate delivery of oxygen and glucose to injured brain), fever and seizures (which may further increase metabolic demand), and hyperglycemia (which may exacerbate ongoing injury mechanisms), as discussed in the following sections.

TBI management focuses on stabilization of primary injury and prevention of secondary injury. We discuss management strategies during the early resuscitation period, as well as postresuscitation intensive care therapeutic goals. It is important to remember that at any period after TBI, the main goal is to avoid hypoxia and hypotension, the two most critical factors in predicting post-TBI outcome (Manley et al., 2001; Brain Trauma Foundation et al., 2007a; McHugh et al., 2007). Presence of prehospital hypoxia (PaO_2 <60 mmHg) and hypotension (systolic pressure <90 mmHg) is strongly associated with a poorer outcome (OR, 2.1 [95% CI, 1.7–2.6] and OR, 2.7 [95% CI, 2.1–3.4], respectively) (McHugh et al., 2007).

Immediate Care After TBI Shares the Same Principles as Advanced Life Support

For TBI patients and patients on advanced life support (ALS), the primary focus is on securing airway, breathing, and circulation (the ABCs of ALS). This may require intubation to maintain adequate oxygenation and fluid resuscitation or use of pressors to maintain perfusion pressure. The patient's heart rate, blood pressure, respiratory rate, and temperature should be continuously monitored. Neurological examination should be performed to assess the GCS score, and should be reassessed at regular intervals. A secondary survey should be performed to assess other extracranial injuries. All patients with TBI should be assumed to have an unstable cervical spine, and precautions should be applied to stabilize the spine (e.g., using a C-collar, "log-roll" precautions) to avoid cervical spinal cord or brain stem impingement during movements of the head. Blood should be sent to check for complete blood count, glucose, electrolytes, blood gases, and pH, as well as toxicology screen. As soon as the patient is stabilized, CT scan of the head should be obtained. CT can reveal skull fractures, intra-axial or extra-axial hemorrhages, significant brain contusions, and edema, which can guide downstream treatment strategies. If initial neurological examination or imaging shows findings suggestive of decompensated ICP elevation and impending herniation (Figure 47.2), then emergent treatment should be started immediately, including head elevation, maneuvers to improve venous drainage, and osmotic therapy.

Surgical Management is Often Necessary for Primary Injury

Timing and indications for surgical intervention after TBI are based on neurological status and radiological findings. Intracranial bleeding extrapleural hematoma (EPH), SDH, SAH causing significant midline shift should be evacuated surgically regardless of hematoma size. In the absence of midline shift, surgical evacuation of hematomas is recommended if the blood volume is large, or if the patient has a GCS less than 8. For penetrating injuries and skull fractures, surgery for superficial debridement and repair of dural tears and CSF leaks are often recommended. However, debridement of deeper tissues and aggressive extraction of intracranial foreign bodies and bone fragments have not been shown to improve outcome or prevent delayed infection, as long as prophylactic broad-spectrum antibiotics are administered in the setting of penetrating injuries. Occasionally, decompressive craniectomy (removal of a substantial portion of the skull) is performed in conjunction with hematoma evacuation to help decrease ICP. However, the efficacy of decompressive craniectomy in outcome improvement is still controversial, and ongoing clinical trials are underway to determine the benefits versus risks of this technique (Jiang et al., 2005; Cooper et al., 2011; Ho et al., 2011; Servadei, 2011).

Targeted Therapies Are Used to Prevent Secondary Injury in the Intensive Care Unit

After the initial resuscitation phase, TBI patients are monitored in the intensive care unit (ICU), where the major goal of care is to maintain cerebral and systemic perfusion, to correct electrolyte and coagulation abnormalities, and to minimize secondary insults.

Maintenance of Adequate Cerebral Perfusion Improves Outcome after TBI

Adequate cerebral perfusion pressure (CPP) is essential to prevent cerebral ischemia or toxic pooling

of inflammatory mediators. Optimal CPP after TBI is between 50 and 70 mmHg, with 60 mmHg being the target (Elf et al., 2005; Jaeger et al., 2010). CPP is equal to mean arterial pressure (MAP) minus ICP (CPP = MAP – ICP). Because CPP cannot be readily measured, MAP and ICP are used as surrogate measures. Goal CPP should be achieved first by reducing ICP, then optimizing MAP.

Patients with TBI should be positioned strategically to minimize ICP elevation. Head of the bed should be elevated to 30°. The neck should be in a neutral position without external compression to ensure adequate venous drainage from the brain to the heart. ICP monitoring is invasive, requiring surgical insertion of a ventricular catheter, but it is indicated in severe TBI (Brain Trauma Foundation et al., 2007b). Ventricular catheters are both diagnostic and therapeutic, allowing drainage of CSF if ICP is too high. In general, CSF drainage is recommended if ICP increases to more than 20 mmHg (Chesnut et al., 2012). If CSF drainage alone is inadequate, then osmotic therapy with hyperosmolar agents (mannitol or hypertonic saline) should be considered. These hyperosmolar agents create an osmotic gradient that draws water across the blood–brain barrier (BBB), thereby decreasing interstitial volume in the brain. Caution should be applied when using these agents, because they invariably trigger diuresis and may lead to an acute decrease in MAP.

MAP can be maintained with fluid infusion and the use of vasopressors. Normal saline is the fluid of choice to maintain euvolemia. Use of albumin is associated with a twofold increase in mortality and should be avoided (Chesnut et al., 2012). Infusion of excessive volume should also be avoided, because hypervolemia may cause further ICP elevations by raising central venous pressure and impeding venous return from the brain. In addition, hypertension may exacerbate intracranial hemorrhage.

Other Targeted Therapies

Many pharmacologic agents and therapeutic strategies have been tested in clinical trials for management of secondary insults after TBI. Unfortunately, no specific neuroprotective agent or strategy has been shown to improve outcome (Maas et al., 2008).

Hyperglycemia or hypoglycemia should be avoided. Hyperglycemia is associated with poor outcome after TBI and should be avoided. However, aggressive treatment of hyperglycemia with insulin infusions has the risk of iatrogenic hypoglycemia, which also leads to adverse outcomes. Glucose control remains a controversial issue in TBI and other critical care arenas. There is a lack of consensus on the exact range of target glucose levels. In general, it is recommended to avoid extremes of hyperglycemia or hypoglycemia.

Normothermia should be maintained. Prehospital hypothermia is strongly associated with poorer outcome after TBI (McHugh et al., 2007). However, hyperthermia has been postulated to exacerbate secondary inflammatory insults. There is no clear evidence, however, that hyperthermia leads to worsened neurological damage or outcome (Childs, 2008). Despite many clinical trials, neither therapeutic hypothermia nor antipyretic treatments have been shown to correlate with improved mortality or morbidity (Henderson et al., 2003; Sydenham et al., 2009). Hypothermia could potentially lead to coagulopathies, metabolic derangements, and increased infections, and is thus not recommended as routine practice.

Early use of antiepileptics may improve outcome. Posttraumatic seizures (PTS) may develop in up to 20% of patients with TBI (Temkin et al., 1990). Seizures increase cerebral blood flow and metabolic demand, which increases ICP and aggravates secondary brain injury (Vespa et al., 2007). Thus, early antiepileptic therapy is recommended, which can reduce the rate of early PTS, but does not prevent later development of PTS (Temkin, 2001).

Glucocorticoids may worsen outcome. Steroids were considered in the treatment of TBI due to postulated potential in reducing inflammation and edema. However, a large clinical trial showed that use of methylprednisolone in early TBI is associated with increased mortality without benefits (Roberts et al., 2004; Edwards et al., 2005). Thus, the use of glucocorticoids is not recommended.

TBIs INCREASE ICP

TBI is often associated with brain edema (or brain swelling), which increases ICP. It is important to note that there are two types of brain edema: cytotoxic brain edema and vasogenic brain edema. Cytotoxic brain edema is caused by disturbances in the osmotic balance of neural cells, such that increased water influx leads to necrosis, as observed in cases of excitotoxicity. Vasogenic brain edema involves disruption of BBB integrity. The BBB is a series of tight junctions between vascular epithelial cells. These tight junctions are dynamic structures that are maintained by the astrocytic endfoot processes that line the vascular epithelium. In pathological states (such as TBI), the tight junctions between epithelial cells are replaced by porous structures that allow the entry of large macromolecules (and their accompanying solvent) into the brain.

To understand the relationship between ICP and brain herniation, it is important to conceptualize the cranium as a rigid vault packed with three major components: brain tissue (~80%), blood (~8%), and CSF (~12%). The physical law of compliance governs the relationship

between ICP and the volume of these three components: an increase in pressure leads to a decrease in volume. Blood and CSF, both being fluids, are much more easily displaced than the solid brain parenchyma. Hence, when intracranial volume increases (e.g., due to acute intracranial bleeding), CSF and blood are the first components to be "pushed out" of the cranial vault. ICP drains from the cranium into the communicating subarachnoid space in the spinal column, whereas venous blood drains back to the heart. The flexibility of CSF and blood volume within the cranium creates a compensatory "cushion," such that increases in intracranial volume do not lead to elevated ICP initially. This "cushioning" effect lasts for as long as there is blood or CSF to be displaced. However, when intracranial volume expands beyond a certain level, this compensatory mechanism becomes exhausted. ICP will now begin to increase steeply with further volume expansion (Figure 47.2), and brain tissue now becomes the only other element left to be compressed by the increased ICP. Such compressive forces can lead to herniation of the brain across various weak points in the brain or across the tentorium. Brain herniation manifests with a wide variety of clinical signs and symptoms, depending on the area of the brain being compressed. Compression of the brain stem can lead to respiratory center depression and cardiac arrest. Other ominous signs suggestive of impending herniation include unilaterally or bilaterally fixed and dilated pupil(s), decorticate or decerebrate posturing, and the Cushing triad (bradycardia, hypertension, and respiratory depression).

Because a TBI that increases ICP is likely to have a poor outcome, it was thought that preventing edema would have therapeutic benefits. However, antiedematous therapy (e.g., steroids, superoxide dismutase, calcium antagonists, and inhibitors of bradykinin and glutamate receptors) has not proved effective in clinical trials conducted after TBI (Unterberg et al., 2004). This suggests that management of functional impairments associated with TBI will require targeting more upstream changes in cell signaling pathways. In line with this, intracranial lesions do not always lead to increased ICP, and increased ICP does not always equate increased risk of herniation. In certain conditions, such as pseudotumor cerebri, ICP can be chronically elevated but well-compensated with decreased ventricular size and intracranial CSF volume. Hence, patients with pseudotumor cerebri are not at significantly increased risk for brain herniation. Neuroimaging can be helpful in assessing the degree of intracranial distortion from elevated ICP. Concerning radiological findings suggestive of decompensated ICP elevation include loss of gray/white distinction, loss of sulci/gyri prominence, asymmetrical compression or enlargement of ventricular space, mass effect (distortion of size or position of normal brain structures), and midline shift (Figure 47.3). These findings should prompt immediate medical or surgical therapy.

COGNITIVE IMPAIRMENTS AFTER TBI RESULT FROM NEURONAL AND SYNAPTIC LOSS

Memories are stored in the connections between neurons (termed synapses). Our ability to form, recall, and lose memories requires the formation, maintenance, and loss of specific synapses. In general, we consider memories to be stored in two broad areas: the hippocampus (for short-term memories) and the cerebral cortex (for working and long-term memories). The cerebral cortex is also responsible for coordinating our conscious behavior based on multiple factors, including sensory information, emotional states, and previous events. Our seamless encoding of conscious experience is attributed to a vast network of synaptic connections within and between the cortex and hippocampus (Figure 47.3). As such, the loss of neurons and synapses is attributable to the cognitive decline observed in many pathological states, including TBI. In fact, impaired short-term memory and attention are characteristic of the majority of all TBIs (Faul et al., 2010). The superficial location of the cerebral cortex makes it particularly vulnerable to TBI.

Neuronal Loss Is an Irreversible Cause of Cognitive Impairment

Neurons are polarized cells with a number of extensive projections. In general, neurons receive information at their dendrites or cell body and transmit information along their axons (Figure 47.4). Although a vast oversimplification, it is generally true that more neurons can allow a greater diversity of information to be encoded. Thus, the loss of neurons disrupts information processing and accounts for the severe changes in behavior observed in several neurodegenerative disorders (e.g., Alzheimer disease, Parkinson disease, and Huntington disease).

Cell death can occur through a variety of mechanisms, such as necrosis, autophagy, apoptosis, and necroptosis. Of these, necrosis and apoptosis are highly relevant to TBI. Necrosis is uncontrolled cell death caused by acute disruption of the plasmalemma. In TBI, necrosis can occur through shearing forces that rip apart axons or osmotic imbalance due to hyperactive ionic conductances that occur during excitotoxicity. Conversely, apoptosis is a form of programmed cell death and is controlled by a variety of intracellular signaling cascades. Briefly, apoptosis occurs when contents in the intramembrane space of the mitochondria leak into the cytoplasm. Notable examples include cytochrome c, Smac/DIABLO, and

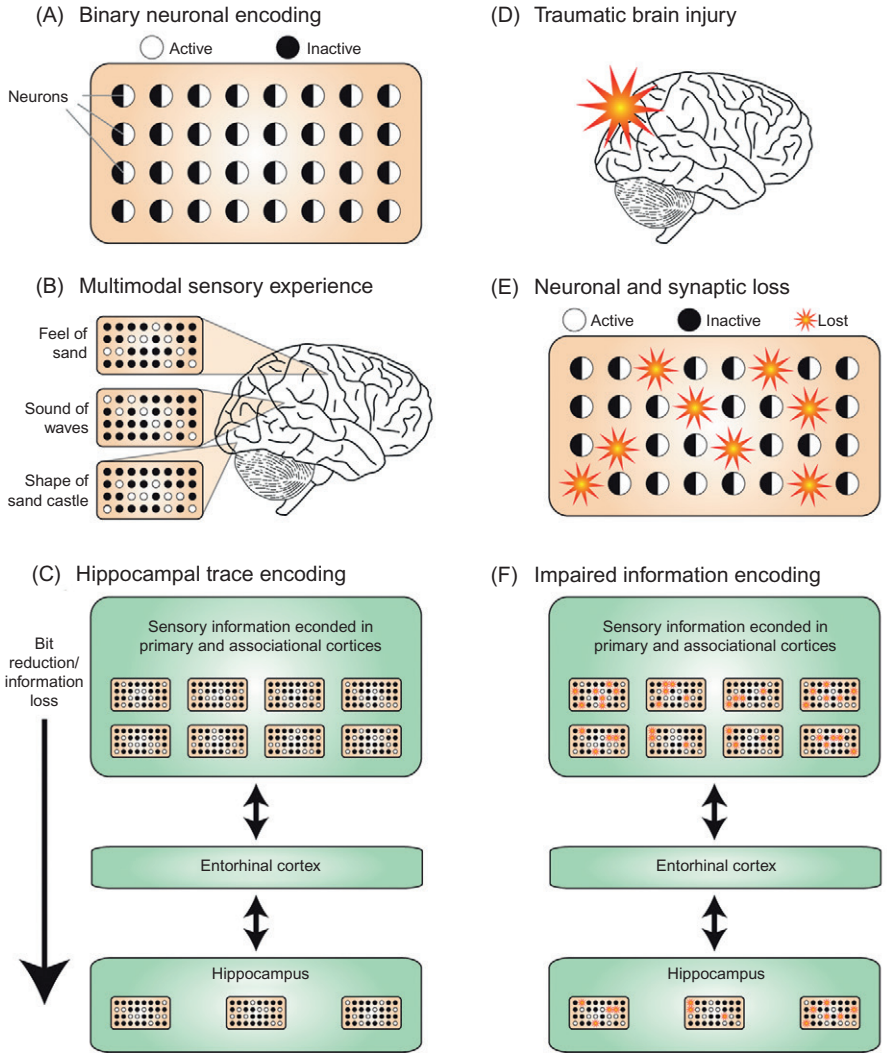

FIGURE 47.3 Cortical and hippocampal networks integrate sensory information. (A) The brain is often thought of as a computer. The binary code used by the brain is the activity (or inactivity) of specific neurons. Even a simple network of 30 neurons can encode more than 100 million (2^{30}) different information states. (B) Different patterns of neuronal activity in distinct brain regions encode a variety of sensory information. (C) Extensive interconnections between the cortex and hippocampus (via the entorhinal cortex) allow the encoding of memory traces and allow us to seamlessly integrate and store a variety of sensory information. Because there are fewer neurons and synapses in the hippocampus than in the cortex, information is lost during the encoding of memory traces, which may contribute to why memories are not as vivid as primary sensory experience. (D) and (E) The loss of neurons that occurs after a TBI reduces the amount of information we can encode. In fact, DAI may also sever connections between brain regions. (F) The result of neuronal loss is impaired information encoding and retrieval. The loss of neurons (and more specifically, synapses) explains the cognitive deficits that occur after a TBI.

Omi/Htra2, which lead to the activation of caspases—cysteine proteases that cleave a variety of intracellular contents. Through autoproteolytic activation, caspases create a feed-forward loop of proteolysis that inevitably leads to cell death. Accordingly, the cell contains the Bcl-2 family of proteins, which regulate mitochondrial permeability and caspase activity. A detailed description of apoptosis and its regulation by Bcl-2 proteins is beyond the scope of this chapter. Readers interested in additional details about the regulatory role of Bcl-2 proteins in apoptosis should read the work by Youle and Strasser (2008).

The loss of neurons through necrotic and apoptotic pathways may explain some of the cognitive impairment that occurs after TBI. Both necrotic cell death and apoptotic cell death are detectable in human cases and animal models of TBI. In controlled cortical contusion rat TBI models, cortical and hippocampal degeneration is evident within hours of injury, and the magnitude of degeneration is related to TBI severity (Sutton et al., 1993). Hippocampal neuron loss is relevant to memory impairments and a feature of the majority of severe TBI cases. A postmortem analysis by Kotapka et al. (1992) demonstrates how extensive this loss can be—most

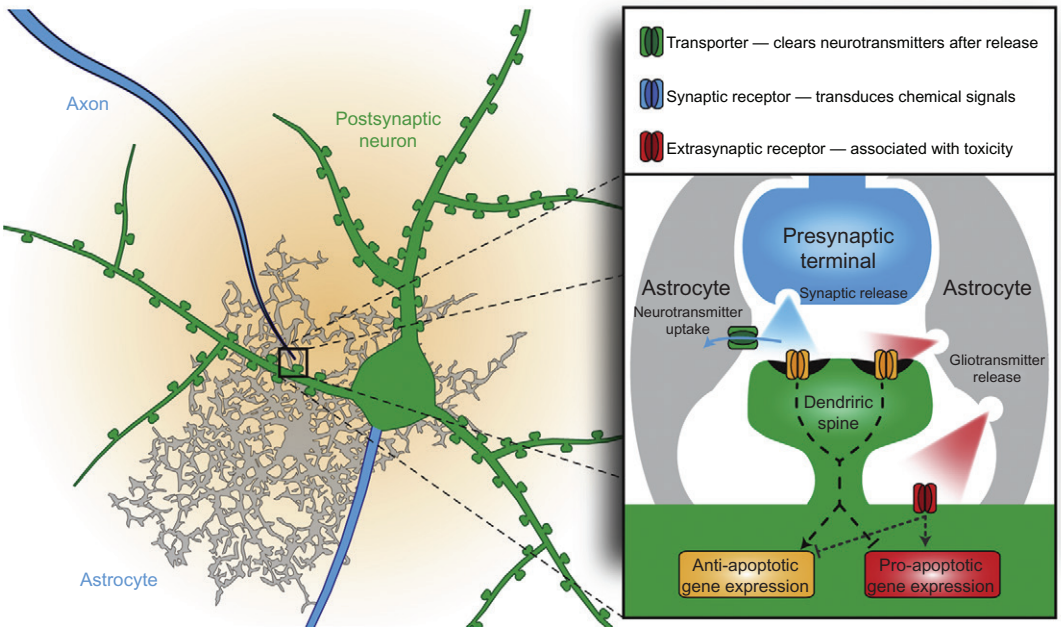

FIGURE 47.4 The synapse is a tripartite structure. Many synapses consist of three components: (i) a presynaptic site; (ii) a postsynaptic site; and (iii) a glial element. Presynaptic sites typically occur on axons and release neurotransmitters in an activity-dependent manner. Neurotransmitters bind to receptors on the postsynaptic site and elicit a cellular response. The activity of neurotransmitters is regulated by astrocytic processes, which express transporter proteins to uptake neurotransmitters such as glutamate. There is also emerging evidence that astrocytes may release neuroactive compounds (called "gliotransmitters") in an activity-dependent manner (called "gliotransmission"). Because of their peripheral location at the synapse, gliotransmitters from astrocytes tend to activate presynaptic and extrasynaptic receptors. Hyperactivation of extrasynaptic NMDA receptors (NMDARs) can lead to apoptotic cell death.

patients with severe, fatal TBI lose more than two-thirds of their neurons in region CA1 (Kotapka et al., 1992). The loss of neurons in the hippocampus after TBI correlates with cognitive impairments in human patients and rodent TBI models (Hicks et al., 1993; Bigler et al., 1997; Tate and Bigler, 2000).

Synaptic Loss is a Potentially Reversible Cause of Cognitive Impairment

In addition to neuronal loss, more subtle alterations in neuronal and synaptic function are observed after TBI. Because these changes do not involve stark cell loss, they are potentially reversible. Before discussing alterations, it is perhaps important to give an overview of synaptic structure and function. Synapses can be either electrical or chemical. We focus on chemical synapses (called synapses for the rest of the chapter) because they are, by far, the most common type of synapse. Synapses are incredibly small structures, with a diameter of 300 ± 150 nm and a gap of only 20 ± 2.8 nm (Ribrault et al., 2011). By and large, synapses comprise three parts: a presynaptic site, a postsynaptic site, and a glial element (Figure 47.4).

Presynaptic sites are typically found along axons and are the site of neurotransmitter release. The postsynaptic site is typically found on dendrites and cell bodies. The postsynaptic site contains a high density of neurotransmitter receptors that bind neurotransmitters and transduce the signal into a cellular response. Postsynaptic sites that occur at dendritic spines are very plastic to allow the storage of new information (i.e., memory formation). Finally, the glial element is an astrocytic projection that is involved in buffering the concentration of ions and neurotransmitters in the extracellular space. Astrocytic projections play an important role in preventing neurotransmitters from leaking outside the synapse or building up to toxic levels—preventing astrocytic uptake of the excitatory neurotransmitter glutamate leads to lethal, convulsive seizures.

All three of these synaptic elements are affected by TBI (Figure 47.5). Presynaptic sites are destroyed by DAI, in which rotational forces cause a shearing of axons. DAI can be measured in vivo using diffusion tensor imaging. Loss of white matter (i.e., axons) scales with TBI severity—moderate-to-severe TBI patients have obvious, global white matter damage, whereas mild TBI patients have more subtle alterations. Axonal damage measured by diffusion tensor imaging also correlates with cognitive impairments (Kraus et al., 2007). Postsynaptic alterations are also obvious after TBI. In controlled cortical impact mouse TBI models, dendritic spine density is reduced in the cortex and hippocampus within days of the injury

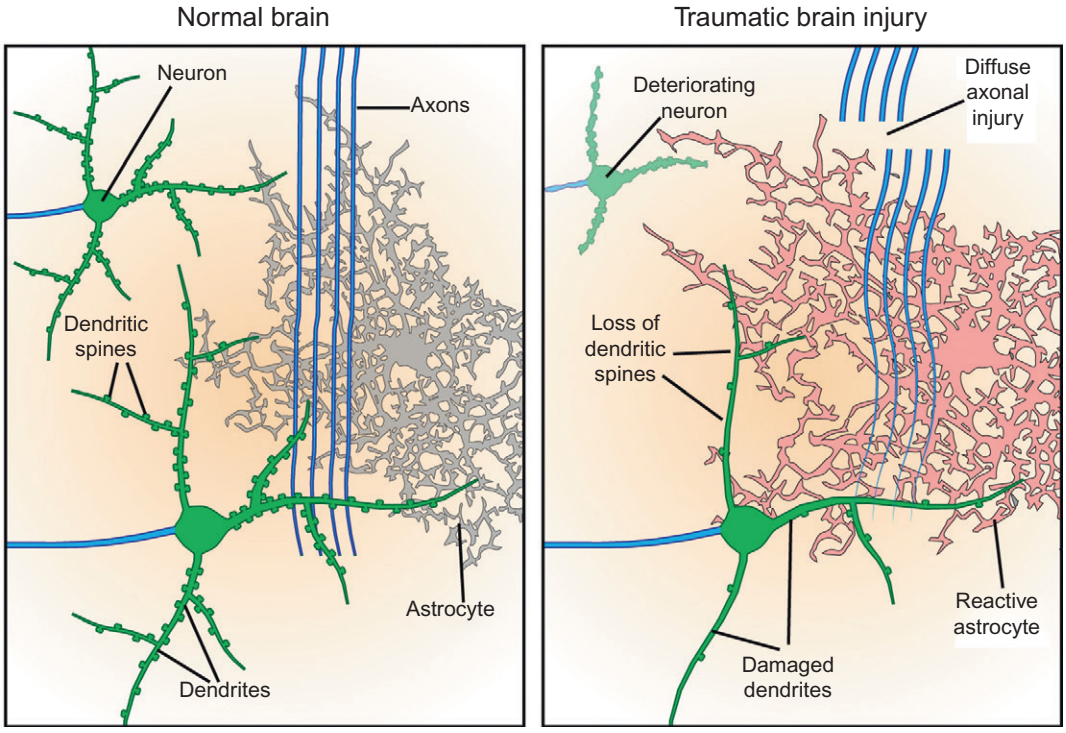

FIGURE 47.5 Traumatic brain injury causes cell death, synapse loss, axon damage, and astrocyte reactivity.

(Gao et al., 2011; Winston et al., 2013). The loss of dendritic spines reduces the number of postsynaptic sites, thus reducing neuronal excitability and restricting release from the presynaptic site. Thus, TBI can compromise synaptic function at presynaptic and postsynaptic sites in the absence of stark neuronal loss. Finally, glial elements are disrupted after TBI. One of the earliest and most reliable alterations is, in fact, reactive astrogliosis. It is not clear whether glial damage occurs before, after, or simultaneously with neuronal damage. However, it is likely that both cell types experience acute injury simultaneously, but neurons are more vulnerable to damage. Markers of neuronal injury are detected earlier than markers of glial injury in the CSF of children after TBI (Berger et al., 2002).

The brain is a dynamic organ, and dendritic spines are perhaps the most dynamic structures in the brain, showing the ability to rapidly retract and grow in an experience-dependent manner (Schubert and Dotti, 2007). In a unilateral fluid percussion injury rat TBI model, the excitatory synapse marker PSD95 is downregulated in the cortex and hippocampus (Campbell et al., 2012a). In harmony with this PDS95 reduction, the density of dendritic spines, particularly in the cortex, decreases acutely (<24 h) after fluid percussion injury (Campbell et al., 2012b). However, after 1 week, the density of dendritic spines recovers. Interestingly, hippocampal neurons have a higher density of dendritic spines 1 week after TBI compared with controls. The significance of this is not clear, although the authors speculate that elevated spine density in the hippocampus may explain the epileptogenic activity reported after TBI.

PRIMARY AND SECONDARY INJURIES INITIATE CELL DEATH THROUGH DISTINCT PATHWAYS

Neurological injuries that result from a TBI are classified as focal versus diffuse and primary versus secondary (Figure 47.6). Focal injuries occur in confined areas of the brain and are caused by contact forces to the head. Diffuse injuries occur over more widespread brain regions and are caused by inertial forces to the head. Primary and secondary injuries are distinguished based on their cause—primary injuries are caused directly by a TBI, whereas secondary injuries result from downstream effects of the original TBI. As a result, primary injuries occur immediately after the TBI and are associated with necrotic cell death. However, secondary injuries may take hours to weeks to manifest and are generally attributable to apoptotic cell death. The physical and cellular basis for these injuries is discussed.

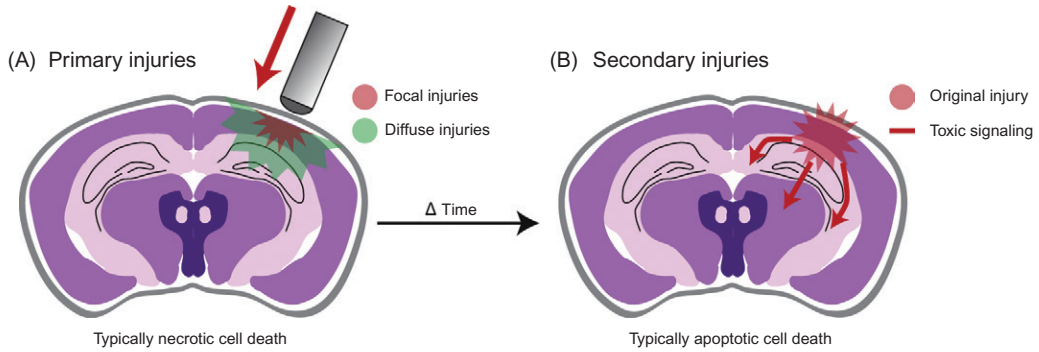

FIGURE 47.6 TBI comprises several types of injury. (A) Primary injuries result from the physical forces of the TBI. Focal damage is caused by contact forces that drive the brain into the skull, whereas diffuse damage is caused by inertial forces (linear and rotational accelerations of the head that shear axons). (B) Over time, secondary injuries emerge due to aberrant cellular signaling cascades that emanate from the initial site of damage.

Initial Primary Injuries Cause Necrosis

The mechanical force of a TBI causes immediate injury due to contact and inertial forces. Contact forces are the forces that prevent the head from moving after impact, which drives the brain into the skull. Even in the absence of skull fracture, movement along naturally occurring ridges and protuberances of the skull causes focal injuries to the cerebral cortex. Inertial forces are the linear and rotational accelerations of the head. Inertial forces cause axonal shearing, particularly at the junction of gray and white matter, which have different densities. This accounts for the diffuse nature of injuries that result from inertial forces. The primary injuries caused by contact and inertial forces are not likely to be treatable due to the limited regenerative capabilities of the central nervous system (CNS). Instead, these injuries must be prevented through the use of protective equipment.

The DAI that occurs during primary injury impairs the propagation of signals from the cell body of neurons to presynaptic terminals. White matter tracts are particularly susceptible to shearing forces incurred during a TBI. DAI is only confirmed by microscopic analysis, although there are many in vivo imaging and detection methods that are used to assess axonal integrity. Diffusion tensor imaging is an MRI variant that measures the diffusivity of water molecules. In white matter, water is unable to diffuse freely and is restricted in certain directions. This directional restriction of diffusion is termed anisotropy. By measuring the fractional anisotropy of different brain regions, white matter maps can be created to trace bundles of axons. Diffusion tensor imaging can readily detect DAI in moderate-to-severe TBI patients, whereas mild TBI patients show some subtle alterations (Inglese et al., 2005; Kraus et al., 2007). Using *in vivo* microdialysis in patients with mild TBI (Glasgow Coma Score = 9), Petzold et al. (2011) monitored axonal injury by detecting neurofilament heavy chain, an intermediate filament found in axons. Extracellular levels of neurofilament heavy chain show two phases of elevation: a pronounced, immediate elevation and a delayed, longer-lived elevation (Petzold et al., 2011). Presumably, these phases reflect primary and secondary neuronal loss: the marked, early elevation is likely due to axonal shearing, which spills out the intra-axonal contents, whereas the later elevation is due to neuronal loss through secondary injuries.

Axons in the CNS may be particularly vulnerable to rotational and translational forces due to the high compaction of myelin. Myelin is a lipid-rich substance that wraps around 0.3- to 2-mm-long segments of axons. The purpose of myelin is twofold: myelin insulates the axon to speed the conduction of action potentials and myelin reduces metabolic demand by decreasing the number of ions that move across the membrane during an action potential. Myelin in the peripheral nervous system (PNS) is moderately compact, providing some cushioning to allow peripheral nerves to withstand compression forces. PNS myelin is synthesized by Schwann cells, which produce an extracellular matrix (ECM) that contains proteins that support axonal regeneration, such as laminin-2 (Chen and Strickland, 2003)—this explains the minimal regenerative properties of the PNS. Myelin in the CNS, however, is highly compacted to allow a greater density of synapses. However, this comes at the cost of providing minimal cushioning in the event of compression forces. CNS myelin is synthesized by oligodendrocytes, which also secrete little to no ECM. As a result, when axons are severed, the myelin sheath does not have the appropriate growth signals to allow regeneration. In fact, CNS myelin contains factors that may actually prevent axonal regeneration (Huang et al., 2005). This lack of regenerative abilities in the CNS highlights the importance of preventing TBIs through the use of proper protective equipment.

Later Secondary Injuries Initiate Programmed Cell Death

Hours or days after the primary injury, secondary injuries develop. Secondary injury is caused by the release of substances that alter synaptic function, blood flow, ionic and neurotransmitter homeostasis, metabolic function, and inflammatory signaling pathways. This section focuses on mechanisms of neuronal loss by secondary injuries; mechanisms of synaptic alterations are discussed in the next section.

Apoptotic Markers Are Detectable Postmortem in TBI Patients and Experimental Models

As discussed, apoptosis is a highly regulated form of programmed cell death that culminates in the activation of caspases. Caspase 3 activation is detectable in the vicinity of cerebral contusions (Petzold et al., 2011), highlighting the importance of maintaining the integrity of BBB. Disruption of the BBB is associated with TBI and a variety of neurodegenerative disorders (Figure 47.2 and Chapter 49). A variety of other pro-apoptotic markers are detectable in the CSF of infants and children after severe TBI, including cytochrome c, caspase 1, and Fas. The increase in pro-apoptotic markers was greater in girls; however, the cause of this gender difference is not clear (Satchell et al., 2005).

The expression of pro-survival and pro-apoptotic genes in cortical neurons was measured using antisense mRNA (aRNA) amplification in individual cortical neurons with fragmented DNA (a sign of cellular damage). After 12 h, pro-survival genes (e.g., neurotrophins, TrkB, and superoxide dismutase) are downregulated in injured neurons. After 24 h, the levels of many pro-survival gene expression return to normal, although by this time the pro-apoptotic genes caspase-2 and bax are upregulated. The study by O'Dell et al. (2000) shows that genetic alterations occur rapidly after TBI and compromise cell health.

If apoptotic pathways are activated after TBI and play a role in the ensuing impairments, then apoptosis inhibitors should offer some therapeutic benefits. Continuous administration of the calpain inhibitor AK295 15 min after injury rescues motor deficits in a fluid percussion injury TBI rat model. However, only marginal improvements were observed in memory function (Saatman et al., 1996). Along these lines, inhibiting formation of the mitochondrial permeability transition pore with cyclosporine A has therapeutic benefits in a variety of TBI models, although utility in human TBI patients has yet to be demonstrated (Okonkwo et al., 1999; Sullivan et al., 2000; Mazzeo et al., 2009). Taken together, these data show a clear connection between TBI and activation of apoptotic pathways.

Glutamate Dysregulation Can Lead to Cell Death Through Necrotic Swelling, Excitotoxic Calcium Elevations, or Hyperactivation of Extrasynaptic NMDA Receptors

The principal excitatory neurotransmitter in the CNS is glutamate. There are two classes of ionotropic glutamate receptors—NMDA and non-NMDA receptors. Non-NMDA receptors desensitize rapidly and show no voltage gating. NMDA receptors (NMDARs), however, desensitize slowly and are only active when the cell is depolarized enough to remove a Mg^{2+} ion that blocks the ion channel. Glutamate levels are tightly regulated by glutamate transporters expressed in neurons and astrocytes.

Persistent glutamatergic activity leads to excitotoxicity through two routes: necrosis and apoptosis (Figure 47.7). In both routes, persistent glutamatergic input depolarizes the cell and allows hyperactivation of NMDA receptors, which desensitize much more slowly than non-NMDA receptors and thus allow more ions to enter the cell. Acutely, as sodium and calcium rush in through ionotropic glutamate receptors, they make the cell hyperosmotic. Acute swelling can lead to rapid necrotic cell loss. Alternatively, if the cell successfully reestablishes ionic imbalance across its membrane, it will deplete its ATP stores. To replenish ATP, mitochondrial activity increases, which elevates the production of free radicals (termed reactive oxygen species, ROS) that lead to oxidative stress. Both oxidative stress and sustained Ca^{2+} elevations can induce cell death by activating apoptotic proteases through formation of mitochondrial transition pores that allow the efflux of pro-apoptotic signaling molecules.

Nilsson et al. (1990) used microdialysis to measure changes in a variety of energy-related metabolites and neurotransmitters in a weight-drop rat TBI model. Two hours after TBI, extracellular glutamate levels increase in relation to TBI severity; mild TBI increases glutamate eightfold, whereas severe TBI increases glutamate 13-fold. Increases in glutamate coincide with higher levels of lactate, the end product of glycolysis (Nilsson et al., 1990). Because astrocytes display higher levels of anaerobic metabolism than neurons, these data suggest that after TBI, neuronal activity is upregulated, which leads to increased glutamate uptake and glycolytic activity in astrocytes in an attempt to buffer this excess glutamate. As discussed, increased levels of glutamate will eventually lead to intracellular calcium increases, which can lead to apoptosis or more subtle alterations in cellular/synaptic function. Readers interested in additional details about the role of glutamate excitotoxicity in TBI should read the work by Arundine and Tymianski (2004).

Because glutamate is a ubiquitous, excitatory neurotransmitter with potentially toxic consequences, the extracellular level of glutamate is tightly regulated. After presynaptic release, glutamate is rapidly taken up by

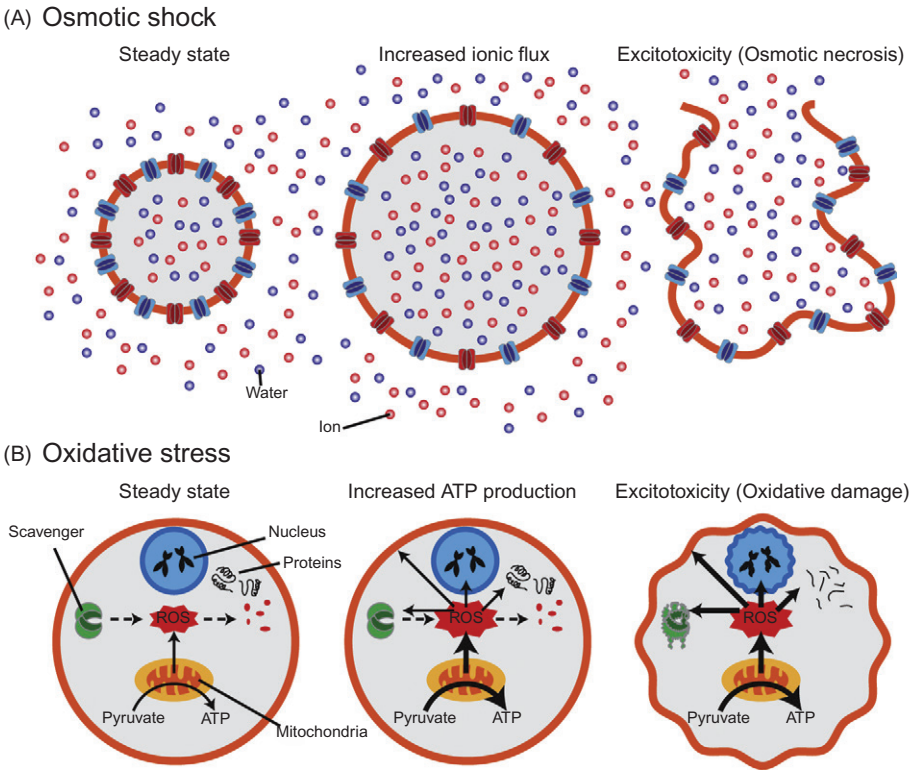

FIGURE 47.7 Excitotoxicity occurs through two routes. Excitotoxicity is caused by excessive glutamatergic signaling. (A) Continuous ionic flux increases the osmolarity of the cell. In extreme cases, this will drive enough water into the cell to cause osmotic necrosis. (B) If the cells survive the massive influx of ions, then ionic imbalance is reestablished in an ATP-dependent manner through a variety of ion pumps. As more ATP is generated through the TCA cycle and oxidative phosphorylation, ROS are produced. High rates of ATP production will produce toxic levels of ROS that cannot be scavenged by intracellular reducing agents (e.g., ROS, glutathione, and Nicotinamide adenine dinucleotide phosphate (NADPH)). ROS oxidize a variety of macromolecules to damage the DNA, proteins, and membranes of the cell. Ultimately, this oxidative damage results in apoptosis.

astrocytes. As discussed, astrocyte processes surround many synapses and play a role in buffering neurotransmitter levels. In the case of glutamatergic transmission, astrocytes regulate extracellular glutamate levels through high-affinity excitatory amino acid transporters (EAATs 1 and 2). For many years, astrocytes were viewed as morphologically simple cells due to their visualization using antibodies against the intermediate filament glial fibrillary acidic protein (GFAP). It is now clear that GFAP occupies a mere 10% of astrocytic volume and that astrocytes extend numerous tortuous processes to sample nearly all of their occupied volume, with essentially no overlap between adjacent astrocytes (Halassa et al., 2007). However, neighboring astrocytes are coupled to one another via gap junctions to create an extensive network with greater buffering capacity and potentially unappreciated signaling capabilities. Readers interested in additional details about astrocytic network functions should read the work by Giaume et al. (2010).

After injury, astrocytes undergo a series of morphological and functional alterations termed reactive astrocytosis, which is characterized by cellular hypertrophy and, in extreme cases, reentry into the cell cycle. Reactive astrocytosis is one of the most reliable markers of neurological damage and occurs within days of TBI. Although reactive astrocytosis is generally thought of as neuroprotective, cellular hypertrophy may lead to glutamate release through volume-sensitive anion channels (Takano et al., 2005). Additionally, the epileptiform activity that occurs after some TBIs will release high levels of glutamate and increase metabolic demands. Because glutamate uptake is driven by ionic gradients that are established in an ATP-dependent manner, depletion of energy stores and reduced metabolic support will necessarily reduce glutamate uptake. Because of their peripheral location at synapses, glutamate released from astrocytes may be particularly toxic due to the activation of extrasynaptic NMDA receptors.

NMDA receptors are found at and away from the synapse. In general, synaptic NMDA receptors are considered neuroprotective, whereas activation of extrasynaptic NMDARs leads to apoptosis (Figure 47.4). This may be a homeostatic adaptation that eliminates leaky synapses (i.e., those that allow glutamate to escape and

active extrasynaptic NMDARs). The details of synaptic versus extrasynaptic NMDA receptor signaling are still not clear, although it does appear that these two pathways have opposing functions on pro-survival, CREB-dependent gene expression and pro-death, FOXO-dependent gene expression. Readers interested in additional details about the cytotoxic effects of extrasynaptic NMDA receptors should read the work by Hardingham and Bading (2010).

ROS and Inflammatory Cytokines are Upregulated After TBI

A commonly reported event that occurs after TBI is an increase in ROS from mitochondria. ROS are highly reactive free radicals (e.g., superoxide and hydroxyl radicals) that damage a range of macromolecules, including lipids, proteins, and DNA. Thus, cells maintain reducing conditions through production of NADPH and glutathione, which work with superoxide dismutase to neutralize ROS. Elevated levels of ROS are indicative of cellular stress and will eventually lead to cell death if left unchecked.

Bayir et al. (2002) published a comprehensive analysis of antioxidant reserve and oxidative damage after TBI. Early on, 1 day after TBI, CSF samples from children and infants with severe TBI show higher levels of the oxidative stress marker F2-isoprostane. F2-isoprostane levels normalized after day 2. However, levels of the antioxidant ascorbate are reduced immediately after TBI (day 1) and continue to decline afterward (up to day 7). Glutathione, an important reducing agent, levels show a biphasic response to TBI: glutathione is elevated immediately after TBI and declines after 5 days (Bayir et al., 2002).

A variety of antioxidant compounds have been used to scavenge ROS in rodent TBI models and human TBI patients (Dohi et al., 2006; Hall et al., 2010). Some ROS scavengers produce positive impacts on survival and neurological outcomes in larger phase III clinical trials (Marklund et al., 2001; Hall et al., 2010). Despite this, ROS scavengers are not currently used as a therapeutic option to treat TBI.

In Neurons, Cell Death Is Also Caused by Attempted Reentry into the Cell Cycle

Neurons are *postmitotic* cells; thus, they no longer undergo cell division. This loss of reproductive capabilities is attributable to their complex cellular morphology. To undergo cell division, neurons must retract their intricate dendritic and axonal arbors—an impossible task. Neurons that reenter the cell cycle invariably fail to complete the task and die. Because of this, neurons actively suppress cell-cycle reentry. This suppression is removed in several neurodegenerative disorders, including TBI.

Cyclin-dependent kinase inhibitors, such as flavopiridol, prevent cell-cycle progression. In a lateral fluid percussion injury rat model of TBI, the expression of genes associated with DNA injury and cell-cycle progression are upregulated. These genetic alterations appear to play a causative role in neuronal cell death, because intracerebroventricular injection of the cell-cycle inhibitor flavopiridol reduces neuron loss. Flavopiridol also reduces the activation of astrocytes and microglia, although it is unclear whether cell-cycle reentry occurs in neurons, glia, or both or whether neuronal dysfunction and glial dysfunction synergize during TBI (Di Giovanni et al., 2005).

POTENTIAL MECHANISMS OF SUBTLE SYNAPTIC IMPAIRMENTS IN TBI

Synapses are maintained in part through neurotrophic signaling. Neurotrophins are a class of proteins that activate receptor tyrosine kinases (Trk receptors) to regulate the growth and maintenance of synapses. Cell growth is stimulated by neurotrophins through their activation of Rho GTPases, a diverse family of proteins that (in general) positively regulate the stability and growth of actin filaments and microtubules. Through their positive impact on cytoskeletal organization, neurotrophins induce the outgrowth of dendrites and axons in a variety of neurons. The importance of neurotrophic support on synaptic maintenance is illustrated in mice that lack brain-derived neurotrophic factor (BDNF), a neurotrophin implicated in learning and memory. BDNF knockout mice develop normal dendritic arbors but fail to maintain them (Gorski et al., 2003). Because dendrites are important postsynaptic sites, this reduction in dendritic arborization reduces the number of possible synaptic contacts.

As discussed, neurotrophic expression is downregulated in neurons with DNA fragmentation after TBI (O'Dell et al., 2000). However, whole brain analysis shows upregulation of neurotrophins within the same time frame in a controlled cortical impact TBI rat model (Oyesiku et al., 1999). Another study using in situ hybridization shows that some neurotrophins increase after mild TBI (e.g., BDNF), whereas others (e.g., NT-3) decrease (Hicks et al., 1999). These data suggest that nondamaged neurons and glia upregulate their expression of neurotrophins to offset the decreased expression in injured neurons. However, upregulation of neurotrophins may be deleterious as high levels of neurotrophins activate the low-affinity $p75^{NTR}$, which is linked to pro-apoptotic and synapse-degenerating signaling pathways.

The loss in postsynaptic sites is regulated by enzymes that control the polymerization state of actin. Work by Campbell et al. (2012a) provides mechanistic insights

into the postsynaptic alterations that occur in a fluid percussion injury rat TBI model. Within 18 h after injury, PSD95 levels in the cortex and hippocampus decline, indicating the loss or shrinkage of postsynaptic sites. Reduction of PSD95 is preceded by an increase in the activity of cofilin—an actin-severing enzyme. When cofilin is activated, it breaks apart actin filaments and causes the shrinkage of dendritic spines (i.e., excitatory postsynaptic sites). Normally, cofilin is held in an inactive state through phosphorylation of Ser3, which disallows interactions between cofilin and actin. However, TBI increases the activation of the protein phosphatase calcineurin, which dephosphorylates and activates cofilin. Because calcineurin activity is regulated by calcium–calmodulin, these results suggest that calcium dysregulation after TBI may cause the loss of excitatory synapses through cofilin-dependent actin depolymerization.

TBIS REPRODUCE PATHOLOGICAL HALLMARKS OF ALZHEIMER DISEASE

Alzheimer Disease Is a Dementia Associated with Aberrant Aβ and Tau Signaling

Alzheimer disease (AD) is a progressive dementia that is classically characterized by amyloid plaques, tau neurofibrillary tangles, and brain atrophy. Amyloid plaques are extracellular aggregates composed mostly of aggregated forms of Aβ. Although plaques are easily detected postmortem, cognitively normal elderly individuals can have high levels of plaques (Buckner et al., 2005). Additionally, a population of Japanese AD patients with a specific mutation in Aβ (E22Δ) exhibited dementia in the absence of plaques (Tomiyama et al., 2008). Therefore, many experts in the field have moved away from the amyloid cascade hypothesis proposed by Hardy and Higgins (1992) and instead believe the soluble oligomer hypothesis, in which diffusible Aβ oligomers (AβOs) attach at or near synapses and alter synaptic function (Hardy and Higgins, 1992; Wilcox et al., 2011). A similar movement has occurred with regard to the microtubule-associated protein tau: soluble tau oligomers are viewed as more toxic than insoluble tau neurofibrillary tangles. Finally, it is absolutely critical to remember that the best correlate with dementia severity is not brain atrophy, but rather synapse loss (Terry et al., 1991). Thus, although most view AD as being typified by plaques, tangles, and atrophy, these are antiquated views.

Aβ is derived by proteolytic processing of the amyloid precursor protein (APP) by a group of proteases called the secretases to produce a number of soluble peptides with diverse cellular functions. Aβ is produced when APP is cleaved outside the membrane by β-secretase (now identified as BACE-1) and within the membrane by γ-secretase. The strongest support for a causative role for Aβ in AD progression comes from human genetics. There are several mutations and duplications in APP and γ-secretase that can lead to heritable familial AD (FAD). FAD-association mutations can increase Aβ production (Swedish, Flemish), promote Aβ polymerization into toxic forms (Osaka and Arctic), or shift the metabolism of APP to produce more Aβ that is 42 amino acids long (Florida, London), two amino acids longer than normal and far more prone to polymerization. It is important to note that one mutation exists that actually decreases the risk of development of AD (Icelandic) by disrupting Aβ production (Jonsson et al., 2012).

Based on studies using tau knockout mice, it appears that Aβ induces synaptic loss and eventually cell death by structural and functional modifications to tau. Normally, tau is localized in axons, where it binds to and stabilizes microtubules. However, when tau is phosphorylated, it leaves the microtubule and activates a protein phosphatase 1 (PP1) through its phosphatase activating domain (PAD). PAD likely acts as an autoregulatory mechanism to limit the time that tau spends away from the microtubule and to prevent tau from becoming hyperphosphorylated—tau hyperphosphorylation is associated with AD progression. In fact, when tau is hyperphosphorylated in AD, it no longer interacts with axonal microtubules and translocates to the cell body and dendrites.

Tau is phosphorylated by a number of kinases, but glycogen synthase kinase 3β (GSK3β) appears to be highly relevant to AD. AβOs indirectly activate GSK3β, thus causing tau phosphorylation. Phosphorylated tau activates PP1 through its PAD. PP1 dephosphorylates both tau and GSK3β, which keep the kinase active and inevitably lead to tau hyperphosphorylation. The functional consequence of tau hyperphosphorylation is "synaptic starving," in which impaired axonal transport disrupts the delivery of critical proteins and energetic substrates to the synapse, eventually leading to synapse loss (Mandelkow et al., 2003). Alterations in intracellular trafficking are likely due to prolonged GSK3β activation (Kanaan et al., 2011). GSK3β phosphorylates the motor protein kinesin, which delivers vesicular cargo to presynaptic and postsynaptic sites. Phosphorylation by GSK3β causes kinesin to dump its cargo, which, in the case of chronic GSK3β activation, can lead to depletion of synaptic components. Thus, the tau–PP1–GSK3β positive feedback, which may be initiated by Aβ, inevitably leads to tau pathology and synaptic dysfunction.

TBI Patients Have Elevated Levels of Aβ

Amyloid pathology is observed in rodent TBI models and human TBI patients. In fact, diffuse amyloid plaques are present in the brains of professional boxers with TBI

at levels comparable with those observed in AD patients (Roberts et al., 1990). APP, BACE-1, presenilin (a component of γ-secretase), and Aβ accumulate in the terminal portion of severed axons after TBI (Chen et al., 2004). By concentrating APP and the secretases together, Aβ production is favored. Increased Aβ production leads to oligomer formation and eventually synaptic dysfunction and loss. Accordingly, Aβ accumulation increases with repetitive head injury and correlates with injury severity (Uryu et al., 2002; Tran et al., 2011). However, attenuating Aβ production with a γ-secretase inhibitor does not prevent the loss of dendritic spines after a controlled cortical impact TBI (Winston et al., 2013). This suggests that although TBI may promote the onset of AD pathology, acute synaptic damage occurs through alternative routes. It is likely that elevated Aβ levels may play a role in the chronic effects of TBI on synaptic structure and function, although this is purely speculative.

Tau Is Hyperphosphorylated After TBI

Because TBIs increase Aβ burden, and because Aβ acts upstream of tau, TBIs also induce tau pathology. Brains from TBI and AD patients show tau hyperphosphorylation at the same amino acid residues (Schmidt et al., 2001), and both occur in cortical pyramidal cells, albeit in different subpopulations (Hof et al., 1992). Neurofibrillary tangles are found in TBI patients years after a single TBI (Johnson et al., 2012). The development of tau pathology after a TBI takes some time, because a single mild TBI does not increase tau pathology after 3 weeks in hTau mice, which express human tau isoforms. Instead, multiple TBIs are required to observe tau pathology, although astrocyte reactivity is apparent 3 weeks after a single TBI (Ojo et al., 2013). Because of the axonal localization of tau, one likely cause of tau pathology in TBI is DAI, which occurs when delicate axons are torn apart during rapid head acceleration (Elson and Ward, 1994). However, based on the time required for tau pathology to become apparent, it is likely that increased Aβ production drives tau hyperphosphorylation through alterations in intracellular signaling.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

TBI is a multifactorial and sporadic neurodegenerative syndrome that is caused by contact and inertial forces that lead to primary injuries that are focal or diffuse, respectively. Early primary injuries cause necrotic cell loss. The surviving cells have compromised cellular function or support, leading to secondary injuries. Secondary injuries lead to aberrant cell signaling, impaired synaptic function, and apoptotic cell death. The mechanisms of apoptotic cell death have multiple factors and include glutamate excitotoxicity, oxidative damage, and reduced neurotrophic support. In the absence of marked cell loss, synaptic loss is also observed after TBI. Synaptic impairments are the result of impaired neurotransmitter homeostasis, which leads to aberrant calcium signaling. Pathological overlaps between TBI and AD also contribute to the loss of synapses and neurons.

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CHAPTER

48

Excitotoxicity, Oxidative Stress, and Neuronal Injury

Snjezana Zaja-Milatovic and Ramesh C. Gupta

INTRODUCTION

Exposure to anticholinesterase (anti-AChE) agents, organophosphates (OPs) and carbamates (CMs) in the form of insecticides and chemical warfare agents (CWAs) affects or threatens much of the world's population. The widespread use and easy accessibility to more than 100 different OPs used as insecticides worldwide result in a huge number of intoxications and several hundred thousand fatalities annually (Gunnell and Eddleston, 2003). Other derivatives of phosphoric acid, nerve agents used in warfare, are considered the most toxic compounds of all chemical weapons. Devastating effects of these agents have been demonstrated during the Iraqi conflict with Iranian troops and Kurdish civilians, as well as a terrorist attack on the Tokyo subway train system that occurred in 1995, resulting in over 5,500 casualties (Nozaki et al., 1995; Nagao et al., 1997). Terrorist attacks involving warfare nerve agents, weapons of mass destruction, or other industrial chemicals present worldwide security threats and health concerns. Therefore, anti-AChE agents represent a significant potential threat not only to the military, but also to the general civilian population.

Pharmacologically, all these compounds are acetylcholinesterase (AChE) inhibitors. Their acute symptoms are attributed to accumulation of acetylcholine (ACh), thus exhibiting cholinergic toxicity. Phosphorylation of the esteratic site of the enzyme diminishes its capacity to catalyze its endogenous substrate ACh (Taylor, 1990). Most OP compounds combine with AChE only at the esteratic sites, and the stability of the bond depends upon the structure of the compound that is attached. OP compounds containing larger alkyl groups may hinder cleavage, leaving the phosphorylated AChE inactivated almost indefinitely. As a result, normal activity recurs only upon the synthesis of a new enzyme. This process is known as *aging*, and its time course varies depending on the nerve agent. Consequently, the hydrolysis of ACh is prevented, leading to accumulation of ACh in the synaptic cleft and overstimulation, followed by the desensitization of muscarinic and nicotinic ACh receptors.

The constantly activated nicotinic cholinergic receptors generate involuntary skeletal muscle contraction, followed by complete depolarization block, the clinical manifestation of which is flaccid paralysis. In a manner similar to the events in the peripheral nervous system (PNS), the accumulation of ACh in central nervous system (CNS) nerve endings causes anxiety, disorientation, and general convulsions, followed by loss of consciousness and respiratory arrest. Anti-AChE agent-induced ACh accumulation at the muscarinic sites also enhances the activity of various secretory glands, leading to excessive salivation, lacrimation, bronchorrhea, diarrhea, and sweating. The severity of poisoning by nerve agents varies from minor cases (mild dyspnea, blurred vision, and glandular hypersecretion) to more severe poisoning, which is characterized by severe dyspnea, skeletal muscle fasciculation, convulsions, and unconsciousness, which occurs soon after an intense exposure of only a few minutes (Goldfrank et al., 1982; Weinbroum, 2005). Thus, depending upon the degree of AChE inhibition, cholinergic stimulation may also lead to respiratory failure, coma, and death.

Convulsions are a major sign of OP nerve agent poisoning (Misulis et al., 1987). OP-induced seizures rapidly progress to status epilepticus (SE), which leads to profound structural brain damage (Lemercier et al., 1983; McLeod, 1985). Excitotoxic levels of glutamate following soman exposure are thought to be involved in the dendritic and synaptic damage as an early toxicological

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response that leads to neuronal dysfunction and memory impairment (Carpentier et al., 1991). Anti-AChE exposure is also associated with oxidative stress, synaptic architecture dysfunction, and cellular deterioration in the brain, especially in the hippocampus (Johnson et al., 2008; Zaja-Milatovic et al., 2009, Milatovic et al., 2010).

EXCITOTOXICITY AND OXIDATIVE INJURY

The most consistent pathological findings in acute experiments with anti-AChE agents include degeneration and cell death in the pyriform cortex, amygdala, hippocampus (where the CA1 region is preferentially damaged), dorsal thalamus, and cerebral cortex. It has been shown that soman-induced seizures produce an increase in extracellular glutamate in the pyriform cortex (Wade et al., 1987) and the cornu ammonis (CA) region of hippocampus (Lallement et al., 1992), followed by activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors in the CA1 region. Moreover, glutamate stimulates the continuous release of ACh (Anderson et al., 1994), contributing to further excitatory stimulation, prolongation of the seizures, and neurodegeneration in vulnerable brain regions (Wade et al., 1987; Lallement et al., 1991, 1992). This excitotoxic injury caused by increased levels of glutamate also causes cognitive dysfunction (Phillips et al., 1998; O'Dell et al., 2000; Faden et al., 2001). Increased synaptic glutamate concentrations following OP exposure also alter glutamate receptor expression (Piehl et al., 1995; Cebers et al., 2001) and perturb NMDA receptor subunit distribution, thus changing the overall physiology of the receptor and the functionality of the hippocampus (Cebers et al., 1999).

Although seizures can induce neuronal death, they may also have nonlethal pathophysiological effects on neuronal structure and function. Dendritic spines represent the structural sites of contact for the majority of excitatory, glutamatergic synaptic inputs into neurons, and they are strongly implicated in mechanisms of synaptic plasticity and learning. NMDA and other glutamate receptor subtypes are clustered in dendritic spines (Rao and Craig, 1997; O'Brien et al., 1998), which serve as integrative units in synaptic circuitry and participate in synaptic plasticity (Yuste and Denk, 1995). The accumulation of glutamate receptor clusters in spines is governed by excitatory synaptic activity and increases when activity is suppressed (Rao and Craig, 1997; O'Brien et al., 1998). Conversely, excitotoxicity produces a rapid and profound loss of dendritic spines in cultured neurons (Halpain et al., 1998), mimicking the loss in dendritic spine synapses in neurological conditions, including epilepsy, aging, and schizophrenia (Jeffrey et al., 1997; Jiang et al., 1998). This suggests that receptor localization at synapses might be critical to excitotoxicity and govern neuronal vulnerability to excitotoxicity.

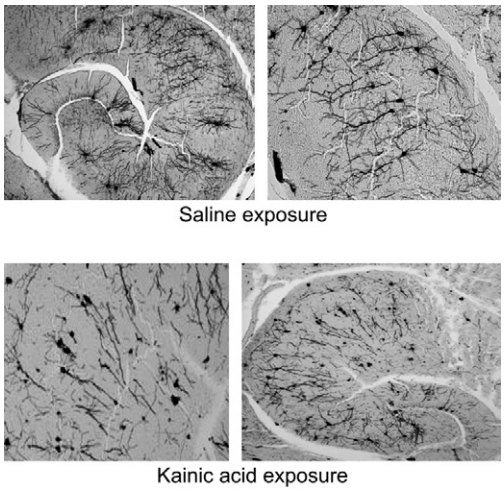

FIGURE 48.1 Photomicrographs of mouse hippocampi with pyramidal neurons from the CA1 hippocampal area of brains 1 h after saline (control) and kainic acid (KA, 1 nmol/5 μL, ICV) injections. Treatment with KA induced degeneration of the hippocampal dendritic system and decrease in the total length of the dendrite and spine density of hippocampal pyramidal neurons.

Earlier studies investigating the role of glutamate receptors in mediating seizure-induced brain damage showed that kainic acid (KA)-induced epilepsy damaged limbic structures in rats (Ben-Ari et al., 1980). Kainate is a rigid analog of glutamate, the principal excitatory neurotransmitter in the CNS, and it is a very potent stimulant of a subset of the ligand-gated ion channel, called KA receptors (Milatovic et al., 2005b). Activation of the KA subtype of ionotropic gluatamate receptors results in sustained epileptic activity in the hippocampus, followed by a selective pattern of neuropathology that is similar to human temporal lobe epilepsy (Schwob et al., 1980; Ben-Ari and Cossart, 2000). Kainate administration and intense seizure activity associated with SE is sufficient to induce oxidative stress, degeneration of hippocampal CA neurons, and hyperexcitability of surviving hippocampal CA neurons (Ben-Ari, 2001; Dong et al., 2003; Zaja-Milatovic et al., 2008) (Figure 48.1).

The hypothesis concerning OP-induced neuronal oxidative injury is that overstimulation of glutamatergic receptors results in sustained epileptic activity in the hippocampus and neuropathologic changes predominantly in the pyramidal neurons. Cell damage is thought to result from intense transient influx of calcium, leading to mitochondrial functional impairment characterized by activation of the permeability transition pores in the inner mitochondrial membrane, cytochrome c-release, depletion of adenosine triphosphate (ATP), and simultaneous formation of reactive oxygen species (ROS) (Cadenas and Davies, 2000; Patel, 2002; Nicholls et al.,

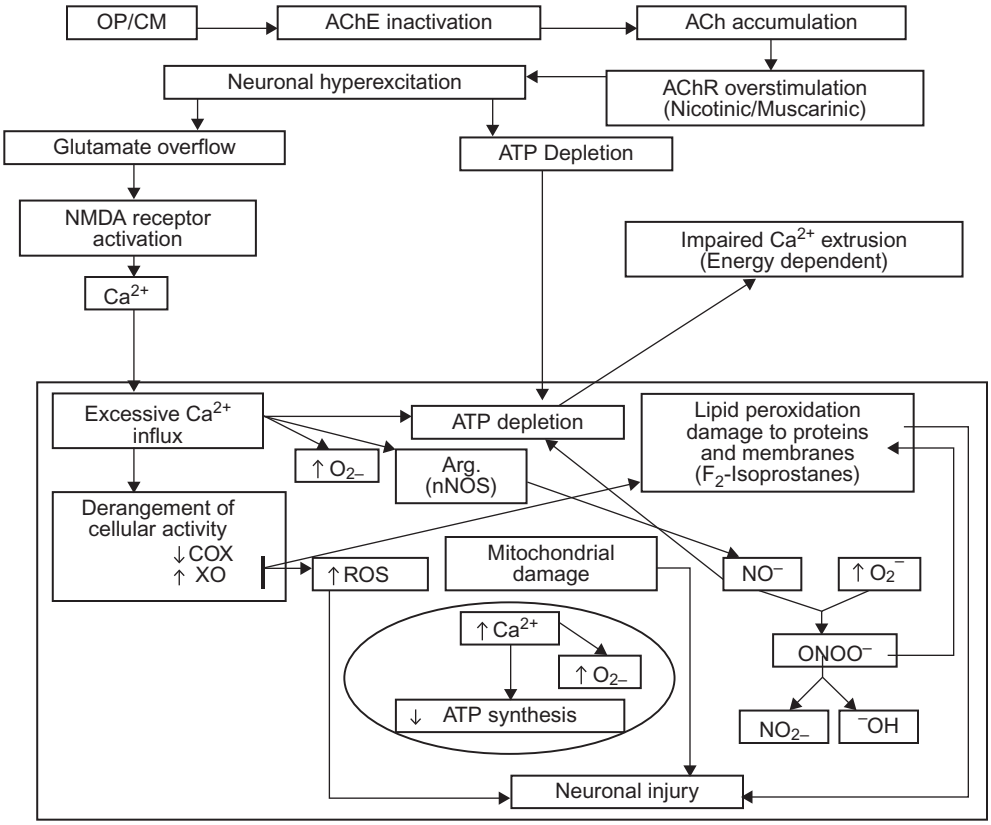

FIGURE 48.2 A schematic diagram showing possible mechanisms involved in an anti-AChE-induced neuronal injury or death by excessive production of ROS/RNS, leading to lipid peroxidation.

2003). In addition, an increase in cytoplasmic calcium ions triggers intracellular cascades through stimulation of enzymes, including proteases, phospholipase A_2, and nitric oxide synthase (NOS), which also leads to increased levels of free radical species and oxidative stress (Lafon-Cazal et al., 1993; Farooqui et al., 2001). Since free radicals are direct inhibitors of the mitochondrial respiratory chain, ROS generation perpetuates a reinforcing cycle, leading to extensive lipid peroxidation and oxidative cell damage (Cadenas and Davies, 2000; Cock et al., 2002). Previous studies have supported a role of oxidative stress and excessive generation of ROS and reactive nitrogen species (RNS) in anti-AChE-induced neurotoxicity (Dettbarn et al., 2001; Gupta et al., 2001a,b, 2007; Milatovic et al., 2005a; Zaja-Milatiovic et al., 2009).

Two radicals that play predominant roles as initiators of lipid peroxidation are the hydroxyl radical (OH^-) and the peroxynitrite radical ($OONO^-$). The superoxide anion radical (O_2^-), which is generated during the electron transport process in mitochondria, is involved in the generation of both OH^- and $OONO^-$. Superoxide dismutase (MnSOD and Cu/ZnSOD) converts O_2 to hydrogen peroxide (H_2O_2), which is then converted to OH^- via the Fenton reaction, catalyzed by Fe^{2+}, Cu^+, or Mn^{2+}. $OONO^-$ is generated from the interaction of NO with O_2^-. A major stimulus for NO production is the elevation of intracellular Ca^{2+}, which binds to calmodulin, resulting in the activation of NOS. Peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects, including lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration, and inhibition of mitochondrial respiration (Virag et al., 2003). Peroxynitrite, which dissipates during oxidation (Wang et al., 2003), has also been found to induce nitration as well as oxidation of adenine, guanine, and xantine nucleosides (Sodum and Fiala, 2001). Low concentrations of peroxynitrite trigger apoptotic death, whereas higher concentrations induce necrosis with cellular energetics [ATP and nicotinamide adenine dinucleotide (NAD)] serving as a switch between the models of cell death. Thus, increased ROS and RNS production has been directly linked to oxidation of proteins, deoxyribonucleic acid (DNA), and lipids, which may cause injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species (Figure 48.2).

LIPID PEROXIDATION AND *IN VIVO* MARKERS OF OXIDATIVE DAMAGE

Due to a high concentration of substrate polyunsaturated fatty acids (PUFAs) in cells, lipid peroxidation is a major outcome of free radical-mediated injury (Montine et al., 2002a,b). A critical aspect of lipid peroxidation is that it will continue until the oxidizable substrate is consumed or termination occurs, making it fundamentally different from many other forms of free radical injury, in that the self-sustaining nature of the process may entail extensive tissue damage (Porter et al., 1995). Decreased membrane fluidity following lipid peroxidation makes it easier for phospholipids to exchange between the two halves of the bilayer, increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels (e.g., K^+ and Ca^{2+}), and damage membrane proteins, inactivating receptors, enzymes, and ion channels (Halliwell and Gutteridge, 2006; Halliwell, 2007). Increases in Ca^{2+} induced by oxidative stress can activate phospholipase A_2, which releases arachidonic acid (AA) from membrane phospholipids. The free AA can then both undergo lipid peroxidation (Farooqui et al., 2001) and act as a substrate for eicosanoid synthesis (Milatovic et al., 2011a). Increased prostaglandin (PG) synthesis is immediately linked to lipid peroxidation because low levels of peroxides accelerate cyclooxygenase (COX) action on PUFAs (Smith, 2005). Phospholipase A_2 can also cleave oxidized AA residue from membranes.

The use of reactive products of lipid peroxidation as *in vivo* biomarkers is limited because of their chemical instability and rapid and extensive metabolism (Gutteridge and Halliwell, 1990; Moore and Roberts, 1998). For these reasons, other more stable lipid products of oxidative damage have generated intense interest in recent years as *in vivo* markers of oxidative damage. These compounds include the F_2-isoprostanes (F_2-IsoPs), F_4-neuroprostanes (F_4-NeuroPs), and isofurans (IsoFs) (Morrow et al., 1990; Fessel et al., 2002; Milatovic and Aschner, 2009; Janicka et al., 2010; Milatovic et al., 2011b).

F_2-IsoPs are PG-like compounds that are produced by a non-COX free radical-catalyzed mechanism involving the peroxidation of AA. Formation of these compounds initially involves the generation of four positional peroxyl radical isomers of arachidonate, which undergo endocyclization to PGE_2-like compounds. These intermediates are reduced to form four F_2-IsoP regioisomers, each of which can consist of eight racemic diastereomers (Morrow et al., 1990). In contrast to COX-derived PGs, nonenzymatic generation of F_2-IsoPs favors the formation of compounds in which the stereochemistry of the side chains is oriented *cis* in relation to the prostane ring. A second important difference between F_2-IsoPs and PGs is that F_2-IsoPs are formed primarily *in situ*, esterified to phospholipids, and subsequently released by phospholipases (Gao et al., 2006), whereas PGs are generated only from free AA (Morrow et al., 1990; Milatovic et al., 2011a).

The measurement of F_2-IsoPs is a method that has been extensively replicated as an efficient means of quantifying free radical damage in *in vivo* models associated with neurodegenerative diseases, including Alzheimer's disease (Montine et al., 1999), inflammation (Milatovic et al., 2003, 2004), metal toxicity (Milatovic et al., 2009, 2011c), and excitotoxicity (Milatovic et al., 2005b; Zaja-Milatovic et al., 2008, 2009). Since AAs present throughout the brain and in different cells in the brain at roughly equal concentrations, F_2-IsoPs reflects damage to brain tissue, but not necessarily to neurons.

Similar studies of lipid peroxidation products have been performed for other substrate lipids. Of particular interest are oxidation products of docosahexaenoic acid (DHA), which have been termed F_4-NeuroPs; (Roberts et al., 1998). In contrast to AA, which is evenly distributed in all cell types in all tissues, DHA is highly concentrated in neuronal membranes (Salem et al., 1986; Montine et al., 2004). Thus, determination of F_4-NeuroPs permits the specific quantification of oxidative damage to neuronal membranes *in vivo* (Montine et al., 2004; Milatovic and Aschner, 2009). In fact, to our knowledge, F_4-NeuroPs are the only quantitative *in vivo* marker of oxidative damage that is selective for neurons.

Another F_2-IsoPs analog may be formed by peroxidation of eicosapentaenoic acid (EPA, C20:5, ω-3) that leads to the production of F_3-IsoPs. Levels of F_3-IsoPs can significantly exceed those of F_2-IsoPs generated from AA, perhaps because EPA contains more double bonds and is therefore more easily oxidizable (Gao et al., 2006). It has also been shown that in the presence of increased oxygen tension in the microenvironment in which lipid peroxidation occurs, an additional oxygen insertion step may take place (Fessel et al., 2002; Milatovic and Aschner, 2009). This step diverts the IsoP pathway to form tetrahydrofuran ring-containing compounds termed IsoFs, which are functional markers of lipid peroxidation under conditions of increased oxygen tension. Thus, measurements of IsoFs represent a much more robust indicator of hyperoxia-induced lung injury than measurements of F_2-IsoPs. Like IsoPs, IsoFs are chemically and metabolically stable, so they are well suited to act as *in vivo* biomarkers of oxidative damage.

The enzymatic and free radical peroxidation of PUFAs which contains at least three double bonds, like AA and DHA, could lead to malondialdehyde (MDA). This product can be generated by thromboxane synthase, but a report from the Biomarkers of Oxidative Stress Study (BOSS) showed that peripheral levels of MDA derive primarily from nonenzymatic peroxidative degradation

of unsaturated lipids (Kadiiska et al., 2005). 4-hydroxy-2-nonenal (HNE) is also reactive aldehyde arising from peroxidation of ω6 fatty acid (Uchida, 2003; Gueraud et al., 2010). HNE is formed under various conditions like auto-oxidation and stimulated microsomal lipid peroxidation (Neely et al., 2005). MDA and HNE are able to covalently modify proteins and alter their functions (Butterfield et al., 2006). In addition to protein modification, these lipid peroxidation products can interfere with synthesis of DNA and ribonucleic acid (RNA), alter cell metabolism and signaling, and mediate brain-induced oxidative damage. Several studies suggest that MDA and HNE can promote the degeneration of cholinergic neurons, Aβ aggregation, and amyloidogenesis (Pedersen et al., 1999; Butterfield et al., 2006).

ROS and RON can react with the DNA molecule and induce purine or pyrimidine base or sugar lesions, nitration and deaminations of purines, and DNA–DNA or DNA–protein cross-links (Dizdaroglu et al., 2002). These processes lead to mutations and impaired transcriptional and posttranscriptional processes and compromise protein synthesis (Colurso et al., 2003). In addition, DNA damage, oxidative phosphorylation, and altered cell metabolism may lead to apoptosis and promote neuronal death (Fishel et al., 2007; Becker and Bonni, 2004). ROS and RNS can also attack amino acids, leading to the formation of carbonyl derivatives (Stadtman and Levine, 2003). Oxidation of protein also leads to protein fragmentation and protein cross-linking. In addition, peroxynitrite and a hydroxyl radical can react with tyrosine and form other indexes of protein oxidation, 3-nitrotyrosine, and ortho-tyrosine, respectively. These protein products are relatively stable, with sensitive assays available for their detections (Chakravarti and Chakravarti, 2007).

ANTI-ACHE-INDUCED SEIZURES, OXIDATIVE INJURY, AND NEURODEGENERATION

Lipid peroxidation, mitochondrial dysfunction, reduced neuronal energy levels, and reduced cytochrome c-oxidase (COx) activity support the contention that anti-AChEs, such as diisopropylfluorophosphate (DFP) and carbofuran (CF), cause neuronal injury by excessive formation of ROS (Yang and Dettbarn, 1998; Milatovic et al., 2000a,b, 2001, 2005a; Gupta et al., 2001a,b). Additionally, our studies showed that seizure-induced cerebral oxidative damage in adult animals is accompanied by alterations in integrity of the hippocampal CA1 dendritic system (Gupta et al., 2007; Zaja-Milatovic et al., 2008, 2009; Milatovic et al., 2010).

A single injection of DFP [1.5 mg/kg, subcutaneously (s.c.)] or another AChE inhibitor, CF (1.5 mg/kg, s.c.), produces toxic signs in rats, including salivation, tremors, "wet dog" shakes, fasciculations, and mild to moderate seizures with rearing and rolling over, with progression to severe seizures within 7–15 min (Milatovic et al., 2006; Gupta et al., 2007; Zaja-Milatovic et al., 2009). Signs of maximal intensity such as severe muscle fasciculations, seizures, and convulsions develop within 15–30 min and last for more than 2 h before tapering off. By 24 h, animals are free of toxic signs. The observed signs are typical of anti-AChE toxicity and reveal the involvement of both the CNS and the PNS (Gupta et al., 2001a,b; Milatovic et al., 2005a). Analysis of brains from saline-treated control rats revealed regional variability in brain AChE activity (cortex, 222.0 ± 10.7; amygdala, 529.2 ± 10.29; and hippocampus 301.2 ± 9.5 μmol/g wet weight). A single acute dose of DFP (1.5 mg/kg, s.c.) suppressed AChE activity to less than 20% in all brain regions, compared to the controls, at 60 min following the exposure. Similarly, 60 min after a single acute dose of CF (1.5 mg/kg, s.c.), AChE was markedly depressed (% remaining activity: cortex, 10.02 ± 1.04%; amygdala, 18.18 ± 1.48%; and hippocampus, 12.75 ± 0.74%) (Gupta et al., 2007). At the time of high AChE inhibition and resultant severe seizure activity, significant increase in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarker of neuronal membranes (F_4-NeuroPs) were seen in the brains of DFP and CF-exposed animals.

While twofold elevations are seen in F_2-IsoPs levels, F_4-NeuroPs levels are more than fivefold higher than that of controls (Figure 48.3). The results confirm the presence of oxidative damage in the cerebrum as a novel aspect of anti-AChE toxicity. The selective increase in F_4-NeuroPs indicates that neurons are specifically targeted by this mechanism.

DFP exposure also caused marked elevation in brain citrulline levels, which indicates NO/NOS activity (Gupta et al., 2001b, 2007). Control levels of citrulline are similar in the hippocampus (247.90 ± 4.10 nmol/g)

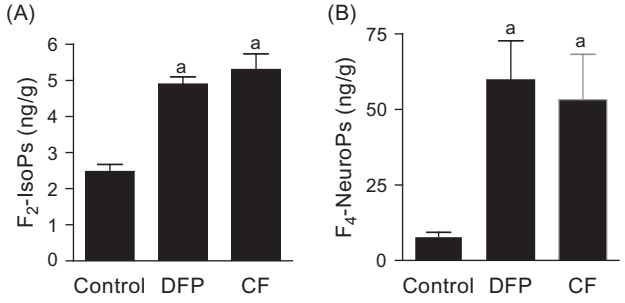

FIGURE 48.3 Effect of DFP (1.5 mg/kg, s.c.) and CF (1.5 mg/kg, s.c.) on F2-IsoPs (A) and F4-NeuroPs (B) levels in rat brain. Values are mean ± SEM (n = 4–6). (a) Significant difference between controls and DFP- or CF-treated rats ($p < 0.05$).

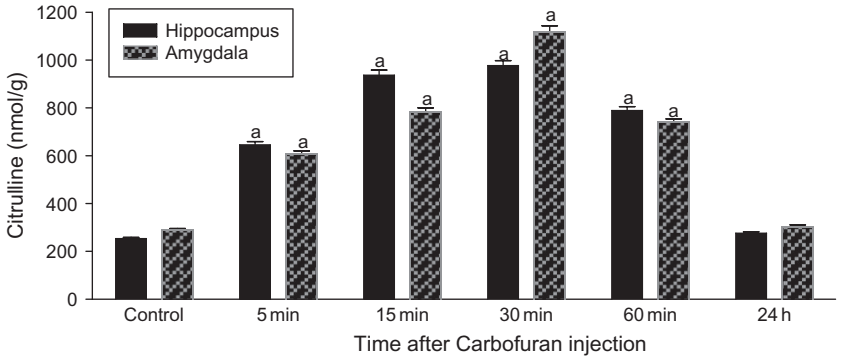

FIGURE 48.4 Citrulline levels in brain regions of rats intoxicated with an acute dose of CF (1.5 mg/kg, s.c.). Values of citrulline are presented as mean ± SEM (n = 4–6). (a) Significant difference between values from controls and CF-treated rats ($p < 0.05$).

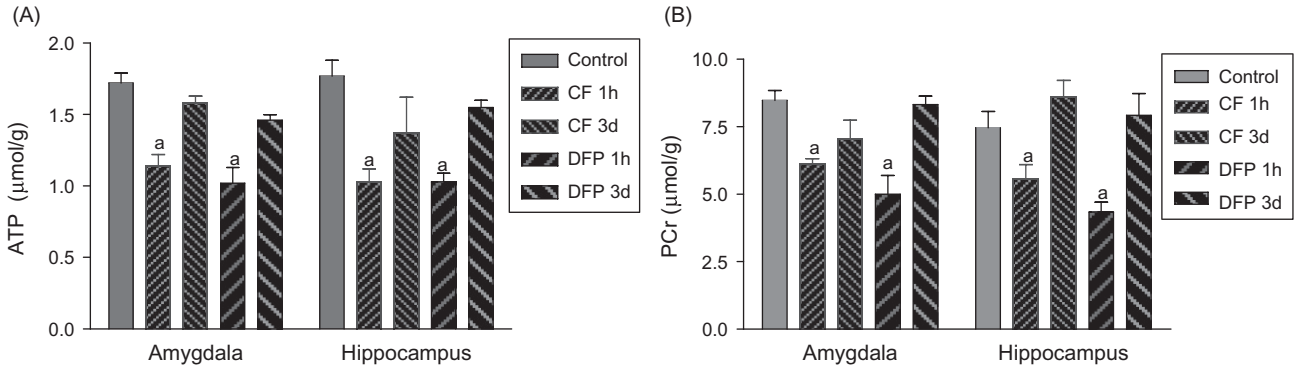

FIGURE 48.5 Levels of HEPs, ATP (A) and PCr (B), in the amygdala and hippocampus of rats intoxicated with an acute dose of CF (1.25 mg/kg, s.c.) or DFP (1.25 mg/kg, s.c.). Rats were sacrificed 1 h or 3 days after CF or DFP injection. Values of ATP and PCr are presented as mean ± SEM (n = 4–6). (a) Significant difference between values from controls and DFP- or CF-treated rats ($p < 0.05$).

and the amygdala (293.20 ± 6.90 nmol/g) (Gupta et al., 2007). Within 5 min of CF injection, the citrulline levels were elevated more than twofold in the investigated brain areas. Within 15 min of CF treatment, the levels of citrulline were significantly higher in both brain regions and were maximally elevated at 30 min post-injection (threefold to fourfold). They remained elevated up to 60 min but returned to control levels when measured 24 h later (Figure 48.4). A similar response was seen following DFP exposure.

Many reports provide evidence that NO impairs mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly COx, thereby causing ATP depletion (Yang and Dettbarn, 1998; Dettbarn et al., 2001; Gupta et al., 2001a,b; Milatovic et al., 2001). Results from our experiments also showed that 1 h after DFP (1.5 mg/kg, s.c.) or CF (1.5 mg/kg, s.c.) treatment, the levels of ATP and PCr were significantly reduced in the hippocampus and amygdala (Figure 48.5). With either DFP or CF treatment, the reduction in ATP and PCr levels was similar in the amygdala and hippocampus. During the course of these excitatory processes, a high rate of ATP consumption, coupled with the inhibition of oxidative phosphorylation, compromises the cell's ability to maintain its energy levels, and excessive amounts of ROS and RNS may be generated. Thus, the combination of impaired synthesis of ATP with its greater utilization during brain hyperactivity appears to result in a significant depletion of ATP. Three days after anti-AChE treatment, significant recovery of ATP and PCr is observed in discrete brain regions (Figure 48.5). The rapid decrease in energy metabolites at the onset of seizures indicates early onset of mitochondrial dysfunction, in turn further increasing ROS production and neuronal injury.

An important question that emerged from previous studies is whether brain hyperactivity, such as seizures, first generates increases in ROS and then causes a decrease in high-energy phosphates (HEPs). The findings revealed that within 5–15 min after CF injection (the time required for onset and development of clinical signs), NO levels increased more than fivefold to sixfold in the cortex and more than twofold to threefold in the amygdala and hippocampus. The maximum increase in NO occurred at 30 min post-injection in all three brain regions. The data also revealed that the maximum

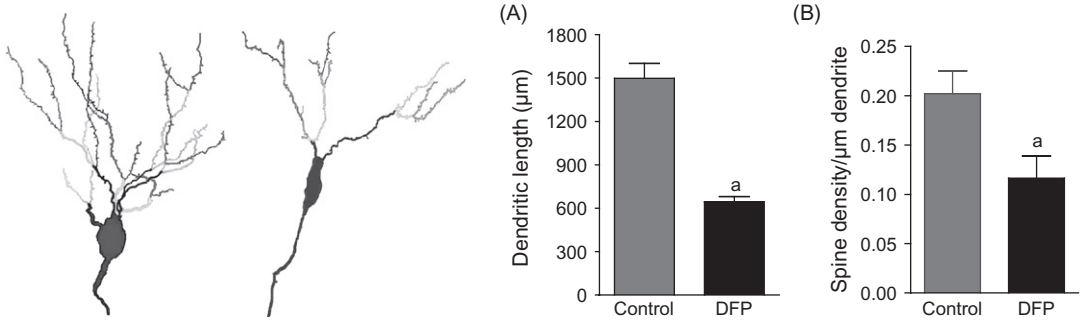

FIGURE 48.6 Morphology and quantitative determination of dendritic length (A) and spine density (B) of hippocampal pyramidal neurons from the CA1 sector of rats treated with saline (control) or DFP (1.5 mg/kg, s.c.) and sacrificed 1 h after the treatment. A total of 4–6 Golgi-impregnated dorsal hippocampal CA1 neurons were selected and spines counted by using the Neurolucida system. (a) Significant difference between controls and DFP-treated rats ($p < 0.05$). Treatment with DFP induced degeneration of the hippocampal dendritic system and decrease in the total length of the dendrite and spine density of hippocampal pyramidal neurons. Tracing and counting are done using the Neurolucida system at 100× magnification under oil immersion (MicroBrightField, VT).

decline in HEPs occurred 1 h after CF injection. This agrees with our previous data showing that a rapid and significant increase in NO precedes increases in lipid peroxidation, mitochondrial dysfunction, and loss of energy metabolites, as well as a reduction of COx activity and an increase in xanthine oxidase (Dettbarn et al., 2006). Therefore, the findings suggest that in the case of CF, the increase in ROS preceded the decrease in HEPs.

Seizures, convulsions, and CNS lesions are typical results of systemic application of sublethal doses of anti-AChE agents (Sparenborg et al., 1992). The most consistent pathological findings in acute experiments include degeneration and cell death in the pyriform cortex, amygdala, hippocampus (where the CA1 region is preferentially destroyed), dorsal thalamus, and cerebral cortex. The early morphological changes in AChEI-induced SE include dendritic swelling of pyramidal neurons in the CA1 region of the hippocampus (Carpentier et al., 1991). Therefore, we have investigated whether seizure-induced cerebral oxidative damage in adult rats is accompanied by alterations in the integrity of the hippocampal CA1 dendritic system. Our results showed that anti-AChE-induced early increases in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarker of neuronal membranes (F_4-NeuroPs) was accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area (Zaja-Milatovic et al., 2009). Anti-AChE-induced brain hyperactivity targeted the dendritic system with profound degeneration of spines and regression of dendrites, as evaluated by Golgi impregnation and Neurolucida-assisted morphometry (Figure 48.6).

Rats injected with DFP show a significant decrease in total dendritic length and spine density compared to pyramidal neurons from the hippocampal CA1 area of control rats (Figure 48.6). Taken together with the biochemical data presented previously, our results suggest that oxidative damage that selectively targets cerebral neurons is a hitherto-unrecognized aspect of anti-AChE toxicity. Results also revealed that anti-AChE exposure is associated with oxidative and nitrosative stress, alteration in energy metabolism, and consequent degeneration of pyramidal neurons from the CA1 hippocampal region of rat brain. Ultimately, the additive or synergistic mechanisms of cellular disruption caused by anti-AChE agents lead to cellular dysfunction and neurodegeneration.

OXIDATIVE DAMAGE AND DENDRITIC DEGENERATION FOLLOWING KA-INDUCED EXCITOTOXICITY

Since excessive presynaptic release of glutamate and activation of NMDA and non-NMDA receptors have a significant role in anti-AChE-induced neurotoxicity, we have investigated the role of glutamatergic excitation, oxidative injury, and neurodegeneration in the model of KA excitotoxicity. We have used intracerebroventricular (ICV) injection of KA, which is known as an experimental model for the investigation of cerebral vulnerability, particularly during acute brain disorders and SE (Schwob et al., 1980; Ben-Ari and Cossart, 2000). The study was designed to investigate whether F_2-IsoPs and F_4-NeuroPs formation correlated with the vulnerability of pyramidal neurons in the CA1 hippocampal area following KA-induced excitotoxicity. Our results showed that ICV KA-induced early increase in biomarkers of oxidative damage, F_2-IsoPs, and F_4-NeuroPs were accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area.

Time-course changes in biomarkers of oxidative damage in the rat model of anti-AChE-induced seizures showed that the highest increase in F_2-IsoPs was

TABLE 48.1 Cerebral Concentrations of F_2-IsoPs and F_4-NeuroPs and Dendritic Degeneration of Hippocampal Pyramidal Neurons Following KA-Induced Seizures in Mice

	F_2-IsoPs (ng/g)	F_4-NeuroPs (ng/g)	Dendritic Length (μm)	Spine Density (Number/100 μm Dendrite)
Control	3.07 ± 0.05	13.89 ± 0.58	1032.10 ± 61.41	16.45 ± 0.55
KA 30 min	4.81 ± 0.19*	34.27 ± 2.71*	363.44 ± 20.78*	8.81 ± 0.55*
KA 60 min	3.40 ± 0.18	18.55 ± 1.26	425.71 ± 23.04*	7.44 ± 0.56*

Data from KA-exposed mice were collected 30 or 60 min post-injection.*One-way ANOVA showed $p < 0.0001$ for each end-point. Bonferroni's multiple comparison test showed significant difference ($p < 0.001$) compared to vehicle-injected control.

evaluated 1 h after the injection of anti-AChE agent or 40 min after the beginning of seizure symptoms (Gupta et al., 2007). In the model of KA-induced excitotoxicity the earliest time point evaluated was 30 min since seizures start immediately after the ICV KA injection. Elevated levels of these *in vivo* markers of oxidative damage are in agreement with our previous findings (Montine et al., 2002c; Milatovic et al., 2005b; Gupta et al., 2007), as well as those of others (Patel et al., 2001), and indicate that KA injection leads to profound cerebral and neuronal oxidative damage in mice.

Our results also showed that the transient rise in F_2-IsoPs and F_4-NeuroPs is accompanied by rapid evolution of dendritic abnormalities, which becomes apparent due to significant decrease in dendritic length and spine density of pyramidal neurons as early as 30 min post-KA injection. However, the recovery in both oxidative damage biomarkers at 60 min after the injection was not paralleled by the rescue of damaged neurons from the CA1 hippocampal area. Extended seizure activity (60 min) induced the same level of dendritic length and spine density decrease when compared to 30 min following KA injection (Table 48.1). Together, these data suggest that both oxidative stress and neurodegeneration occur as an early response to seizures, but they do not determine whether oxidative stress is a cause or an effect of seizure-induced CA1 cell damage. Neuronal damage processes triggered by sustained seizure activity may occur as a continuum, last longer than formation of oxidative lipids, and, although not evident by the markers, may already be in progress when the peak increases in F_2-IsoPs and F_4-NeuroPs occur. Thus, we investigated dynamic changes in lipid peroxidation and dendritic structures immediately after seizures occur, but future studies over the longer period should be able to determine the long-term course of these spine and dendritic changes. It is very likely that the spine loss seen in our study is the initial phase of more chronic spine loss and progressive neurodegeneration reported in other studies (Muller et al., 1993; Jiang et al., 1998; Zeng et al., 2007).

In vivo data have also established that KA induced a significant increase (more than twofold) in citrulline concentrations 30 min following the injection (Zaja-Milatovic et al., 2008). Although we did not determine whether increased citrulline originated from a combination of NOS isozymes or one in particular, our data agree with the results from the models of anti-AChE toxicity and activated innate immunity (Milatovic et al., 2003, 2004; Gupta et al., 2007) and indicate that a subset of NOS activity also contributes to cerebral oxidative damage in the model of KA-induced excitotoxicity.

NEUROINFLAMMATION AND OXIDATIVE INJURY

Neuronal injury caused by seizures is accompanied by an inflammatory reaction involving gliosis, and induction of inflammatory mediators, including PGs, cytokines, cell adhesion proteins, and matrix metalloproteinases (Jourquin et al., 2003; Zaja-Milatovic et al., 2009). Sustained release of inflammatory mediators and increased oxidative and nitrosative stress activate additional microglia, promoting their proliferation and resulting in further release in inflammatory factors. Owing to this sustained nature of inflammation, the blood–brain barrier (BBB) may be compromised, increasing infiltration of peripheral macrophages into the brain parenchyma, further perpetuating the inflammatory process (Rivest, 2009). Animals exposed to soman at doses producing convulsions exhibit a rapid increase in active astrocytes and the accumulation of glial fibrillatory acidic protein (GFAP; Zimmer et al., 1997).

Activation of innate immunity occurs simultaneously with several pathogenic processes and responses to stressors and injury, thereby greatly confounding any clear conclusion about cause-effect relationships. For these reasons, we have adapted a simple but highly specific model of isolated innate immune activation: ICV injection of low-dose lipopolysaccharide (LPS). LPS

TABLE 48.2 Cerebral Oxidative Damage and Dendritic Degeneration in Mice

	24 h	24 h	72 h	72 h
	ICV Saline	**ICV LPS**	**ICV Saline**	**ICV LPS**
F_2-IsoPs (ng/g tissue)	3.26 ± 0.19	4.77 ± 0.26*	3.13 ± 0.11	2.98 ± 0.17
F_4-NeuroPs (ng/g tissue)	13.91 ± 1.17	58.50 ± 5.98*	12.30 ± 1.18	16.80 ± 0.96
Dendritic length (μm)	1018 ± 113	324 ± 37*	848 ± 60	1030 ± 61
Spine density (spine no./100 μm dendrite)	16.89 ± 1.67	5.86 ± 0.57*	17.09 ± 1.13	16.77 ± 0.87

Effects of ICV saline (5 μL, control) and ICV LPS (5 μg/5 μL) treatment determined at 24 and 72 h following exposure.
Each value represents mean ± SEM (n = 4–6).
*One-way ANOVA showed $p < 0.001$ for each end-point. Bonferroni's multiple comparison test showed significant difference ($p < 0.01$) compared to vehicle-injected control.

specifically activates innate immunity through a Toll-like receptor (TLR)-dependent signaling pathway (Imler and Hoffmann, 2001; Akira, 2003). Activation of proteins (CD14 and adaptor protein MyD88) signals transduction cascade, primarily via NF-κB activation but also through c-Fos/c-Jun-dependent pathways, culminate in the generation of effector molecules, including bacteriocidal molecules. Free radicals generated by NAD phosphate oxidase and myeloperoxidase (MPO), as well as cytokines and chemokines are known to attract an adaptive immune response (Milatovic et al., 2004).

We have employed an ICV model and identified the molecular and pharmacologic determinants of LPS-initiated cerebral neuronal damage *in vivo* (Montine et al., 2002a; Milatovic et al., 2003, 2004). Interestingly, the degree of oxidative damage in this model was equivalent to what we observed in diseased regions of brain from patients with degenerative diseases (Reich et al., 2001). Results from our studies with mice showed that a single ICV LPS injection induced delayed, transient elevation in both F_2-IsoPs and F_4-NeuroPs 24 h after exposure and then returned to baseline by 72 h post exposure (Table 48.2; Milatovic et al., 2003). While others have shown that altered gene transcription and increased cytokine secretion occur rapidly and peak within a few hours of LPS exposure, it is likely that the delay in neuronal oxidative damage observed in our experiments is related, at least in part, to the time required to deplete antioxidant defenses.

To address if oxidative damage is related to neurodegeneration, we directly examined the dendritic compartment of neurons, which is largely transparent to the standard histological techniques used so far to investigate ICV LPS-induced damage. Using Golgi impregnation and Neurolucida-assisted morphometry of hippocampal CA1 pyramidal neurons (Leuner et al., 2003; Milatovic et al., 2010), we first determined the time course of dendritic structural changes following ICV LPS in mice. Our results show a time course similar to neuronal oxidative damage, with maximal reduction in both dendrite length and dendritic spine density 24 h post LPS and, remarkably, a return to baseline levels by 72 h (Table 48.2). Thus, these data strongly imply that neuronal oxidative damage is closely associated with dendritic degeneration following ICV LPS. We and others have shown that primary neurons enriched in cell culture do not respond to LPS (Minghetti and Levi, 1995; Xie et al., 2002); therefore, our results also showed that LPS-activated microglial mediated paracrine oxidative damage to neurons.

It is becoming increasingly evident that neuroinflammation and associated oxidative damage plays a crucial role in the development and progression of brain diseases. Glia, and particularly microglia, are central to mediating the effects of neuroinflammation. Emerging evidence suggests that the number of activated microglia and the release of inflammatory mediators from these cells increase with age. This amplified or prolonged exposure to inflammatory molecules, including cytokines, chemokines, ROS, and PGs in the aged brain may impair neuronal plasticity and underlie a heightened neuroinflammatory response.

SUPPRESSION OF SEIZURE-INDUCED OXIDATIVE INJURY AND NEURODEGENERATION

Antioxidants

Antioxidants [e.g., vitamins, glutathione (GSH), selenium, zinc, creatine, and arginine] and antioxidant enzymes (e.g., superoxide dismutase, catalase, GSH reductase, and GSH peroxidase) exert synergistic actions in scavenging free radicals. A large body of literature (e.g., Fang et al., 2002) supports the notion that antioxidants play an important role in preventing many human

diseases (e.g., cancer, atherosclerosis, stroke, rheumatoid arthritis, and neurodegeneration). Vitamin E has been recognized as one of the most important antioxidants. Vitamin E inhibits ROS-inducing generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFAs in membrane phospholipids, from oxidative damage of cellular proteins and DNA, and from membrane degeneration (Topinka et al., 1989). Vitamin E mainly acts as a chain-breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage (Van Acker et al., 1993). In addition, vitamin E regulates ROS production (Chow et al., 1999), maintains oxidative phosphorylation in mitochondria, and accelerates restitution of high-energy metabolites (Punz et al., 1998). Decreased levels of vitamin E in response to hyperoxia or treatment with convulsants reported in studies (Onodera et al., 2003; Mori et al., 2004; Rauca et al., 2004) suggest that vitamin E in the brain is consumed to prevent oxidative damage. Vitamin E also prevented metasystox (OP insecticide)-induced changes in lipase activity and lipid peroxidation in the brain and spinal cord of rats (Tayyaba and Hasan, 1985).

A synthetic spin-trapping agent such as phenyl-*N*-*tert*-butylnitrone (PBN) is also capable of scavenging many types of free radicals. This compound is widely used to trap ROS in a variety of physical, chemical, and biological studies using electron paramagnetic resonance spectrometry. PBN is known to be concentrated in the mitochondria, where it reacts with ROS and forms stable adducts, and thereby maintains normal levels of energy metabolites. Numerous *in vitro* and *in vivo* experiments have shown the beneficial effects of PBN on the prevention of neuronal degeneration. Protective effects are described in experimental models of brain ischemia/reperfusion (Carney and Floyd, 1991; Fetcher et al., 1997; Gido et al., 1997), excitotoxicity (Lancelot et al., 1997; Milatovic et al., 2002), inhibition of NOS induction (Miyajima and Kotake, 1995), and in different models of seizures (He et al., 1997; Thomas et al., 1997). Additional findings also corroborate that PBN effectively prevents neurodegeneration in Parkinson's disease (Sack et al., 1996; Frederiksson et al., 1997), Alzheimer's disease and anti-AChE neurotoxicity (Sack et al., 1996; Gupta et al., 2001a,b). Thus, PBN has been proven to rescue neurons in multiple experimental injury models. Other pharmacological properties of spin-trapping agents have been described that could influence the outcome of oxidant injury. These have been described for PBN as reversible Ca^{2+} channel blockade in vascular muscle, causing vasodilatation (Anderson et al., 1993); direct effect on striatal function, including inhibition of excitation–contraction coupling (Andersen et al., 1996); and induction of hypothermia (Pazos et al., 1999).

Previous studies have shown that antioxidant pretreatment suppressed DFP- or CF-induced alterations in HEP, their metabolites, and citrulline levels, supporting the possibility that increased generation of ROS/RNS contributes to the depletion of energy phosphates (Gupta et al., 2001a,b). PBN or vitamin E treatment alone did not alter the levels of HEPs, their major metabolites, or citrulline in any of the brain regions. Vitamin E pretreatment suppressed the depletion of HEP and their metabolites and increased citrulline levels without preventing seizures (Gupta et al., 2001a). The protective efficacy provided by vitamin E against DFP- or CF-induced changes in energy metabolites was of varying degrees in different brain regions and could partly be due to pharmacokinetic variables involved in attaining different levels of vitamin E in different brain regions. However, PBN pretreatment 1 h before the anti-AChE agent protected mitochondria and maintained the cellular level of high-energy metabolites, but it also prevented DFP- or CF-induced convulsions and seizures (Gupta et al., 2001a,b). This could primarily be due to a protective interaction of PBN with AChE, sufficient to protect a critical fraction of AChE against phosphorylation by DFP or carbamylation by CF (Zivin et al., 1999; Milatovic et al., 2000a,b). We have also shown that AChE inhibitor-induced increases in NO (citrulline) were significantly prevented by PBN and by vitamin E (Gupta et al., 2001a). There is evidence that suggests that PBN inhibits the induction of inducible NOS (iNOS) by reducing the expression of iNOS protein (decrease in mRNA expression), thus preventing the overproduction of NO (Miyajima and Kotake, 1997).

The efficacy of the spin-trapping agent PBN and the antioxidant vitamin E was tested to suppress increase in NO and lipid peroxidation and prevent neurodegeneration of pyramidal neurons in the CA1 hippocampal area in the model of KA-induced excitotoxicity (Zaja-Milatovic et al., 2008). Vitamin E or PBN alone did not alter basal citrulline and F_4-NeuroPs levels or dendritic arborization. However, vitamin E and PBN suppressed KA-induced increases in citrulline and cerebral and neuronal markers of oxidative damage, F_2-IsoPs and F_4-NeuroPs, respectively (Figures 48.7 and 48.8).

Importantly, vitamin E and PBN completely suppressed both reduction in dendrite length and reduction in spine density of pyramidal neurons from the CA hippocampal area from KA-exposed mice (Figure 48.9).

A close concordance was found between these results, showing that protection of the cerebrum from neuronal oxidative damage also protected hippocampal CA1 pyramidal neurons from dendritic degeneration. These agents did not alter kainate-induced seizure severity, indicating that the protective effect of vitamin E and PBN is most likely mediated by scavenging ROS and preventing lipid peroxidation and consequent neuronal damage, not by a specific effect on seizures *per se*. Furthermore, since antioxidants minimize lipid peroxidation following an

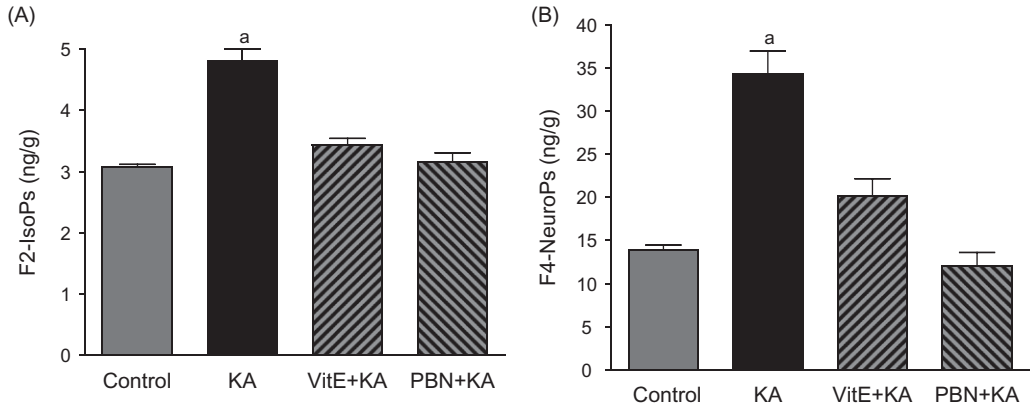

FIGURE 48.7 Ipsilateral cerebral F2-IsoPs (A) and F4-NeuroPs (B) concentrations following ICV KA with or without vitamin E (Vit E) or PBN pretreatment. Brains from mice exposed to KA were collected 30 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $p < 0.0001$ with Bonferroni's multiple comparison tests significant for KA versus control, vitamin E + KA or PBN + KA treatment.

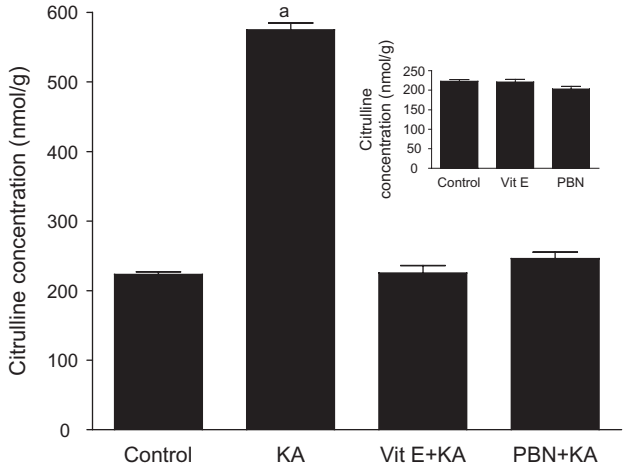

FIGURE 48.8 Ipsilateral cerebral citrulline concentrations following ICV KA with or without vitamin E or PBN pretreatment. Brains from mice exposed to KA were collected 30 min post-injection ($n \geq 5$ for each group). One-way ANOVA had $p < 0.001$ with Bonferroni's multiple comparison tests significant for KA versus control, vitamin E + KA, or PBN + KA treatment.

increase in α-tocopherol and PBN, then a parallel reduction in neuronal damage provides strong evidence that oxidative stress and lipid peroxidation in a causal way mediate seizures and the corresponding injury. One limitation to the potential therapeutic application of this type of substance is that to be effective, the drugs need to be administered prophylactically before the onset of seizures. Future research should address the efficacy of these agents in preventing seizure-induced oxidative and dendritic changes and potentially reducing resultant neurocognitive deficits when they are administered at higher concentrations, either during or possibly even after seizures.

NMDA Receptor Antagonist (Memantine)

As excitotoxicity-induced neuronal damage in the model of anti-AChE-induced seizures is explained by the excessive release of glutamate that activates both NMDA and non-NMDA postsynaptic receptors, antagonism of the excitotoxicity mechanism may protect the CNS from the deleterious effects of anti-AChE agents. Several NMDA receptor antagonists have been shown to exert anticonvulsant effects against nerve agent-induced seizures when administered either as a pretreatment or after the seizure has been initiated, usually terminating the convulsions after an initial period of epileptical activity (Shih, 1990; Sparenborg et al., 1992). NMDA receptor antagonists do not modify the events responsible for the early phase of the seizure, but they block the subsequent recruiting of glutamate receptor activation, and hence the maintenance of seizure activity and irreversible functional and structural brain damage.

Among promising candidates as antidotes against CNS intoxication by OP nerve agents, memantine has been shown to pose both antiexcitotoxic and antiepileptic properties. Memantine is an uncompetitive NMDA receptor antagonist, clinically used for the treatment of Alzheimer's disease, Parkinson's disease and spasticity, in the absence of serious side effects (Lipton, 2005; Ozsuer et al., 2005). From a series of rat *in vivo* experiments, it is evident that pre-administration of memantine significantly protects AChE activity from inhibition caused by AChE inhibitors, including OP and CM insecticides and OP nerve agents (Gupta and Kadel, 1990; Gupta and Dettbarn, 1992; McLean et al., 1992; Gupta and Dekundy, 2005). By now, it is well established that memantine exerts various pharmacological effects by multiple pharmacological mechanisms: (i) blockage of nicotinic ACh receptor-ion channel complex (Masuo et al., 1986), (ii) reduced reflex excitability of both flexors

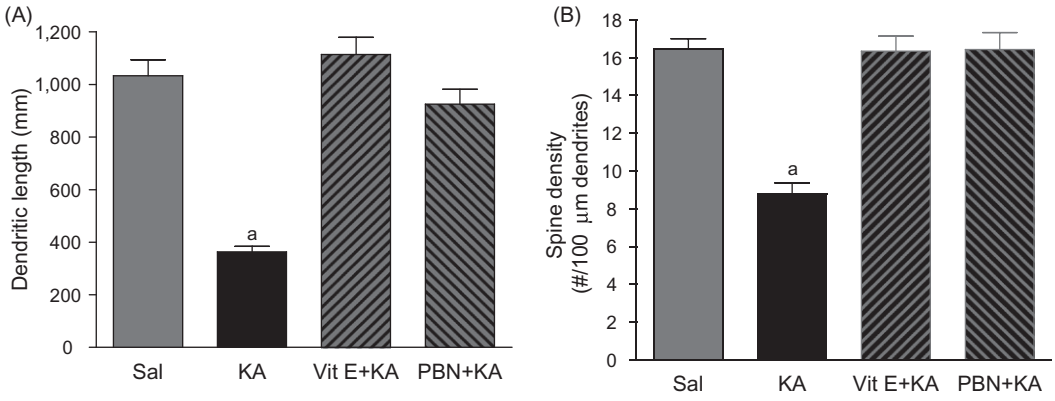

FIGURE 48.9 Dendritic length (A) and spine density (B) of pyramidal neurons from the CA1 hippocampal area of mice following ICV KA with or without vitamin E or PBN pre-treatment. Brains from mice exposed to KA were collected 30 min post-injection ($n \geq 5$ for each group). One-way ANOVA had $p < 0.001$ with Bonferroni's multiple comparison tests significant for KA versus control, vitamin E + KA or PBN + KA treatment.

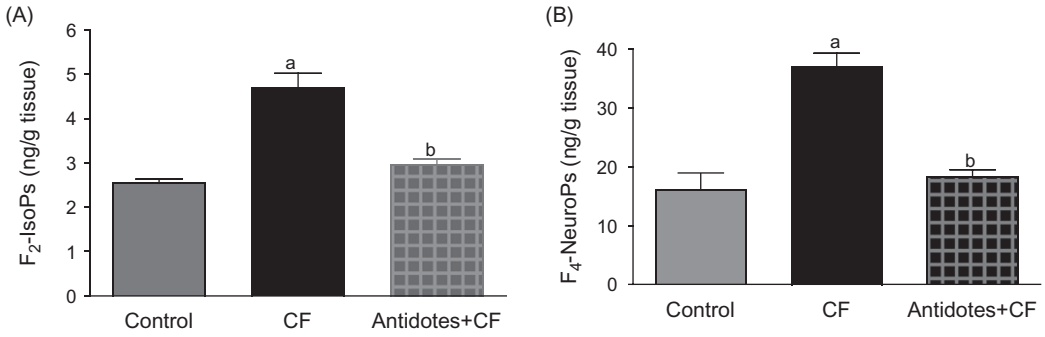

FIGURE 48.10 Cerebral F_2-IsoPs (A) and F_4-NeuroPs (B) concentrations following CF (1.5 mg/kg, s.c.) with or without antidote pretreatment (memantine, 18 mg/kg, and atropine, 16 mg/kg, given prophylactically, 60 and 15 min, respectively, before CF administration). Brains from rats exposed to CF were collected 60 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $p < 0.0001$ with Bonferroni's multiple comparison tests significant for CF versus control (a), and for CF versus antidote + CF (b).

and extensors (Wand et al., 1977), (iii) prevention of neural hyperexcitability (McLean et al., 1992), (iv) central muscle relaxation (Grossman and Jurna, 1997), and (v) prevention of AChE inhibitor-mediated energy loss from muscle cells (Milatovic et al., 2005a). Memantine is also able to prevent the pathogenic calcium influx caused by continuous mild activation by low-level glutamate. On the other hand, memantine allows the physiological activation of the NMDA channels by high concentrations of glutamate, a phenomenon necessary for synaptic plasticity underlying normal learning and memory (Parsons et al., 1999).

Previous studies have also demonstrated that memantine treatment significantly reduces lipid peroxidation (Figure 48.10) and alterations in citrulline and HEP levels in muscles and brain of rats intoxicated with CF (Milatovic et al., 2005a; Gupta et al., 2007; Zaja-Milatovic et al., 2009).

No significant alterations in biomarkers of neuronal damage, citrulline, HEP, and their metabolite levels were seen in any of the brain regions receiving memantine and atropine. In addition, memantine and atropine exposure did not induce any alteration in neuronal morphometry, but when given as pre-treatment, it did provide protection against CF-induced morphometric changes in hippocampal neurons (Figure 48.11). Memantine, in combination with atropine, completely suppressed reduction in both dendrite length and in spine density of pyramidal neurons from the CA1 hippocampal area from CF-exposed rats (Figure 48.11).

In conclusion, the data demonstrated that synergistic mechanisms of cellular disruption caused by anti-AChE agents led to cellular dysfunction and neurodegeneration. It has also been demonstrated that preventing CF-induced neuronal hyperactivity by pretreatment with memantine and atropine blocks pathways associated with oxidative damage in rat brain. The documented ability of memantine therapy to reduce free radical generation and lipid peroxidation, prevent HEPs, and attenuate the morphological injury provides further support for the role of ROS and RNS in anti-AChE-induced seizures.

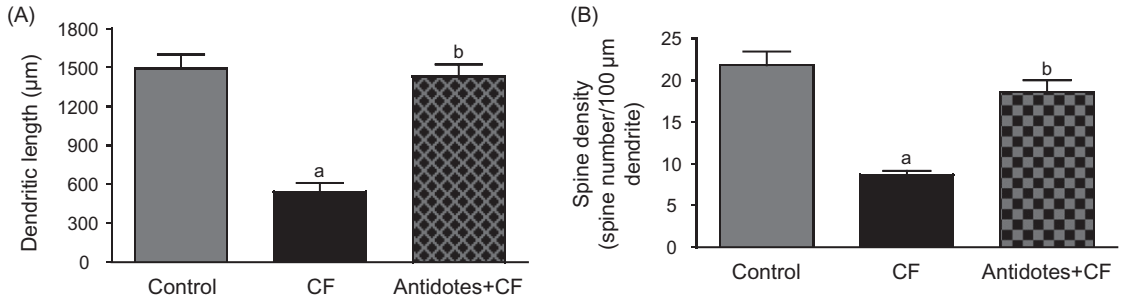

FIGURE 48.11 Dendritic length (A) and spine density (B) of pyramidal neurons from the CA1 hippocampal area of mice following CF (1.5 mg/kg, s.c.) with or without antidote pre-treatment (memantine, 18 mg/kg and atropine, 16 mg/kg given prophylactically, 60 and 15 min, respectively, before CF administration). Brains from rats exposed to CF were collected 60 min post-injection ($n \geq 5$ for each group). One-way ANOVA had $p < 0.0001$ with Bonferroni's multiple comparison tests significant for CF versus control (a), and for CF versus antidote + CF (b).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Exposure to OP nerve agents induces seizures, rapidly progressing to SE and profound structural brain damage. The progression of events includes initial high cholinergic activity followed by activation of glutamatergic neurons as a result of release of glutamate. Moreover, glutamate stimulates the continuous release of ACh, contributing to further excitatory stimulation, prolongation of the seizures, and excitotoxic neurodegeneration in vulnerable brain areas. The ensuing neuronal damage is thought to result from intense transient influx of calcium, which leads to mitochondrial functional impairment, cytochrome c-inactivation, depletion of ATP, simultaneous formation of free radical species, and oxidative stress. Therefore, control of excitotoxicity and oxidative stress, better understanding of the mechanisms of noncholinergic-mediated activities, and pathways that protect or promote neuronal survival are essential for the development of efficacious treatments and preventive therapies associated with OP exposures.

We have explored mechanisms associated with OP-induced neurotoxicity by probing their effects on oxidative stress and associated dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. We have also investigated different pathways to attenuate biomarkers of oxidative damage associated with anti-AChE exposure and the extent to which such attenuation is accompanied by rescue from neurodegeneration. Results from our studies suggest that vitamin E, PBN, and memantine efficiently suppress oxidative injury. Future studies should be directed at deciphering the mechanisms of protection, addressing the ability of these agents to attenuate OP neurotoxicity via radical scavenging, AChE inhibition, suppression of neuroinflammation, glutamate antagonism, or any combination. Additional studies should also determine whether a combination of these treatments improve the therapeutic index against OP poisoning (compared to administration of each alone). Complementary studies should also investigate not only the prophylactic, but also the therapeutic effects of these neuroprotectants. Successful identification of safe and effective neuroprotectants that suppress noncholinergic activities associated with anti-AChE exposure will provide new pharmacological modalities to protect and treat both the acute and delayed effects of nerve agent poisoning.

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CHAPTER

49

Blood–Brain Barrier Damage and Dysfunction by Chemical Toxicity

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INTRODUCTION

Direct evidence for the existence of the blood–brain barrier (BBB) came at the dawn of the twentieth century from the observations of Nobel Laureate Paul Ehrlich. When he injected colored dyes, including trypan blue, into the blood stream, they leaked out of capillaries in most regions of the body to stain the surrounding tissue, but the brain and spinal cord remained unstained. Further support for the existence of the BBB came from the work of the Russian neurophysiologist Lina Stern in 1921, who called this barrier a "hematoencephalic" barrier. Since then, doubts have been cast over the actual existence of the BBB, until the emergence of electron microscopic data demonstrating the presence of the tight junctions between the endothelial cells that form brain capillaries (Abbott et al., 2010; Palmer, 2010). The BBB is formed and maintained through a dynamic interaction between cerebral and endothelial cells constituting the anatomical basis of the BBB and other neighboring cells, such as astroglia, pericytes, perivascular microglia, and neurons (Deli et al., 2005). The cross-talk between these cells endows endothelial cells with a unique BBB phenotype comprising not only the morphological barrier of endothelial tight junctions, but also the enzymatic and metabolic barriers, as well as the uptake and efflux transport systems (Abbott et al., 2010).

The term BBB describes a series of mechanisms that control the internal environment of the brain (Saunders et al., 2008). Stability of this environment is essential for normal brain development and function. Underlying the cellular mechanisms that determine the brain's internal environment is a fundamental physical barrier at the level of intercellular tight junctions between cells forming the interface between blood and brain (BBB) and in the choroid plexuses (blood–cerebrospinal fluid barrier, BCSFB). These tight junctions severely attenuate or occlude movement of chemicals and proteins through intercellular spaces between endothelial cells in the BBB and epithelial cells in the choroid plexus BCSFB.

The BBB formed by the endothelium of cerebral blood vessels is one of the three main barrier sites protecting the central nervous system (CNS). The barrier is not a rigid structure, but rather a dynamic interface with a range of interrelated functions, resulting from extremely effective tight junctions, transendothelial transport systems, enzymes, and regulations of leukocyte permeation, which thereby generates the physical, transport, enzymatic, and immune regulatory functions of the BBB (Abbott and Friedman, 2012). In addition, recent studies have revealed important stages, cell types, and signaling pathways involved in BBB development. Several enzymes (monoamine oxidase, epoxy hydrolase, endopeptidases, acetylcholinesterase, dopa decarboxylase, γ-glutamyl transpeptidase) are known to be present in endothelial cells and are important elements of the BBB phenotype constituting the so-called metabolic barrier, and they participate in the regulation of brain penetration of drugs (Fenstermacher, 1989; Pardridge, 2002). The role of the BBB as a metabolic barrier was further ascertained by the presence of mitochondria in cerebral endothelial cells (Fenstermacher, 1989). Thus, the BBB may be considered a physical as well as a metabolic barrier.

Because the involvement of brain barriers is apparent in neurodegenerative diseases, neurotrauma, chemical-induced neurotoxicity, and delivery of drugs of use and abuse, renewed interest in this area of research has emerged (Neuwelt et al., 2008; Palmer, 2010). In physiological conditions, the BBB regulates the exchange of nutrients, waste, and immune cells between the blood

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and the nervous tissue of the CNS and is the most important component preserving CNS homeostasis and neuronal function (Abbott et al., 2010). Dysfunctional brain barrier mechanisms contribute to the pathology of neurological conditions, ranging from trauma to neurodegenerative diseases, and provide obstacles for successful delivery of potentially beneficial pharmaceutical agents (Saunders et al., 2008; Palmer, 2010; Erickson and Banks, 2013). Interestingly, modulations in the BBB can be the cause and/or consequence of non-CNS diseases, such as diabetes, chronic inflammatory pain, and obesity, but they are not discussed in this chapter because of chapter size constraints. This chapter describes, in brief, the structure and function of the BBB and modulations of its components and permeability by chemical warfare agents (CWAs) and other toxicants, Gulf War Illness (GWI), stress, blasts, excitotoxicity, and neurodegenerative diseases.

STRUCTURE AND FUNCTION OF THE BBB

The brain develops and functions within a strictly controlled environment resulting from the coordinated action of different cellular interfaces located between the blood and the extracellular fluids (interstitial fluid and the cerebrospinal fluid (CSF)) of the brain (Strazielle and Ghersi-Egea, 2013). The barrier between the blood and the brain or spinal cord parenchyma proper, referred to as the BBB, is formed by the endothelium of the cerebral microvessels. Several layers exist between the blood and brain: capillary endothelial cells, a basement membrane consisting of type IV collagen, fibronectin and laminin that completely cover the capillaries, pericytes embedded in the basement membrane, and glia/astrocytes that surround the basement membrane (Figure 49.1). Each of these layers could potentially restrict the movement of solutes (Hawkins et al., 2006; Alvarez et al., 2013).

de Boer et al. (1998) described that the BBB has narrow tight junctions, has no intercellular clefts, has minor pinocytotic activity, is not fenestrated, has a continuous basement membrane, contains many mitochondria, and has high electrical resistance (1,500–2,000 Ohm $\times$ cm^2). Next to the microvascular endothelial cells, pericytes, microglia, and neurons influence the functionality of the BBB, and there are also leukocytes (lymphocytes and monocytes) and, in the surrounding larger vessels, perivascular macrophages and mast cells. In addition to endothelial and epithelial cells, astrocytes and pericytes are involved in structure, functions, and regulation of brain barriers (Abbott et al., 2010; Armulik et al., 2010; Daneman et al., 2010; Mizee et al., 2013).

The BBB is to be distinguished from a second barrier component located between the blood and the ventricular CSF, and thus is called the blood–CSF barrier (BCSFB). The BCSFB interface is formed by the tight epithelium of the choroid plexuses, which are specialized structures projecting in all four ventricles of the brain and are responsible for the active secretion of CSF. The BBB develops a large surface area of exchange between the blood and the neutrophil, with an average of 100 cm^2/g of brain tissue in the adult mammal. In a 1-month-old rat, the apical surface area in contact with CSF has been estimated to be 75 cm^2 and close to the surface area developed by the BBB (Keep and Jones, 1990).

BBB tightness is maintained by various intercellular junctions, such as tight junctions, adherence junctions, gap junctions, and syndesmos, from which the tight junctions seem to be the most important ones in restricting passive hydrophilic transport. The BBB presents more possibilities for the transport of small molecules (molecular weight 400–600) compared with large ones, keeps the composition of electrolytes constant in the interstitial fluid of the brain, and prevents the passage of water-soluble drugs and proteins from the blood to the brain.

The BBB closely regulates the exchange of molecules in and out of the brain parenchyma to maintain its optimal homeostasis for a chemical environment that allows adequate brain functioning (de Vries et al., 2012). The ionic stability, neurochemical environment, and normal functioning of the brain are crucially dependent on the integrity of the brain barrier systems (Saunders et al., 2008). Without this stability, complex functions performed by the brain would be impossible. Saunders et al. (2008) further emphasized that dysfunction of brain barrier mechanisms in a variety of pathologies is more than a disruption of the normal (tight junction) diffusion restraint and that such dysfunction might be a part of the disease process, rather than a consequence. In a recent report, Erickson and Banks (2013) described that the BBB dysfunction is a cause as well as a consequence of neurological diseases, such as Alzheimer disease (AD).

IN VIVO AND *IN VITRO* MODELS TO STUDY THE BBB

The movement of compounds from the circulating blood into the brain is strictly regulated by the brain capillary endothelial cells, which constitute the BBB. The importance of the BBB is not only in the passage of toxicants but also in therapeutic drugs and antidotes. Brain capillary endothelial cells are connected to each other by continuous tight junctions and have a low number of pinocytotic vesicles. Several proteins (such as P-glycoproteins) and enzymes (such as monoamine oxidase, dopa decarboxylase, acetylcholinesterase, and others) are expressed by brain endothelial cells

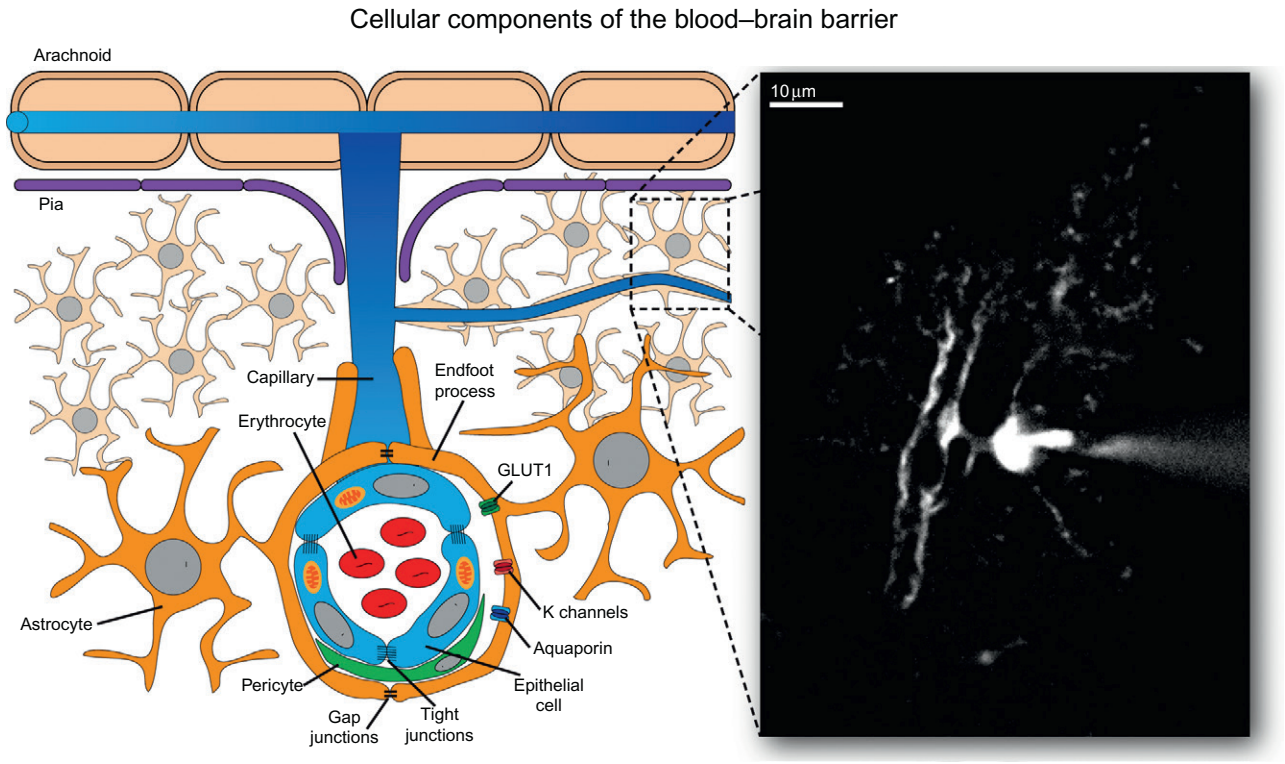

FIGURE 49.1 The BBB is a multicellular interface. The brain is a highly vascularized organ; blood vessels in the arachnoid space enter the brain and form a network of highly branched capillaries. Vascular epithelial cells are linked together through tight and adherens junctions. These intercellular junctions restrict the entry of blood-borne molecules from entering the CNS and form the BBB. Interactions between the epithelial cells are maintained by astrocytes, a type of glial cell that surrounds more than 99% of the vascular epithelium. Astrocytes ensheath blood vessels with specialized processes called endfoot processes, which express a variety of transporter proteins to maintain osmotic balance and uptake glucose from the blood. This intimate relationship between astrocytes and the vasculature is illustrated in the inset figure, which shows an astrocyte in an acute brain slice filled with a fluorescent dye. The patch pipette and blood vessel are visible to the right and left of the astrocyte, respectively. The buffering capacity of astrocytes is dramatically improved through a series of gap junctions between neighboring astrocytes.

and constitute the so-called metabolic barrier. Because of these characteristics, the BBB limits or impairs the delivery of certain drugs to the CNS. So, to understand the crossing of compounds of various classes, drugs, and antidotes, various *in vivo* and *in vitro* models have been developed.

In Vivo Model

Birngruber et al. (2013) reported a cerebral open flow microperfusion as a new membrane-free technique for measuring substance transport across the intact BBB in Sprague-Dawley rats. This *in vivo* technique is based on a probe that is inserted into the brain, thereby rupturing the BBB. The BBB is usually reestablished within 15 days, which then allows sampling of interstitial brain fluid under physiological conditions. This technique allows monitoring of BBB permeability, which can be useful for measuring pharmacokinetics across the BBB and pharmacodynamics in the brain. Using tracers, such as Evans blue (EB), horseradish peroxidase, and [^{131}I] albumin, breakdown of the BBB in humans and experimental animals has been studied under many conditions, such as hypoglycemia, hypertension, seizures/convulsions, and inflammation (Öztaş, 1996).

In Vitro Models

In vitro reconstituted models of the BBB from different mammalian species have been used since the late 1970s. However, their comparison is difficult because of the different species and methods used for isolation, culture, coculture, and characterization of the models. Lundquist et al. (2002) confirmed that the epithelial cells might not represent a valid and reliable *in vitro* BBB model, because results obtained on epithelial monolayers correlated poorly with *in vivo* BBB permeability values. Bowman et al. (1983) introduced the first *in vitro* BBB filter model. The insert was made of nylon mesh and polycarbonate tubing, and bovine brain endothelial cells were seated on it for studying the effect of calcium-free medium and osmotic shock on sucrose flux. Since then, a variety of

chambers and inserts from different materials and with diverse pore size have become commercially available. Garberg et al. (2005) used an *in vitro* model for BBB permeability based on the use of a continuous cell line and to investigate the specificity of this model. These authors developed a coculture procedure that mimics the *in vivo* situation by culturing brain capillary endothelial cells on one side of a filter and astrocytes on the other. Under these conditions, endothelial cells retain all the endothelial cell markers and the characteristics of the BBB, including tight junctions and enzymes activities (γ-glutamyl transpeptidase and monoamine oxidase). Raub (1996) identified the signaling pathways involved in the barrier-enhancing effects of C6 glioma cells, suggesting that the action is not mediated through cAMP, but rather by protein kinase C (PKC) activation via phospholipase D, independent of intracellular calcium increase.

Deli et al. (2005) presented permeability data from various *in vitro* BBB models by measuring transendothelial electrical resistance (TEER) and by calculation of permeability coefficients for paracellular or transendothelial tracers. These authors summarized the results of primary cultures of cerebral microvascular endothelial cells or immortalized cell lines from bovine, human, porcine, and rodent origin. This also described the effect of coculture with astroglia, neurons, mesenchymal cells, blood cells, and conditioned media, as well as the physiological influence of serum components, hormones, growth factors, lipids, and lipoproteins on the BBB function.

The strong correlation between the *in vivo* (Oldenhorf method) and *in vitro* (coculture) drug transport, the relative ease with which such cocultures can be produced in large quantities, and the reproducibility of the system provide evidence for an efficient system for the screening of drugs that are active in the CNS (Dehouck et al., 1997). These authors suggested that the coculture method is a useful system for investigating passive diffusion, carrier-mediated transport, and P-glycoprotein-dependent drug transport. The *in vitro* permeabilities of propranolol and cyclosporine A were parallel with indications from *in vivo* extraction, showing that transporters and P-glycoprotein are expressed in the coculture system. For further details, readers are referred to the work by Deli et al. (2005), who reviewed various *in vitro* models covering bovine, human, porcine, and rodent (murine and rat) brain endothelial cell-based systems. Bovine systems provide a high yield of brain endothelial cells sufficient for pharmacological screening, and they are widely used in basic as well as in applied research. Mouse brain yields the least endothelial cells compared with other species.

Some examples of the modulators of BBB permeability in *in vitro* models are: both cAMP elevator peptide hormone adrenomedulin and calcitonin gene-related peptide decrease paracellular permeability; a glucocorticoid hormone, hydrocortisone, improves the barrier properties; insulin exerts a tightening effect on tight junctions; and catecholamines (adrenaline and noradrenaline) increase the sodium fluorescein flux.

In essence, *in vitro* models have been widely used in pharmacological research for screening drugs and drug candidate molecules for either modifying BBB permeability or investigating brain penetration (Deli et al., 2005). This area of research is very important for permeability screening during drug development in the pharmaceutical industry.

GENDER DIFFERENCES IN THE BBB

For more than a quarter of a century, evidence has suggested that the BBB differs in males and females in terms of morphology, metabolism, and permeability. Interest arose from previous studies suggesting that women had higher cerebral blood flow than men, and the differences were more pronounced in the frontal regions (Mathew et al., 1986). Follow-up studies further confirmed the finding of higher cerebral blood flow in females as compared with males (Rodriguez et al., 1988).

In animal studies, although Saija et al. (1990) found no substantial difference in the permeability of the BBB between male and female rats, fluorescein penetrated to a greater extent in the brains of females as compared with males, but only into those regions that reside outside the BBB. Öztaş (1998) investigated the gender effects on the BBB permeability during bicuculline-induced seizures in female and male rats and found the extravasation of EB-stained albumin in a more pronounced manner in the brains of females as compared with males. In a similar study, Öztaş (1996) noted that disruption of the BBB permeability during pentylenetetrazol-induced seizures was asymmetric between the right and left hemispheres in female rats but not in male rats. Interestingly, ovariectomy decreased the BBB permeability during seizures, suggesting the involvement of estrogen because endothelial cells have been shown to contain estrogen receptors (Colburn and Buonassivi, 1978).

Furthermore, the distribution of serotonin is different in the brain and in the cerebral endothelial cells of males and females (Fischette et al., 1983), and serotonin is an important modulator of BBB permeability (Sharma and Dey, 1986). The sex hormone-related differences in the serotonin and other neurotransmitter levels in endothelial cells may cause different responses under pathological conditions. Öztaş (1998) hypothesized that if BBB permeability can increase more easily in females, then this increased breakdown may result in higher incidences of neurodegenerative diseases, such as AD and multiple sclerosis (MS) in women.

THE BBB IN YOUNG AND ADULT BRAINS

Structure and function of the BBB substantially differ in young and adult brains. In general, the BBB is immature in fetuses and newborns. This may be partly because of the fact that the blood vessels in the immature brain are more fragile than in the adult. Astrocytes in the developing brain are also responsible for the induction of tight junctions in the cerebral endothelial cells as well as other features of brain barrier mechanisms (Janzer and Raff, 1987). Saunders et al. (1999a,b) provided evidence that barriers to proteins at blood–brain and blood–CSF interfaces (tight junctions) are present from very early in development. Immunocytochemical and permeability findings revealed that proteins are largely excluded from extracellular space in the developing brain. In addition to tight junctions present at the BBB and BCSFB, the immature brain also has a mixture of other junctions present at the outer CSF–brain barrier (plate junctions, strap junctions, and wafer junctions). These barriers are not present in the adult (Saunders et al., 1999b). It was suggested that both the functional and the morphological properties of the BBB develop progressively from the onset of intraneural vascularization. However, the morphological characteristics of the BBB do not fully develop until the neonatal period. Because the BBB in fetal and early neonatal life is not fully developed, it allows for the diffusion of blood-borne macromolecules and toxins that are normally excluded from the mature CNS. Apparently, the fetus and the neonate are at greater risk for brain injury from toxicants, such as CWAs, metals, pesticides, and other environmental contaminants.

TRANSPORT OF MOLECULES ACROSS THE BBB

Brain capillary endothelial cells form the BBB. They are connected by extensive tight junctions and are polarized into luminal (blood-facing) and abluminal (brain-facing) plasma membrane domains (Hawkins et al., 2006). A pivotal function of the endothelial cells is to express transporters at the BBB and regulate the selective transport and metabolism of substances from blood to brain (Daneman et al., 2010). These transporters may be utilized to target specific molecules for delivery into the brain for therapeutic purposes. Figure 49.2 depicts various transport mechanisms for amino acids, glucose, proteins, and other molecules.

There are two major pathways for molecules and cells to cross the BBB: the paracellular (junctional) route and the transendothelial route (Deli et al., 2005). One of the hallmarks of the BBB phenotype is the restrictive paracellular pathway, which is regulated by interendothelial tight junctions. Tight junctions not only restrict paracellular flux but also maintain the polarity of enzymes and receptors on luminal and abluminal membrane domains. The most important integral tight junction proteins include occludin, claudin-1, claudin-5, and junctional adhesion molecules. Daneman et al. (2010) identified several tight junction molecules whose expression was found at the BBB, including marveld2, cingulin-like-1, and pard3, that might play a crucial role in the formation of BBB tight junctions.

It is established that the brain environment signals brain endothelial cells to form the BBB, but the identity of these signals is unknown. Paracellular permeability is regulated by diverse signaling cascades (Krizbai and Deli, 2003). Among many signaling pathways, some are noteworthy, such as Wnt/β-catenin signaling (Leibner et al., 2008; Daneman et al., 2010), sonic hedgehog (Shh) signaling (Alvarez et al., 2011), intracellular stabilization signals mediated VE-cadherin (Rudini et al., 2008; Taddei et al., 2008), and retinoic acid signaling (Mizee et al., 2013). Daneman et al. (2010) have generated a comprehensive data set describing the transcriptome of the BBB, which will provide a valuable resource for understanding the development and function of this crucial barrier, as well as its role in modulating CNS function.

The transendothelial pathways also exist at the brain microvasculature. In contrast to peripheral endothelium, the rate of pinocytosis is minimal, and free membrane diffusion applies mainly to small lipophilic molecules, for example, ethanol or nicotine (Pardridge, 2002). Daneman et al. (2010) utilized ingenuity pathway analysis (IPA) software to analyze the CNS endothelial enriched transcripts to identify signaling pathways that are enriched at the BBB or in peripheral endothelial cells. Various transport systems are present at the BBB to transport compounds in and out of the brain (de Boer et al., 1998). Active transport systems can be divided into three groups. First is carrier-mediated bidirectional transport, which is responsible for nutrient uptake in the brain. These transporters include glucose transporter GLUT-1, monocarboxylic acid transporter MCT1, large neutral amino acid transporter LAT1, or sodium-coupled nucleoside transporter CNT2 (and others). Second is efflux transport, which is unidirectional and delivers metabolites and xenobiotics from brain to blood. Third is receptor-mediated transport by endocytosis and transcytosis, which is important for the brain supply of peptides and proteins, such as low-density lipoproteins, transferrin, leptin, and insulin (Pardridge, 2002). Bidirectional transporters include glucose transporter GLUT-1, monocarboxylic acid transporter MCT1, large neutral amino acid transporter LAT1, or sodium-coupled nucleoside transporter CNT2 (and others) (Pardridge, 2002). P-glycoprotein and MRP-1 multidrug resistance proteins, brain multidrug resistance proteins (ABCG2/BCRP), or organic anion-transporting

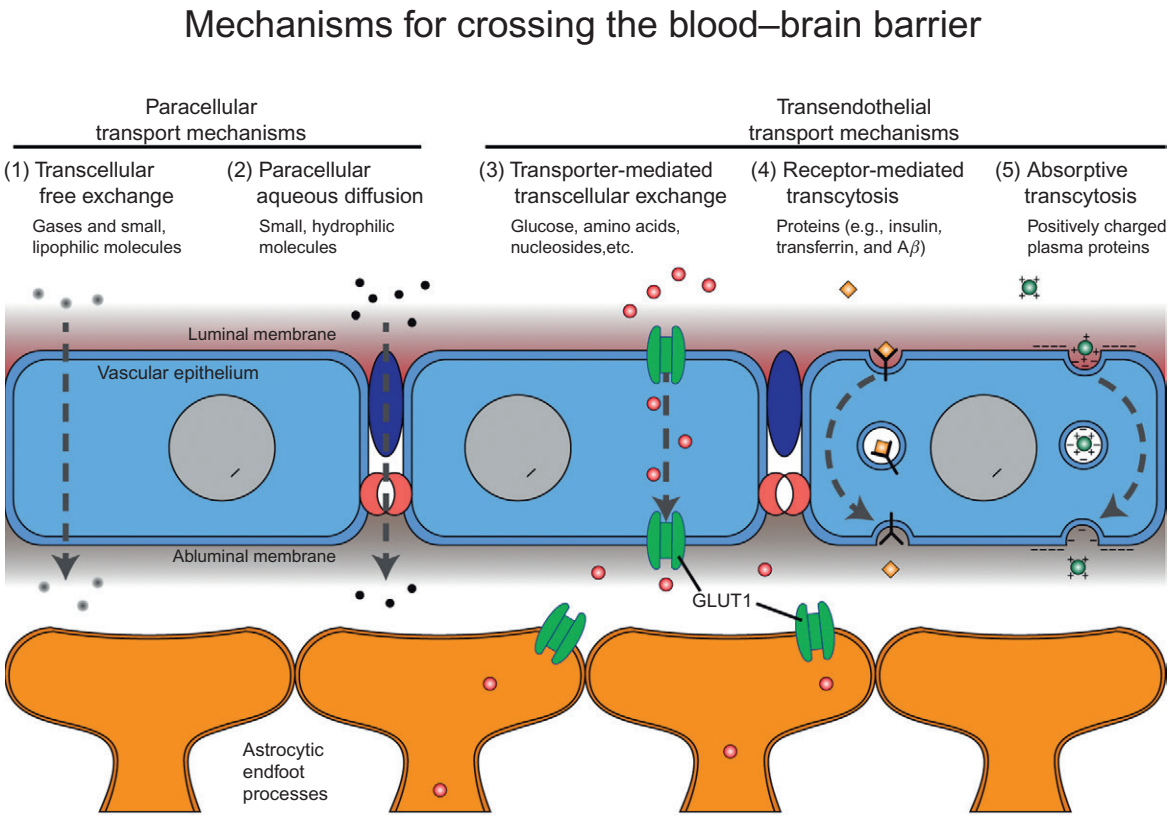

FIGURE 49.2 **Transport across the BBB occurs through a variety of mechanisms.** Gases and small lipophilic molecules enter and exit the CNS freely through transcellular exchange. Very small hydrophilic molecules are able to permeate the tight junctions (dark blue ovals) and adherens junctions (salmon crescents) between vascular epithelial cells that restrict the entry of larger molecules into the CNS. Instead, larger hydrophilic molecules are transported through transmembrane pores, receptor-mediated transcytosis, or absorptive transcytosis. After crossing the BBB, solutes are then transported through the astrocytic endfoot processes and shuttled to neurons.

polypeptide OATP2 belong to the rapidly growing group of efflux transporters at the BBB (de Boer et al., 2003; Chan et al., 2013).

A number of cellular and molecular factors can influence transport of molecules across the BBB. For most solutes and macromolecules, permeability is largely dependent on their lipophilicity. Hydrophilic solutes and macromolecules are believed to cross the barrier through specific carrier mechanisms or facilitated diffusion (Aschner, 1998). Some of these carriers are symmetrically distributed both on the luminal and abluminal membranes of the endothelial cells, whereas others have an asymmetric distribution. For example, the carriers for the essential neutral amino acids, which are required in the brain for neurotransmitter synthesis, are localized on both luminal and abluminal membranes. In contrast, the carrier for the amino acid glycine appears to be located only on the abluminal membrane. The function of this asymmetric distribution is to remove glycine from the CNS and to keep its concentration low in the brain. The polar distribution of proteins maintains amino acid homeostasis in the brain. The existence of two facilitative transporters for neutral amino acid on both membranes provides the brain access to essential amino acids. By now, it is well-established that the BBB participates in the active regulation of the amino acid content of the brain. These comprise various amino acid transport systems (System L1, System ASC, System A, acidic amino acid transporter, peptide transporter) (Begley, 1995). In addition to these transporters, P-glycoprotein (Pgp) is one of the transporters of great interest (Chan et al., 2013).

In recent studies, Daneman et al. (2010) and Geier et al. (2013) stated that the transporters and neuroprotective function of the BBB present major challenges for therapeutic drug delivery to the CNS. Critical to this function, BBB membrane transporters include the ATP-binding cassette (ABC) transporters, which limit drug penetration across the BBB, and the less studied solute carrier (SLC) transporters. These authors demonstrated expression of profiling of 359 SLC transporters and immunoassays in microvessels at the human BBB. In some situations, to be more effective, osmotic opening of the BBB has been used clinically to enhance entry of water-soluble drugs from the blood into the brain.

Daneman et al. (2010) also asserted that IPA software can be used to identify metabolic pathways enriched at the BBB or in peripheral endothelial cells. Glycolysis/gluconeogenesis and amino acid metabolism are enriched at the BBB, suggesting that brain endothelial cells may be intimately involved in the production of energy metabolites and amino acids for neurons, a function that has been thought to be uniquely served by astrocytes (Bélanger et al., 2011).

EFFECTS OF TOXIC AGENTS ON THE BBB

Breakdown of the BBB in humans and experimental animals in various conditions (stress, hypertension, convulsions, seizures, ischemia, hypoglycemia, inflammation) has been well-documented. The BBB is incapable of preventing the exchange of toxins/toxicants from the blood to the brain when its integrity (structure, function, or permeability) is compromised by chemicals or their toxicity.

Anticholinesterase Nerve Agents

Drewes and Singh (1985) reported that the cerebral transendothelial carrier-mediated transport of glucose and amino acids was not affected in mongrel dogs poisoned by soman, an irreversible acetylcholinesterase (AChE) inhibitor. Carpentier et al. (1990) investigated acute changes in BBB permeability to proteins using EB-labeled serum albumin and plasmatic gamma-immunoglobulin G (IgG) as indicators in rats. An increased BBB permeability to the EB–albumin complex was macroscopically observed in two-thirds of the convulsive rats intoxicated by 85 µg/kg of soman. Soman produced seizures and reversible BBB opening to a greatest extent after 30–60 min of paroxysmal electroencephalographic (EEG) discharges when signs of cerebral hyperactivity (epileptic EEG pattern, hyperoxia) were also at their height. Topographically, the protein leakage was bilateral and restricted to anatomically defined brain structures, some of which were sites of parenchymal edema and neuronal damage. Vascular damage occurred approximately when toxic symptoms began to reduce and was nearly concomitant to the highest level of brain oxygenation and to the maximal intensity of seizures. Interestingly, the first signs of increased BBB permeability were shown to precede the onset of edema. Carpentier et al. (1990) detected the BBB opening in the amygdaloid complex and in some cortical regions (cingulum, entorhinal, and piriform complex). The thalamus was the most frequently and intensely affected structure, and the hippocampus remained free of exudated immunoreactive IgG.

Observations from various studies suggest that soman-induced brain alterations are predominantly related to seizures or brain hyperactivity or to a direct cytotoxic action of soman or acetylcholine (ACh) itself, or because of ChE inhibition (McDonough et al., 1987).

Domer et al. (1983) found increased permeability of the BBB by systemic administration of ACh. Of course, brain hyperactivity alone appears inadequate to be responsible for the BBB opening. Obviously, the short duration of the transient protein leakage (Carpentier et al., 1990) contrasted with the well-known long-lasting brain AChE inhibition induced by soman (Petrali et al., 1985). Several other anti-ChE compounds, such as physostigmine and paraoxon, are also known to produce the BBB opening for macromolecules that was seizure-dependent, reversible, and unrelated to brain ChE inhibition. Ashani and Catravas (1981) observed that in soman-intoxicated rats, induced damage to BBB integrity was significantly reduced, despite a high degree of AChE and BChE inhibition, and protected from seizures by nembutal or atropine. In essence, endothelial AChE or BChE plays no role in BBB opening, although it may function as an "enzymatic barrier" to ACh.

Although the exact mechanism is unknown, various contributing factors, such as increased electrical activity, oxidative/nitrosative stress, decreased energy supply and store, deleterious action of excitatory amino acids, enhanced calcium intrusion, and brain edema, seemed to play significant roles in the brain damage (Misulis et al., 1987; Carpentier et al., 1990; Solberg and Belkin, 1997; Gupta et al., 2001a,b; Zaja-Milatovic et al., 2009; Prager et al., 2013). Other mechanisms in soman-induced damage to BBB integrity may be related to vasoactive substances (ACh, amines, amino acids, peptides, free radicals, and steroid hormones of the pituitary adrenal axis) and vasogenic events (acidosis, increased blood flow, and hypertension).

OP nerve agents, which are small lipophilic molecules, can easily penetrate the BBB by free diffusion and thereby inhibit AChE in the CNS (Mercey et al., 2012). Increased BBB permeability by OP nerve agents or other ChE inhibitors may lead to their enhanced entry into the brain, resulting in greater AChE inhibition and possibly resulting in subsequent maintenance of seizures and aggravation of their pathological consequences, such as edema and neuronal loss in certain brain structures. Evidently, increased BBB permeability may facilitate the entry of an antidote (oxime class) to the brain, which otherwise has limited access because of the BBB.

Oxime Reactivators of AChE Inhibited by OPs and the BBB

AChE reactivating oximes can be categorized into four groups: charged or noncharged and one-ring or two-ring compounds (Worek and Thiermann, 2013; Esposito et al., 2014). Commonly used AChE reactivators

(2-PAM, MMB-4, and HI-6) against OP nerve agents/pesticide-induced AChE inhibition are permanently charged cationic compounds that do not appreciably cross the BBB (Melchers et al., 1994; Cassel et al., 1997; Mercey et al., 2012; Esposito et al., 2014). These oximes reactivate AChE in peripheral sites, but they are not effective in the CNS and, consequently, they provide little or no protection against the neurological/neurotoxic effects of OP nerve agents. However, BBB-penetrating uncharged oximes, such as monoisonitrosoacetone (MINA), diacetylmonooxime (DAM), and dihydropyridine 2-pralidoxime (pro-2-PAM), act centrally and abrogate brain OP-induced seizure activity. But these compounds have a much lower propensity for reactivation of ChEs in peripheral tissues and blood compared with 2-PAM and other quaternary oximes, and they are too toxic for use (Skovira et al., 2010; Demar et al., 2010). Okuno et al. (2008) examined the BBB penetration of novel PAM-type oximes (alkylPAMs) using brain microdialysis with LC-MS/MS. Findings revealed that 4-[(hydroxyimino) methyl]-1-octylpyridinium bromide (4-PAO) may be effective for the reactivation of inhibited ChE in the brain; however, its toxicity was greater than that of 2-PAM. Recently, Okolotowicz et al. (2014) reported that uncharged amidine-oxime reactivator, (z)-*N*-((E)-1-(dimethylamino)-2-(hydroxyimino)ethylidine)butan-1-aminium chloride, is more lipophilic, chemically and metabolically stable, nontoxic, and can penetrate the BBB in animals and protect from the toxicity of nerve agents. Recently, Chambers et al. (2013) have synthesized some novel phenoxyalkyl pyridinium oximes that are more lipophilic than currently approved oximes against OP nerve agent surrogates. Being more lipophilic, these oximes may penetrate the BBB easily and thereby reactivate the OP-inhibited AChE. Furthermore, Esposito et al. (2014) have developed QSAR models to understand and improve physicochemical properties for the reactivation of OP-inactivated AChE, and these novel oximes may have easy access to cross the BBB as well.

Other strategies to deliver oximes across the BBB may include facilitative transport where sugar-oxime conjugates could be transferred by glucose transporters (Mercey et al., 2012; Bhonsle et al., 2013). Bhonsle et al. (2013) developed and validated molecular models for AChE reactivation by sugar oximes, which appear to be relatively nontoxic with a guinea pig LD_{50} of 1,590 mg/kg. By now, it is well-demonstrated that sugar oximes are relatively better reactivators of AChE inhibited by OPs compared with monoamine quaternary pralidoxime derivatives.

In OP nerve agent poisoning, a combination of AChE reactivating oximes provides better therapeutic efficacy compared with an individual oxime (Kassa et al., 2010, 2011a,b). Also, oximes (pralidoxime, trimedoxime, obidoxime, HI-6, HLo-7) provide greater neuroprotection against OP nerve agents when given in combination with an antimuscarinic drug (e.g., atropine sulfate) and an anticonvulsant drug (e.g., diazepam). In such scenarios, oximes alone or in combination, with and without atropine sulfate/diazepam, need to be evaluated at the BBB for their mechanisms of transport.

Karasova et al. (2010) evaluated 30 AChE reactivating oximes for BBB penetration using an *in vitro* model. Findings revealed that monoquaternary AChE reactivators were able to penetrate the BBB, and their molecular structure and molecular weight appeared to be the influencing factors for passive transport. Regarding the transport of bisquaternary oximes, the connecting linker plays a key role in penetrating into the BBB, for example, short linkers tend to facilitate penetration. Furthermore, the location of the oxime group on the pyridine ring influences passive transport into the brain. The optimum position of the oxime group was found to be at position four (i.e., para).

In essence, novel oxime reactivators (alone or in combination) that are capable of crossing the BBB and efficiently reactivating ChEs within the CNS are of great interest for protection of military personnel and civilians from nerve agents and OP pesticides.

NMDAR Antagonist Memantine and the BBB

Currently, an NMDA receptor (NMDAR) antagonist memantine is widely used in neurodegenerative diseases, but is more commonly used in moderate to severe AD. In a series of experimental studies, memantine has also been shown to protect animals from seizures, lethality, AChE inhibition, oxidative/nitrosative stress, high-energy phosphates depletion, cytotoxicity, and morphological alterations in the dendritic system of the brain against OP nerve agents or their surrogates (McLean et al., 1992; Gupta et al., 2007; Zaja-Milatovic et al., 2009). To protect or reverse OP-induced effects in the brain, memantine has to cross the BBB. Yet, the exact mechanism by which memantine crosses the BBB remains unknown. Recently, Mehta et al. (2013) identified the putative transporter involved in memantine disposition in the brain in mice. The findings implicate the involvement of an organic cation transporter regulated by proton antiport mechanisms in the transport of memantine across the mouse BBB, possibly the organic cation/carnitine transporter, OCTN1. Furthermore, memantine brain uptake was markedly reduced by various cationic transporter inhibitors (such as amantadine, quinine, tetraethylammonium, choline, and carnitine), suggesting the need for further investigation in patients using memantine and other medications (multidrug regimens). In the case of OP nerve agent poisonings, memantine is given in combination with atropine sulfate, which does not easily penetrate the BBB, and the interaction of memantine and atropine sulfate needs to be investigated at the BBB.

Melatonin and the BBB

CWAs are known to generate excess free radicals and cause excitotoxicity and neuroinflammation. Melatonin (*N*-acetyl-5-methoxytryptamine), being an indirect antioxidant and free radical scavenger, can modulate and control oxidative stress. Melatonin is also involved in vasomotor control and adrenal function. It possesses antiexcitatory actions, regulates immune function and energy metabolism, and exerts anti-inflammatory properties. Melatonin is highly lipophilic and, consequently, easily crosses cell membranes, including the BBB. Taking all these properties into consideration, melatonin could be an excellent and suitable candidate molecule to prevent CWA-induced tissue damage (Pita et al., 2013).

Drugs of Abuse-Induced BBB Damage

Currently, a large number of drugs of abuse are consumed by civilians as well as military personnel around the world. Among these illicit drugs, cocaine, morphine, and amphetamines (methamphetamine (METH), amphetamine, and methcathinone) are most commonly used (Multani et al., 2013). Because of its small size and lipophilicity, METH readily crosses the BBB by nonspecific diffusion. METH can induce BBB dysfunction in rodents, particularly in the limbic region including the hippocampus (Bowyer and Ali, 2006). Recently, Multani et al. (2013) described BBB disruption by using protein tracers, such as EB, iodine, and albumin immunohistochemistry of IgG1 in the cortex, hippocampus, thalamus, hypothalamus, cerebellum, and amygdala, and METH-induced neurotoxicity. The appearance of albumin immunoreactivity in the neuropil and leakage of serum albumin into the brain tissue had been observed as a consequence of METH-induced BBB breakdown, further leading to neuronal damage, myelin degeneration, reactive astrocytosis in the parietal and occipital cortices, extensive degeneration of pyramidal cells, and activation of microglia in the amygdala and hippocampus of rats (Sharma et al., 2007). It is suggested that, in addition to direct damage of monoaminergic nerve terminals, the deregulation of the BBB in these areas potentially contributes to widespread METH-induced neurotoxicity. METH directly damages dopaminergic and serotonergic nerve terminals, but also induces BBB dysfunction, which is thought to contribute to its neurotoxicity (Silva et al., 2010). Martins et al. (2013) provided mechanistic evidence that BBB breakdown was attributable to endothelial nitric oxide synthase (eNOS) activation and enhanced transcytosis. The neurotoxic effects of METH-induced BBB damage have been linked to hyperthermia, because METH causes dose-dependent temperature increases. Antioxidant H-290/51 pretreatment prevented hyperthermia, neuronal damage, myelin degradation, glial response, and leakage of serum albumin into brain tissue, establishing the role of free radicals in BBB damage and oxidative stress in METH neurotoxicity (Multani et al., 2013).

ElAli et al. (2012) reported that repeated administration of low-dose METH induces a transient parenchymal stress response, reflected by JNK1/2 and p38 MAPK activation, accompanied by the induction of pro-inflammatory cell adhesion molecules ICAM-1 and VCAM-1 in cerebral microvessels. Although tight junction proteins occludin and claudin-5 were expressed at high levels, a differential regulation of ABC transporters was observed after METH administration. The luminal ABCB1, which carries its substrates from the vessel into the blood, was upregulated, whereas the abluminal ABCC1, which carries its substrates from the vessel into the brain (Kilic et al., 2008), was downregulated in brain capillary cells (ElAli et al., 2012). These studies confirmed that METH modifies the structure of the BBB. JNK signaling has been shown to be involved in BBB breakdown associated with cerebral ischemia and subarachnoid hemorrhage. Recently, Urrutia et al. (2013) demonstrated that METH-induced changes in BBB integrity were attributable to JNK1/2-mediated activation of MMP-9 and laminin degradation. These effects were observed after acute exposure to METH at doses comparable with those used by consumers, for example, doses ingested typically in the range of 50–500 mg.

Metals

Metals, such as manganese (Mn), lead (Pb), and mercury (Hg), are transported on endogenous carriers, which otherwise function in the transport of essential macromolecules (Aschner, 1998; Song et al., 2014). Mn binds readily to transferrin without displacing iron (Fe) in plasma. Brain areas (pallidum, thalamic nuclei, and substantia nigra) with high Mn levels differ from those with high levels of transferrin receptors (nucleus accumbens and caudate putamen), suggesting that perhaps these sites may accumulate Mn through neuronal transport. Like Fe, Mn-loaded transferrin is taken up by receptor-mediated endocytosis at the luminal membrane of brain capillaries.

Pb can enter the CNS by more than one mechanism. The transport of Pb may occur either via the exchange of $PbCO_3$ with an anion or via exchange of an anion-ternary complex of $PbCO_3$ with another anion. Kinetic studies with ^{203}Pb continuously infused intravenously into adult rats revealed that ^{203}Pb uptake into different brain regions was linear with time up to 4 h after infusion (Bradbury and Deane, 1993). In the absence of organic ligands for Pb, the metal readily entered the CNS. However, the presence of albumin, L-cysteine, or EDTA during the vascular perfusion completely abolished the measurable uptake of ^{203}Pb. It was also suggested that Pb may passively enter the CNS in the form of $PbOH^+$.

It is well-established that Pb accumulates in the choroid plexus of humans as well as animals, suggesting that the choroid plexus is a primary target for Pb-induced neurotoxicity. Behl et al. (2009) demonstrated that exposure to Pb results in a significantly increased accumulation of intracellular amyloid-β ($A\beta_{1–40}$) in rat choroid plexus tissues *in vivo* and in immortalized choroidal epithelial Z310 cells *in vitro*. Several mechanisms may lead to an increased Aβ level at the BCSFB: a diminished expulsion of Aβ molecules from the plexus cells to the extracellular milieu; an increased uptake of Aβ from the CSF, blood, or both; an increased synthesis of Aβ; and/or a reduced metabolism or degradation of Aβ. Pb-induced inhibition of the production of LRP1 (a key intracellular Aβ transport protein in the choroid plexus) may be responsible for the accumulation of Aβ, and may be a major risk factor for AD.

Developing fetuses and neonates are most sensitive to methyl mercury (MeHg)-induced neurotoxicity, because MeHg is more readily transported across the immature BBB and because of its inhibitory effects on cell division. Aschner et al. (1990) demonstrated that MeHg conjugated to cysteine is transported across the BBB via the neutral amino acid transport L-system. It was reported that the structural similarity of the L-cysteine–MeHg conjugate with the structure of methionine suggested that, because of the broad specificity of the L-system, it should transport cysteine–MeHg conjugates efficiently across the BBB.

Copper (Cu) and Fe are trace minerals that are essential for normal brain function. They play important roles as catalysts, gene expression regulators, and second messengers. Recently, Monnot et al. (2011) investigated how BBB and BCSFB regulated Cu transport and how Fe levels altered brain Cu homeostasis. The findings demonstrate that both the BBB and BCSFB contribute to maintaining a stable Cu homeostasis in the brain and CSF. Cu appears to enter the brain primarily via the BBB and is subsequently removed from the CSF by the BCSFB. Fe deficiency has a more profound effect on brain Cu levels than Fe overload. Fe deficiency increases Cu transport at the brain barriers and prompts Cu overload in the CNS. The BCSFB plays a key role in removing excess Cu from the CSF.

BACTERIAL TOXIN-INDUCED BBB DAMAGE

Bacteria, their cell wall components, and their toxins can induce severe damage and dysfunction to the BBB (Deli et al., 2005). Lipopolysaccharide (LPS) is the primary endotoxin involved in inflammatory processes, sepsis, and multiorgan failure caused by Gram-negative bacteria like *Escherichia coli* (*E. coli*) or *Haemophilus influenzae*. LPS has been found to affect the CNS and damage the BBB by either direct or indirect means (Wispelwey et al., 1988; Shukla et al., 1996). Wispelwey et al. (1988) reviewed several studies dealing with LPS-induced damage to the BBB. In one study, intracarotid injections of *E. coli* LPS in rabbits caused diffuse breakdown of the BBB. In a second study, intracarotid LPS injections in rabbits were followed by an injection of colloidal Fe 4 h later. Electron microscopy of the brains of the LPS-treated animals revealed a large quantity of Fe within the endothelial cells, basement membrane, and glial process; however, in the saline controls, the Fe remained confined to the vascular lumen. In a third study, microscopic evaluation of brains from cats that had received intracisternal injection of *E. coli* LPS revealed evidence of profound inflammatory cell infiltration and microcirculatory impairment in both meningeal and cortical microvessels. Free radicals and nitric oxide (NO) appear to play a role in the LPS-induced increase in BBB permeability because stimulation with LPS not only produces superoxide radicals, but also enhances NO production. Minami et al. (1998) investigated the roles of NO and prostaglandins (PGs) in the development of damage to the BBB induced by LPS by using NOS and cyclooxygenase (COX) inhibitors. These authors concluded that both the NO produced by NOS (especially by iNOS) and the PGs produced by COX participate in the LPS-induced increase of BBB permeability. In *in vitro* BBB models, LPS induced a concentration-dependent and time-dependent increase in monolayer permeability (de Vries et al., 1996; Gaillard et al., 2003). Interestingly, glial cells protected cerebral endothelial cells from LPS-mediated injury in a coculture model.

Pertussis toxin, a virulence factor of *Bordetella pertussis*, severely compromises the integrity of brain endothelial monolayers in a dose-dependent and time-dependent manner and is possibly mediated by the PKC pathway (Brückener et al., 2003).

Meningitis-causing bacteria interact with brain endothelium and can cross the BBB as live bacteria either transcellularly or paracellularly and subsequently multiply inside the CNS (Kim, 2008). *Bacillus anthracis*, the etiologic agent of anthrax, has been shown to penetrate the BBB *in vivo*, and expression of the anthrax toxins was essential for this transmigration (Van Sorge et al., 2008). A more recent study demonstrated that anthrax toxins contributed to BBB disruption, invasion, trafficking, and the development of meningitis during live bacterial infection (Ebrahimi et al., 2011).

GWI AND THE BBB

After the first Persian Gulf War, many soldiers reported a variety of symptoms designated as Gulf War

Illness (GWI). The long-term symptoms include chronic fatigue, musculoskeletal pain, and cognitive–psychological disturbances, such as memory loss, confusion, inability to concentrate, mood swings, irritability, and somnolence. Among several factors, the use of pyridostigmine bromide (PB) pills, given to protect troops from the effects of AChE-inhibiting OP nerve agents (such as sarin, soman, tabun), and pesticides used during deployment, is highly likely (Amourette et al., 2009). PB is a reversible AChE-inhibiting carbamate that has been recommended by most military health services for prophylaxis against intoxication with irreversible AChE-inhibiting nerve agents. The toxic signs associated with PB are due to overstimulation of nicotinic and muscarinic receptors in the peripheral nervous system (PNS). Because of its quaternary amine structure, PB has limited access to the CNS. So, no effects on the CNS are described at doses currently recommended, unless BBB permeability is compromised.

Stress can disrupt the BBB and thereby can increase the neurotoxicity induced by chemicals in many cerebral areas (Sharma and Dey, 1986). Therefore, involvement of stress during PB treatment may allow PB to enter the brain and produce inhibition of brain AChE activity. It has been hypothesized that combat stress combined with PB treatment may have induced central penetration of PB, leading successively to the following: brain AChE inhibition; stimulation of muscarinic ACh receptors; rapid induction of c-fos oncogene; and selective regulatory effects on the long-lasting activities of genes involved in ACh metabolism (Amourette et al., 2009). Experimental studies have shown an increase in BBB permeability and inhibition of brain AChE activity after exposure to a combination of stress and PB (Friedman et al., 1996). At high doses, PB crosses the BBB in the hypothalamus to induce cholinergic and noncholinergic changes in nonstressed mice (Ropp et al., 2008). It is also suggested that the neurological symptoms of GWI are linked to neurodegeneration in some discrete brain areas, as clearly demonstrated in rats subjected to daily restraint stress and combined exposure to a repellent (*N*,*N*-diethyl-*m*-toluamide; DEET), an insecticide (permethrin), and PB (Abdel-Rehman et al., 2004).

EFFECTS OF BLASTS ON THE BBB

In recent military conflicts, the incidence of explosive blast-induced traumatic brain injury (TBI) has substantially increased. BBB disruption associated with TBI results in brain edema and increased cerebrovascular permeability, both of which affect morbidity and mortality in patients with head injury (Ling et al., 2009; Shetty et al., 2014). Brain edema after TBI is thought to be initiated by BBB rupture, permitting the influx of protein-rich exudate through compromised endothelial tight junctions that may lead to delayed neuronal dysfunction and degeneration (Shlosberg et al., 2010). In TBI, elevation of glutamate results in neuronal death primarily because of NMDAR-mediated excitotoxicity (Zhou and Sheng, 2013). In a recent study, Hue et al. (2013), using an *in vitro* model, reported the impact of primary blast on the BBB. By multiple measures, the barrier function of an *in vitro* BBB model was disrupted after exposure to a range of controlled blast loading conditions. Trans-endothelial electrical resistance (TEER) decreased acutely in a dose-dependent manner that was most strongly correlated with impulse, as opposed to peak overpressure or duration. Significantly increased hydraulic conductivity and solute permeability after injury further confirmed acute alterations in BBB function. Compromised zonula occludens-1 (ZO-1) immunostaining identified a structural basis for BBB breakdown. After blast exposure, TEER remained significantly decreased 2 days after injury, followed by spontaneous recovery to preinjury control levels by day 3. A study conducted on breachers (a military and law enforcement population that is routinely and repeatedly exposed to low-level blasts) showed changes in serum brain biomarker levels (ubiquitin C-terminal hydrolase-L1, αII-spectrin breakdown product, and glial fibrillary acidic protein), neurocognitive performance, and self-reported symptoms, suggesting brain injury and possibly damage to the BBB (Tate et al., 2013).

EXCITOTOXICITY, STRESS AND THE BBB

Glutamate excitotoxicity has been linked to chronic neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), MS, and Parkinson disease (PD), as well as in ischemia and TBI (Deli et al., 2005; Marmiroli and Cavaletti, 2012). Škultétyová et al. (1998) reported stress-induced increase in BBB permeability in control and monosodium glutamate-treated rats. Glutamate administration in neonatal rats causes reversible changes in BBB permeability and known neurotoxic lesions. These investigators evaluated whether glutamate repeatedly administered to neonatal rats influences properties of the developing BBB with consequences on adult BBB function. In control rats, 30 min of immobilization stress resulted in increased endogenous albumin extravasation in the hypothalamus, hippocampus, brain stem, and cerebellum, but not in the cortex and striatum. Basal levels of albumin in adult glutamate-treated rats (4 mg monosodium glutamate/g body weight, intraperitoneal, five times during neonatal period) were significantly lower in the hypothalamus compared with that in controls. Stress-induced increase in albumin levels was

lower in the brain stem, higher in the hypothalamus, and similar in other brain regions in glutamate-treated rats in comparison with controls. In conclusion, short-lasting immobilization stress increased BBB permeability in some, but not all, brain regions. Glutamate treatment of neonatal rats resulted in low basal albumin levels in the hypothalamus but did not exert a pronounced influence on adult BBB function. BBB vulnerability in glutamate-treated rats during stress exposure was increased in the hypothalamus and decreased in the brain stem.

In *in vitro* studies, although no permeability change was found in the case of basolateral application on a bovine coculture (Gaillard et al., 1996), apical glutamate treatment increased the flux of 70 kDa FITC-dextran (Collard et al., 2002) and decreased TEER (Sharp et al., 2003) in human brain endothelial monolayers. These findings support that brain endothelial cells express functional glutamate receptors (Krizbai et al., 1998; Sharp et al., 2003).

BRAIN BARRIERS AND CNS DISEASES

Involvement of brain interfaces, such as BBB and BCSFB, has been implicated in various neurodegenerative diseases, such as AD (Deane and Zlokovic, 2007; Zisper et al., 2007; Agyare et al., 2013; Burgmans et al., 2013), PD (Shaltiel-Karyo, et al., 2013), motor neuron disease (Garbuzova-Davis et al., 2007), and MS (Minagar and Alexander, 2003; van Horssen et al., 2007; Basivireddy et al., 2013). Modulation of the BBB permeability has also been reported in stroke, TBI, epilepsy (Abbott and Friedman, 2012; de Vries et al., 2012), autism, schizophrenia and other psychiatric disorders (Shalev et al., 2009; Palmer, 2010), GWI (Amourette et al., 2009), and edema, hypoxic-reoxygenation, or ischemic conditions (Deli et al., 2005; Kaur and Ling, 2008).

Impaired cognitive function and short-term memory are characteristic clinical features of AD. Underlying pathological features of this disease include neuronal and synaptic loss in the cerebral cortex as well as amyloid-β (Aβ)-containing diffuse and neuritic plaques (senile plaques), intraneuronal neurofibrillary tangles, and cerebral amyloid angiopathy (Zisper et al., 2007; Agyare et al., 2013). A prevailing hypothesis in the AD field was the amyloid cascade hypothesis that Aβ deposition in the CNS initiates a cascade of molecular events that cause neurodegeneration, leading to AD onset and progression. However, because of poor correlation between insoluble amyloid and cognitive impairment, increased *in vitro* toxicity of Aβ in the presence of antifibrillogenic agents and the synaptic localization of soluble oligomeric polymers, many experts now support the soluble Aβ oligomer cascade hypothesis (reviewed in Wilcox et al., 2011). Amyloid plaques may instead act as Aβ sinks, plaques that are surrounded by a halo of oligomers, which then attach to synapses and eventually cause synapse loss (Koffie et al., 2009). The loss of synapses is highly relevant to cognitive impairments, and is the best correlate with AD dementia severity (Terry et al., 1991).

Zisper et al. (2007) provided evidence that in advanced AD, plasma proteins like prothrombin can be found within the microvessel wall and surrounding neuropil, and that leakage of the BBB may be more common in patients with at least one APOE4 allele. Agyare et al. (2013) demonstrated that DutchAβ40 shows preferential accumulation in the BBB endothelial cells because of its inefficient blood-to-brain transcytosis. Consequently, DutchAβ40 establishes a permeation barrier in the BBB endothelium, prevents its own clearance from the brain, and promotes the formation of amyloid deposits in the cerebral microvessels. In a recent review, Burgmans et al. (2013) presented ample evidence for interplay between Aβ and BBB function in AD and reiterated that accumulation of the Aβ and disruption of the BBB can initiate cerebral microangiopathy, which has frequently been associated with vascular dementia. Although Aβ and BBB dysfunction have both been associated with AD and vascular dementia, respectively, they coexist in most demented patients. In fact, increasing evidence suggests that Aβ and BBB disruption may interact and facilitate each other in their effect on neurodegeneration. Recently, Erickson and Banks (2013) published a review of BBB dysfunction as a cause and consequence of AD. Paganetti et al. (2014) demonstrated that oral treatment with pirenzepine dose-dependently reduced brain Aβ level by its enhanced clearance in AβPPS1, hAβPP$_{st}$, and AβPP/PS1 transgenic mice by selective inhibition of muscarinic ACh receptors on endothelial cells of brain microvessels at the BBB.

It appears that the BBB does not play a major role in the etiology of PD, but its disruption may be beneficial in drug development. The neuropathological hallmarks of PD are progressive loss of dopaminergic neurons in the substantial nigra pars compacta accompanied by inclusions termed Lewy bodies and dystrophic Lewy neuritis in surviving neurons. The main constituent of the Lewy bodies is the α-synuclein protein. The etiology of these proteins is thought to involve major conformational changes leading to their misfolding, followed by production of β-sheet structures that have a strong tendency to aggregate into small oligomers and protofibrils that elongate into mature fibrils (Leong et al., 2009). One of the main obstacles in drug development is the inability of most drugs to pass across the BBB into the CNS. Mannitol, a nonmetabolized FDA-approved osmotic diuretic mediator, can also be used to open the BBB by producing osmotic shrinkage of the endothelial cells and mechanical separation of the tight junctions that form the BBB. Shaltiel-Karyo et al. (2013) demonstrated that mannitol interferes with α-synuclein aggregation without

exerting adverse effects, and suggested that mannitol administration in combination with other drugs could be a promising novel approach for treating PD or other brain-related diseases.

In neurologic disorders such as MS, epilepsy, capillary cerebral amyloid angiopathy, and AD, a profound dysfunction of the BBB is apparent (de Vries et al., 2012). In stroke and TBI, acute BBB dysfunction causes vasogenic edema with the danger of transtentorial herniation, and the effects of chronic BBB impairment are involved in neuroinflammatory disorders, such as MS, AD, and epilepsy (de Vries et al., 2012). Studies have led to the belief that BBB disruption represents an early event in MS lesion formation, preceding the massive infiltration of leukocytes (mainly T lymphocytes and monocyte-derived macrophages), leading to myelin degradation and nervous tissue destruction (Minagar and Alexander, 2003). *In vitro* and *in vivo* animal studies and patient tissue studies showed a significant involvement of the disruption of BBB integrity and function in MS pathology. Alterations not only involved the modulations of the tight junction but also included a reduced expression of the efflux pumps and the ABC transporters (Kooij et al., 2011).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The CNS is composed of various interfaces, such as the BBB, the BCSFB, and the blood–spinal cord barrier (BSCB). Brain capillary endothelial cells form the tight junctions and are referred to as the BBB. Currently, there are many *in vivo* and *in vitro* models to understand the mechanistic aspects of BBB functionality and permeability. Under physiological conditions, brain barriers protect the brain from pathogens, toxins, toxicants, proteins, and neurotransmitters. Disruption of the normal function of the BBB to circulating solutes and toxicants is usually the result of widened interendothelial junctions and/or alterations in one of the transporters localized within the BBB. Structure and function of the BBB and its permeability can be modulated by CWAs, toxins/toxicants, adverse conditions, and induced pathological conditions. Disruption of the BBB can also be the cause and/or consequence of neurodegenerative diseases, such as AD and MS. In future studies, novel *in vitro* and *in vivo* methods need to be developed to screen the compounds with properties of easy access to the BBB and those that can be used as therapeutic agents against CWAs.

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CHAPTER

50

Neuropathologic Effects of Chemical Warfare Agents

Randall L. Woltjer

INTRODUCTION

Nerve agents exert their effects through binding and irreversible inactivation of acetylcholinesterase (AChE), the enzyme that hydrolyzes acetylcholine (ACh), leading to a toxic accumulation of ACh at nicotinic (skeletal muscle and preganglionic autonomic) receptors, muscarinic (mainly postganglionic parasympathetic receptors), and central nervous system (CNS) synapses. Exposure to symptomatic doses of these agents leads to characteristic neuropathologic effects that have been examined in a variety of animal models. In addition, it is becoming increasingly recognized that even lower levels of exposure to these agents, even in the absence of seizures or other manifestations of acute toxicity, may be associated with more subtle forms of toxicity. Historically, this has been of concern in organophosphate (OP) and carbamate insecticide exposure among farm workers; however, it is likely that use of nerve agents in civilian populations or against military personnel would likely result in a range of exposures that stem from both the proximity of various groups to the site of deployment as well as the persistence of residues of some agents in the environment. This chapter will describe what is known about the neuropathologic consequences of both symptomatic and asymptomatic exposure to these agents and touch upon underlying mechanisms that may account for some of the regional specificity of their effects in nervous tissues.

BACKGROUND

Common nerve agents developed for chemical warfare purposes include the G-series (so named because they were first developed by German scientists in the mid-1930s) and V-series (a designation of ambiguous origin) weapons. The G agents includes tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF); the V-series includes *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX), first synthesized by the British in 1954, which is approximately an order of magnitude more potent than agents of the G-series. The other V agents include Russian VX and Chinese VX. The G-series compounds are volatile liquids at room temperature, are soluble in both fat and water, and are absorbed readily through the eyes, respiratory tract, and skin. V-series agents are oilier, and therefore toxic mainly via skin exposure, and pose a lower inhalation hazard than G agents. However, their persistence in the environment has implications for chronic exposures, including low-level exposures, that may also lead to more subtle toxicities.

Nerve agents are organic esters of phosphorus-based acid derivatives that are potent inhibitors of AChE. Many toxicological and medical studies have been conducted since their development, and it has been helpful to classify exposure-associated health effects in terms of four general clinical classifications (Brown and Brix, 1998). These are:

1. Acute cholinergic effects that occur within minutes to hours, which may be lethal or lead to at least some degree of recovery.
2. Long-term neurological and neuropsychological effects that sometimes follow recovery from acute toxicity, and which may persist for years.
3. A delayed peripheral polyneuropathy, known as *organophosphate-induced delayed neuropathy (OPIDN)*, from which recovery may be poor.

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4. A delayed syndrome of muscle weakness (so-called intermediate syndrome) that occurs within days of recovery from severe acute effects and that is typically reversible.

In addition, new research supports the hypothesis that even asymptomatic exposures to these agents, especially if they occur over a prolonged period of time, may lead to effects on nervous tissue. This would be an obvious concern in the event that populations are exposed to nerve agents, as unrecognized exposure and repeated low-dose exposure during the course of inappropriately conducted decontamination procedures could lead to these effects. Furthermore, the possibility of additional susceptibilities of pregnant women and children to low doses of nerve agents in these circumstances are additional areas of concern that we know little about.

Nonneurologic effects of nerve agent exposure have also been reported and include psychiatric and cardiac disease (Brown and Brix, 1998). However, this chapter will focus on the known neuropathologic effects, which are highly correlated with the cognitive effects of nerve agent exposure (Myhrer et al., 2005). For the purpose of this chapter, the clinical classification of exposure effects presented here will be used as an approximate scaffold for the description of lesions, predominantly in the CNS, that have been encountered in approximately equivalent stages of exposure in animal models. In addition, the limited amount of data regarding lesions encountered in humans who have been exposed to these agents will be reviewed.

ACUTE EFFECTS OF SYMPTOMATIC EXPOSURE

Ischemic/Hypoxic Injury

A large number of studies using experimental animals, including rodents, cats, and nonhuman primates, have been carried out to characterize the neurobehavioral effects of nerve agent exposure and their histopathologic correlates in brain tissue (Petras, 1981, 1994; Lemercier et al., 1983; Churchill et al., 1985; Carpentier et al., 1990; Hymowitz et al., 1990; Baze, 1993; Kadar et al., 1995). Acute exposure to nerve agents is associated with a range of clinical symptoms, varying from abnormal movements and salivation, to limb tremor and muscle fasciculation, to convulsions. In general, axonal degeneration was present in the cerebrum of all animals with convulsions and in the majority of animals with tremor and fasciculations. In a variety of animals, especially rodents, the test subjects that survived seizures tended to manifest extensive bilateral brain neuronal necrosis affecting predominantly the forebrain, thalamus, tegmentum, and spinal cord. In monkeys, the striatum, globus pallidus, substantia nigra, amygdala, hippocampus, and optic pathway (including the lateral geniculate bodies, pretectum, and superior colliculi) also sustained injury that progressed to necrosis.

Acute injury may be attributable to several mechanisms. Ischemic hypoxia may derive from respiratory insufficiency during prolonged seizures, and evidence of cellular ischemia is present in the brains of exposed animals. This consists of shrinkage of the cell soma and proximal dendrites, cytoplasmic microvacuolation due to mitochondrial swelling, dispersion of Nissel substance (cytoplasmic ribonucleic acid/RNA), increased cytoplasmic eosinophilia, nuclear changes (including displacement of the nucleus to an eccentric position in the neuron), shrinkage, and darkening. Generally, these nerve agent–associated lesions were described as being indistinguishable from those associated with brain ischemia or anoxia (Brown and Brierley, 1966, 1972; Brierley et al., 1973; McLeod et al., 1984).

Subsequent data argued against the hypothesis that hypoxic injury, at least in isolation, primarily accounts for the patterns of injury observed with acute nerve agent–induced brain injury. Although the presence of prolonged seizure activity is highly correlated with nerve agent–associated brain damage (Lemercier et al., 1983; McDonough and Shih, 1997; Carpentier et al., 2000), the changes in blood or brain oxygenation preceding or during nerve agent–induced seizures are minimal (Clement and Lee, 1980; Lynch et al., 1985; Carpentier et al., 1990; Goldman et al., 1993), and in rat experimental models, increases in regional blood flow and glucose uptake are actually observed (Pazdernik et al., 1985; Samson et al., 1985; Goldman et al., 1993). In addition, hippocampal slices exposed in culture to soman at levels that elicited spontaneous epileptiform activity were found to have earliest morphologic changes that recapitulated those seen in soman-treated intact animals; these included nuclear indentations and a decrease in nuclear size (Sikora-VanMeter et al., 1985, 1987). These findings differed from the dilated endoplasmic reticulum and mitochondrial alterations observed in hippocampal slices maintained in hypoxic conditions, and they support the argument that some other aspect of seizure activity, apart from hypoxic injury, contributes mechanistically to at least part of the damage to cerebrum associated with nerve agent exposure.

Nerve Agent-Associated Excitotoxic Injury

Within minutes after exposure to nerve agents, there is a marked decrease in AChE activity and associated rise in ACh. The earliest seizure activity begins in the absence of other significant neurotransmitter alterations and is prevented by anticholinergic drugs. These observations suggest that seizure-associated neuropathologic findings that occur upon nerve agent exposure are

caused primarily by a mechanism of cholinergic toxicity. However, if seizures progress untreated, other neurotransmitter systems display secondary alterations, and the involvement of these has been invoked in models of injury to the cerebrum that involve mechanisms of excitotoxic injury. Specifically, they refer to the involvement of glutamate, an excitatory amino acid transmitter that increases intracellular calcium mobilization. In excitotoxicity, overstimulation of glutamatergic synapses leads to marked neuronal calcium dyshomeostasis that in turn leads to neuronal injury (reviewed in Aarts and Tymianski, 2004). Excitotoxic injury contributes to neuronal pathology in a variety of conditions, including epilepsy, stroke, traumatic brain injury, multiple sclerosis, and an assortment of neurodegenerative diseases.

Protein expression changes support the involvement of glutamate receptors in nerve agent exposure. Hippocampal lysates from soman-exposed guinea pigs were found not to differ in their content of neurofilament or synaptophysin, but showed significant increases in subunits of a variety of glutamate and *N*-methyl D-aspartate (NMDA) receptors (Johnson et al., 2008) that mediate excitatory neurotransmission. Experiments in which nerve agents were injected directly into various brain sites established that the areas most sensitive to seizure induction lie generally in the ventrolateral aspects of the forebrain that contain high concentrations of AChE (McDonough et al., 1987, 1993). Following activation of these areas, seizures can propagate to sites distant from the area of injection, and these areas may demonstrate morphologic evidence of neuronal injury. Importantly, pre-treatment or early post-exposure treatment of experimental animals with anticonvulsants (for example with the benzodiazepine diazepam) blocks nerve agent–associated seizures that in turn prevents or diminished neuropathologic effects (Martin et al., 1985; McDonough et al., 1989, 1995; Hayward et al., 1990; Clement and Broxup, 1993). This occurs in the absence of a direct effect on cholinergic processes. Moreover, a pro-drug derivative of the 2-pralidoxime oxime, which produced a dose-dependent reactivation of OP-inhibited AChE, was found to block seizures 16–33% of the time, and seizure inhibition was highly correlated with neuropathologic effects of OPs in guinea pigs (Shih et al., 2011). A similar correlation of anti-seizure efficacy and prevention of markers of brain tissue injury was reported with the use of a kainate receptor antagonist (Figueiredo et al., 2011). These observations, taken together, suggest strongly that excitotoxic mechanisms contribute largely to the structural changes observed in cerebrum upon nerve agent exposure. Abrogation of these mechanisms by anticholinergic drugs within 20–40 min of the onset of seizures is sufficient in most cases to significantly diminish neuropathologic lesions (McDonough and Shih, 1997). The failure of anticholinergic drugs to prevent nerve agent–associated pathologic changes after this time period has been attributed to the recruitment and dominance of noncholinergic mechanisms of excitotoxicity, and perhaps to secondary loss of the integrity of the blood-brain barrier (McDonough and Shih, 1997).

Additional Acute Effects of Nerve Agents on Brain Tissue

Review of the literature on excitotoxicity suggests a variety of subsequent mechanisms by which calcium dyshomeostasis leads to neuronal dysfunction and injury. It is conceivable that pathologic effects of nerve agents could also arise via additional, as yet incompletely characterized, mechanisms that are independent of excitotoxicity, or that involve a variety of self-reinforcing cycles of brain tissue injury. A toxicogenomic study of messenger RNA (mRNA) expression changes after acute sarin exposure provided evidence for increased free radical stress and neuroinflammation in the cerebrum, as well as anticipatable changes in the expression of genes involved in calcium flux and binding, neurotransmission, synaptic activity, and glial activation (Damodaran et al., 2006). In a more recent study, increases in transcription of especially inflammatory markers were found to be widespread throughout the cerebrum (Spradling et al., 2011). One factor secreted by activated glia during the first days after OP exposure is vascular endothelial growth factor, and angiogenesis, the formation of new blood vessels, has also been described in the months after soman administration in mice (reviewed by Collombet, 2011). Consistent with an etiologic role for inflammation in OP-induced injury, one recent report showed that alpha-linolenic acid, an omega-3 fatty acid with antidepressant and anti-inflammatory properties, protects against soman-induced neuropathologic effects (Pan et al., 2012). Roles for oxidative injury and neuroinflammation have been hypothesized in a host of other neurologic conditions, including neurodegenerative diseases, and the demonstration of their involvement in nerve agent–associated neuropathologic changes in the brain may suggest additional prophylactic or therapeutic opportunities that are being explored in other neurologic disease. Hence, the possibility of modulation of the effects of AChE inhibitors on brain tissue by antioxidant and anti-inflammatory drugs has been the subject of investigations by a number of research groups (e.g., Gupta et al., 2000, 2001a,b; Dettbarn et al., 2001; Klaidman et al., 2003; Milatovic et al., 2006).

The morphologic manifestations of immune activation upon exposure of soman-treated rats include a rapid increase in glial fibrillary acidic protein staining, which is a marker of gliosis, as well as subsequent (1–4 h post-exposure) microglial activation in susceptible areas, including the piriform cortex and hippocampus (Zimmer

et al., 1997). This is accompanied by increased expression of central neuroinflammatory genes (Levy et al., 2004). These observations have led to the development of a novel class of anti-inflammatory, anti-cholinesterase (anti-ChE) drugs that may have utility in treating nerve agent poisoning (Amitai et al., 2005). In a rat model of AChE inhibitor intoxication, markers of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were found to be elevated in susceptible regions of brain within 15 min to 1 h of exposure (Gupta et al., 2007; Zaja-Milatovic et al., 2009); this was accompanied by dendritic damage to the hippocampus, a sensitive morphologic marker of neuronal injury (Gupta et al., 2007; see also Figure 50.1). Consistent with an excitotoxic contribution to neurotoxicity, the NMDA receptor antagonist memantine, in combination with the anticholinergic drug atropine, prevented increases in ROS and RNS, as well as dendritic damage. Taken together, these findings suggest that novel combination therapies that target a variety of processes of nerve agent toxicity may offer enhanced protection, or at minimum may offer a degree of protection if post-exposure treatment is delayed past the point at which anticholinergic drugs have lost much of their effectiveness.

PROLONGED EFFECTS OF SYMPTOMATIC EXPOSURE

A number of studies describe the long-term effects of nerve agent exposure on the cerebrum (Lemercier et al., 1983; Kadar et al., 1992; McDonough et al., 1998; Collombet et al., 2005a, 2006; Grauer et al., 2008). Inflammatory changes are pronounced and may occur in two phases—namely, an initial response to cholinergic cell death, as well as a second, delayed inflammatory process that begins approximately 1 month after exposure and persists for at least several months (Grauer et al., 2008). Morphologically, reactive changes that may be seen with many etiologies of brain tissue injury may be observed if survival is sufficiently prolonged after exposure. Subpial astrogliosis with hyperplastic, swollen astroctyes may be observed, as may reactive microgliosis with microglial lipidosis. Focal calcification of brain parenchyma may also be observed, along with localized associated gliosis.

Other long-term changes may be more particular to nerve agent exposure. After acute soman exposure in mice, neuronal degeneration in the hippocampus begins within 1 day. After initial neuronal losses, a subpopulation of degenerating neurons persists for approximately 30–90 days before many of these are lost as well (Collombet et al., 2006). These losses are accompanied by a dramatic decline in spatial memory, with a slight recovery at 90 days that may reflect partial neuronal regeneration (Collombet et al., 2006). Similar changes were described in the amygdala (Collombet et al., 2008).

Neurogenesis has also been described in cerebrum in response to focal or global ischemia and epilepsy-associated neurotoxicity (Nakatomi et al., 2002; Scharfman et al., 2000). After soman-induced toxicity, neural progenitor cells in the subgranular zone of the dentate gyrus and the subventricular zone of the cerebrum undergo a transient reduction (Collombet, 2011), followed by a more prolonged expansion (Collombet et al., 2005a) and migration to sites of brain injury (Collombet et al., 2005b), where they engraft and differentiate into neurons and astrocytes within approximately 34 days after exposure (Collombet et al., 2005b). Whether neurotrophic treatments that have been proposed and are under investigation in models of neurodegenerative diseases may have a place in the therapy of nerve agent exposure remains a speculative matter, but one that will surely be investigated in the future.

The only published study of the brain structural effects of nerve agent exposure in a human population, focused on long-term changes induced in victims of a sarin attack in the Tokyo subway system that occurred in 1995 (Yamasue et al., 2007). In this event, which resulted in 12 deaths, approximately 5,500 victims were exposed to sarin, and all patients exhibited typical symptoms of acute intoxication. A variety of somatic, ocular, and cognitive symptoms were reported to persist among victims even 5 years after the attack (Kawana et al., 2001). Magnetic resonance imaging revealed decreased regional brain volumes in the insular cortex, neighboring white matter, and hippocampi of victims (Yamasue et al., 2007); and reduced regional white matter volume was significantly correlated with decreases in serum cholinesterase levels and with victim symptoms (Yamasue et al., 2007). Although these findings are compatible with sites of susceptibility reported in the nonhuman primate studies cited previously, the findings reported were from purely imaging studies, and the precise histopathologic correlates of these findings remain unknown.

ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY

OPIDN is defined as a delayed-onset central and peripheral distal sensorimotor polyneuropathy caused by exposure to nerve agents (Brown and Brix, 1998), typically within 1–2 weeks, and no more than 4 weeks, after exposure. Symptoms attributable to effects on sensory (numbness, tingling, and pain) and motor (fatigue, weakness, and paralysis) functions are present and display a typical axonal length-associated pattern (e.g., predominantly lower extremities, with upper extremities affected at higher agent exposure). No treatment exists, and recovery is slow and rarely complete.

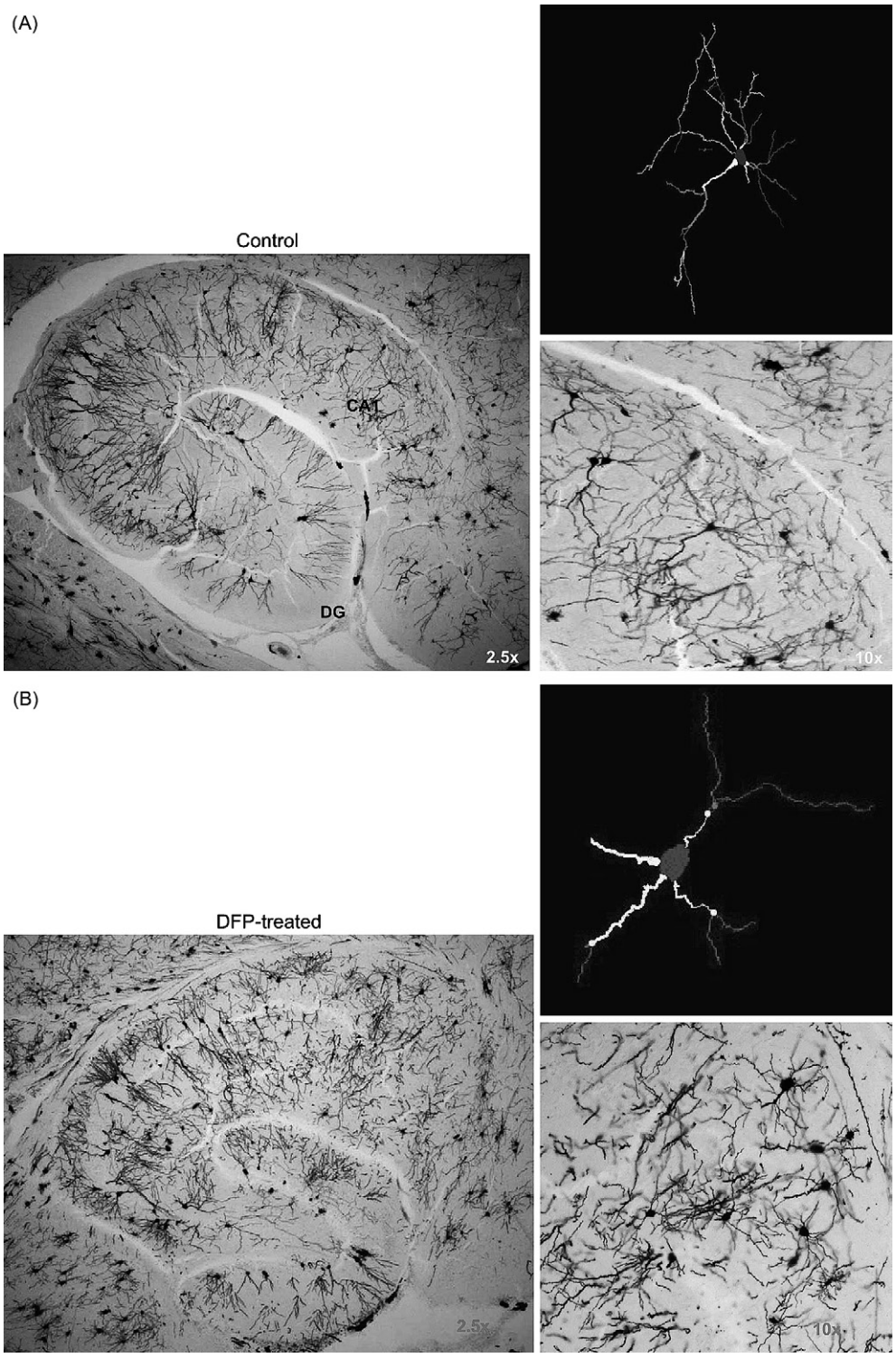

FIGURE 50.1 Photomicrographs of rat hippocampi with pyramidal neurons and their tracings from CA1 hippocampal area (DG, dentate gyrus) of rat brains 1 h after saline (control, panel A) or diisopropyl phosphorofluoridate (DFP, 1.5 mg/kg, s.c.) injections (panel B). DFP is an OP compound which acts as a potent AChE inhibitor with similar structure to OP nerve agents. Morphological correlates of anticholinesterase exposure were investigated using the structural integrity of the CA1 dendritic system, the neuronal compartment most sensitive to both age-related and disease-related degeneration (Uylings and de Brabander, 2002), as an experimental endpoint. Golgi impregnation of 50-μm-thick rat brain sections from paraffin-embedded blocks were carried out according to the manufacturer's specifications (FD Rapid GolgiStain Kit). Golgi-impregnated pyramidal neurons with no breaks in staining along the dendrites from CA1 sector of hippocampus were selected and spines counted according to the methods by Leuner et al. (2003). Tracing and counting were carried out with a Neurolucida system at × 100 objective magnification under oil immersion (MicroBrightField, VT). In each panel, the lower left view is a low (2.5× original objective) magnification of the hippocampus, the lower right view shows dendritic structure at higher magnification (10× original objective), and the upper right view shows a Neurolucida tracing of dendritic structure. Anti-AChE-induced seizures is accompanied by rapid evolution of dendritic abnormalities, apparent in a significant decrease in the dendritic length and spine density of pyramidal neurons as early as 1 h after DFP. Dendritic degeneration seen in this model of DFP-induced seizures is similar to neurodegeneration of pyramidal neurons in CA1 hippocampal area in mouse models of kainic acid (KA)–induced excitotoxicity (Zaja-Milatovic et al., 2008) and activated innate immunity (Milatovic et al., 2003). *Source: Courtesy of Dr. Dejan Milatovic, Department of Pediatrics, Vanderbilt University School of Medicine.*

OPIDN has been attributable to inhibition of neuropathy target esterase (NTE), rather than AChE, as inhibition of AChE is not necessary for the development of OPIDN (Pope et al., 1993; Wu and Casida, 1996; Jamal, 1997). Hence, since nerve agents target AChE much more than NTE (Gordon et al., 1983), the degree to which OPIDN would emerge as a significant consequence of nerve agent exposure alone is questionable; rather, doses that could potentially lead to OPIDN would, as a practical matter, cause lethal acute anti-AChE effects first. As a practical matter, unlike many other OPs, VX has not been shown to induce OPIDN and is reported to be at least 1,000 times less effective than sarin in inhibiting NTE (Gordon et al., 1983). However, several therapies, such as the anticonvulsant agent diazepam, that are used to minimize or prevent injury to the central nervous effects of nerve agents have less significant effects in the periphery; specifically, they have no effect on the targeting of NTE at peripheral sites. Therefore, it is conceivable that OPIDN could be a significant effect in treated survivors of nerve agent exposure. Indeed, exposure of humans to OP insecticides has led in some instances to OPIDN, even in the absence of symptoms associated with acute toxicity (Barns, 1975). Experimentally, OPIDN has been induced in hens treated with subsymptomatic doses of OPs if the time of dose administration is not prolonged (Barns, 1975).

Morphologically, OPIDN is manifested as classic neuropathologic lesions associated with Wallerian-type, or dying-back degeneration of axons, with secondary demyelination. Briefly, this begins with swelling of the axolemma (the membrane of the axon), and is followed by granular disintegration of intraaxonal organelles and cytoskeleton, including the microtubular network. Schwann cells react rapidly, stopping synthesis of new myelin initially, and clearing degenerating myelin debris and subsequently recruited macrophages. In the recovery phase, which may be initiated within days after injury, Schwann cells proliferate and release neurotrophic factors that encourage the growth of proximal axonal sprouts from intact axons. This pattern is a nonspecific response to axonal injury and in the absence of a history of exposure to nerve agents, the clinical and neuropathologic differential diagnosis is fairly broad; it includes amyotrophic lateral sclerosis, as well as a variety of toxic neuropathies attributable to exposure to heavy metals, solvents, and other substances (Barrett and Oehme, 1985; Longstreth, 1994). Hence, the history of nerve agent exposure is critical in establishing the diagnosis.

OP-ASSOCIATED MUSCLE WEAKNESS

The term *intermediate syndrome* refers to a delayed onset of muscle weakness that occurs shortly after recovery from severe acute effects of OP exposure and is typically associated with full recovery within approximately 4 weeks (Marrs, 1993). Although details of the mechanism by which this occurs are not known, it is believed to be attributable to the accumulation of ACh at the neuromuscular junction (Karalliedde and Henry, 1993). Consistent with this, morphologically, one finds a necrotizing myopathy that appears somewhat localized to regions of nerve endplate regions (Ariens et al., 1969; Fenichel et al., 1972, 1974; Kawabuchi et al., 1976; Good et al., 1993). Ultrastructural studies reveal vacuolated myofibers in association with nerve terminals containing swollen mitochondria (Petras, 1981, 1994). In these studies, a large degree of variability both between experimentally treated animals and within affected muscles was observed; hence, it is clear that unknown additional factors mediate susceptibility. Separation of nerve terminals from the muscle surface was attributable to progressive myofiber vacuolar swelling and insertion of Schwann cell or macrophage processes into the region. This appears likely to be incompatible with effective neurotransmission at this site, and the reversibility of this process may account for the transient nature of OP-associated muscle weakness.

EFFECTS OF SUBSYMPTOMATIC EXPOSURE TO NERVE AGENTS

The classic neuropathologic lesions of nerve agent exposure, as described previously, are greatly associated with the induction of clinically and electrographically apparent seizure activity. Recently, however, concern has been raised about even low levels of exposure to nerve agents that may not be sufficient to result in symptoms at the time of exposure, but may nevertheless lead to clinical symptoms or neuropathologic effects after long delays, perhaps in the context of chronic or repeated exposure. One scenario in which this might occur invokes an urban exposure to an agent such as VX, followed by incomplete decontamination with prolonged evaporation from persistently contaminated surfaces (Bloch-Shilderman et al., 2008). In addition, practical experience with nerve agent exposure has shown that most episodes involve small or asymptomatic doses (for example, to emergency and medical treatment personnel), as opposed to acute symptomatic toxicity (Levin and Rodnitzky, 1976; Morita et al., 1995; Gray et al., 1999).

Concern about effects of low doses of nerve agents are founded in part on observations in populations, such as agricultural workers, with chronic occupational exposure to OP compounds, such as pesticides. Many reports relate neurologic changes to these exposures, and relevant biochemical alterations have been described in these subjects, including changes in antioxidant status (López et al., 2007). Other investigators have shown

biochemical changes, including muscarinic receptor decreases and mRNA cytokine expression changes in affected cerebrum in animal models (Henderson et al., 2002). In a rat model of subsymptomatic exposure, repeated exposure to sarin at a dose that induced a 30% inhibition of erythrocyte AChE led eventually to ataxic gait, increased stereotyped behavior, and increased CNS excitability following administration of the convulsive drug pentamethylenetetrazol (Kassa et al., 2001). Repeated low-dose sarin exposure also led to a disrupted electroencephalogram (EEG) sleep pattern (Shih et al., 2006) and decreased locomotor activity and cognitive performance tests in rats and mice (Russell et al., 1986; Nieminen et al., 1990; Sirkka et al., 1990; Baille et al., 2001). Interestingly, some endpoints, such as temporal perception, were initially impaired by low-dose soman treatment but led to tolerance later, whereas others, such as nociceptive sensory and perceptual thresholds, became elevated throughout the course of treatment (Russell et al., 1986). In nonhuman primates treated with subtoxic doses of sarin (Burchfiel et al., 1976), as well as humans with symptomatic but limited exposure to nerve agents (Metcalf and Holmes, 1969; Wadia et al., 1974; Duffy et al., 1979), EEG changes were significant and persisted many months after exposure.

No study of subsymptomatic nerve agent exposure found significant neuropathologic lesions, as determined by standard histopathologic techniques, to be associated with behavioral or biochemical endpoints. However, it remains to be determined whether more sensitive morphologic endpoints, such as hippocampal dendritic structure (as illustrated in Figure 50.1), are altered by exposures of this nature. Having said that, the data presented here illustrate the difficulty of determining safe levels of nerve agent exposure in human populations.

It is conceivable that the prenatal period may represent a time of special sensitivity to OP exposure. Recently, a review of epidemiologic and animal data pertaining to chlorpyrophos, a common OP insecticide, was undertaken (Li et al., 2012), with the conclusion that there was insufficient evidence to conclude that adverse neurobehavioral effects occurred in humans exposed to this agent. This, of course, leaves open the question of the developmental effects of higher doses of this agent or the effects of other agents. Reviews of animal studies suggest mixed results for prenatal exposure and behavioral effects on rodents exposed during the first few days of life. Morphologic effects on brain tissue were similar to those described in adult animals and occurred when significant AChE inhibition was present in brain tissue or red blood cells. Interestingly, red cell AChE inhibition was more pronounced in dams than in offspring, suggesting a maternal protective effect (Maurissen et al., 2000). The longer-term effects of lower doses were not determined.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The neuropathologic effects of chemical warfare agents (CWAs), particularly nerve agents, have been characterized and related to a variety of central and peripheral clinical syndromes. The most prominent of these are neuronal losses at characteristic sites in the cerebrum that follow nerve agent–induced seizure activity, with accompanying glial reaction and a subsequent, incomplete neuroregenerative response. Recent progress in the scientific understanding of excitotoxic, neuroinflammatory, and oxidative processes that may contribute to the development of neurotoxicity provides a conceptual link to other neurologic diseases. Therapies that prove successful in preventing or treating these diseases might also be translated into neuroprotective strategies that might be useful in treating populations exposed to CWAs. Conversely, if novel, effective treatments for nerve agent exposure can be developed, the possible value of their application to a host of neurological illnesses, especially age-related neurodegenerative diseases that are becoming increasingly prevalent in Western societies, should be considered.

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CHAPTER

51

The Effects of Organophosphates in the Early Stages of Human Muscle Regeneration

Tomaz Mars, Katarina Mis, Sergej Pirkmajer, Maja Katalinic and Zoran Grubic

INTRODUCTION

Organophosphate (OP) poisoning is a complex clinical condition. Increased acetylcholine (ACh) concentration in the cholinergic synapses attributable to acetylcholinesterase (AChE) inhibition leads to altered signaling in these synapses, causing various pathological effects, including failure of respiratory muscles and cardiac arrhythmias. Hypoxia that develops as a combination of impaired exchange of gases in the lungs and bradycardia is an especially threatening complication. However, there is now substantial evidence that inhibition of AChE is not the exclusive mechanism underlying a wide variety of adverse consequences of OP exposure described previously (Terry, 2012). It is known that cholinesterases (ChEs) are not the only targets of OPs and that various intracellular mechanisms are modified because of direct or indirect OP actions (Jett and Lein, 2006; Terry, 2012; Lima et al., 2013). For instance, DNA microarray analysis of cells treated with OP diazinon disclosed overwhelming modulation (upregulation or downregulation) of a wide range of genes (Mankame et al., 2006). Although still poorly understood, such noncholinergic OP actions might directly or indirectly modify functioning of the complex intracellular mechanisms and importantly contribute to the effects of OP poisoning.

OP poisoning frequently results in myopathy (Preusser, 1967; Wecker et al., 1978). Its pathophysiology is complex and is a combination of muscular hyperactivity and consequent alterations in oxidative processes in the muscle fibers. During sustained seizures and fasciculations, the flow of oxygen through the muscle is greatly increased at a time when the demand of ATP is greater than the rate of its generation. Under such conditions, production of reactive oxygen species is greatly increased and exceeds the protective capacity of the cellular defense system so that their damaging effects result in muscle injury (Dettbarn et al., 2001, 2006; Gupta et al., 2002).

Skeletal muscle responds to the injury by the activation of the regeneration process (Bischoff, 1979). Muscle regeneration shares its basic features with embryonic myogenesis, although it also differs from it in some respects (Charge and Rudnicki, 2004; Collins, 2006; Bentzinger et al., 2012; Yin et al., 2013). The regeneration process starts from the dormant mononucleated satellite cells located between the basal lamina and cell membrane of the adult skeletal muscle fiber. Final outcome of the muscle damage therefore depends not only on the extent of the damage itself, but also on the efficiency of the regeneration process. Especially with high OP intake, typically found with suicide attempts, poisoning is often complicated by respiratory failure combined with metabolic and respiratory acidosis (Emerson et al., 1999). These patients must be treated in intensive care units and often require mechanical ventilation. Critically ill patients frequently have development of skeletal muscle dysfunction (de Letter et al., 2001; Bolton, 2005) that often persists even after hospital discharge. Insufficient muscle regeneration might directly contribute to such myopathies (Prelovsek et al., 2006); therefore, it is important to know whether OPs interfere in any way with the mechanisms involved in this process. This aspect of

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OP poisoning has not been approached yet in human muscle. In this chapter, we provide evidence that the mechanisms underlying early stages of human muscle regeneration are affected by OPs.

Recently, we have identified the neuropathy target esterase–related enzyme (NRE) as a novel OP target in human skeletal muscle. NRE is highly homologous to the neuronal neuropathy target esterase (NTE), which was originally identified as a target enzyme for highly toxic OPs that cause delayed paralyzing syndrome (Richardson et al., 2013). If targeted by OPs *in vivo*, NRE might be involved in OP poisoning-related myopathy.

One of the key steps in the process of muscle regeneration is myoblast proliferation, which decisively determines the mass of regenerated muscle tissue. Our investigations of the effects of OPs on muscle regeneration were therefore focused on these early precursors of muscle fibers. All experiments were performed in the *in vitro* model in which the process of human muscle regeneration is genuinely reproduced. We describe various OP influences on the early precursors of muscle development and discuss their potential effects on muscle regeneration. Because AChE is the most thoroughly characterized target of OPs, we also approached the question of its role and expression in the mononuclear myoblasts. Diisopropylphosphorofluoridate (DFP) was used throughout our studies.

REGENERATION PROCESS IN HUMAN SKELETAL MUSCLE

The process of human muscle regeneration in many respects follows the process of embryonic development of skeletal muscle (Figure 51.1A). The earliest myogenic precursors in this development are mononuclear myoblasts (Charge and Rudnicki, 2004; Bentzinger et al., 2012; Yin et al., 2013). These still mononucleated, but already committed, cells proliferate several times to reach sufficient density before they fuse into multinucleated myotubes. Once formed, myotubes enter into a long-term and complex process of muscle differentiation that results in development of fully functional mature myofiber. An important step in the transition from myotube to myofiber is its innervation and formation of the complex structure of the neuromuscular junction (NMJ) (Emerson and Hauschka, 2004; Witzemann et al., 2013).

All these steps are repeated during the postnatal regeneration process, except that adult mononuclear myoblasts are derived from the satellite cells. These primordial cells failed to enter developmental and differentiation process during embryonic myogenesis, but retained the capacity to enter myogenic process and self-renewal when activated by the muscle damage (Collins, 2006). As such, they serve as mononuclear muscle precursors of muscle fibers formed *de novo* during muscle regeneration (Figure 51.1A).

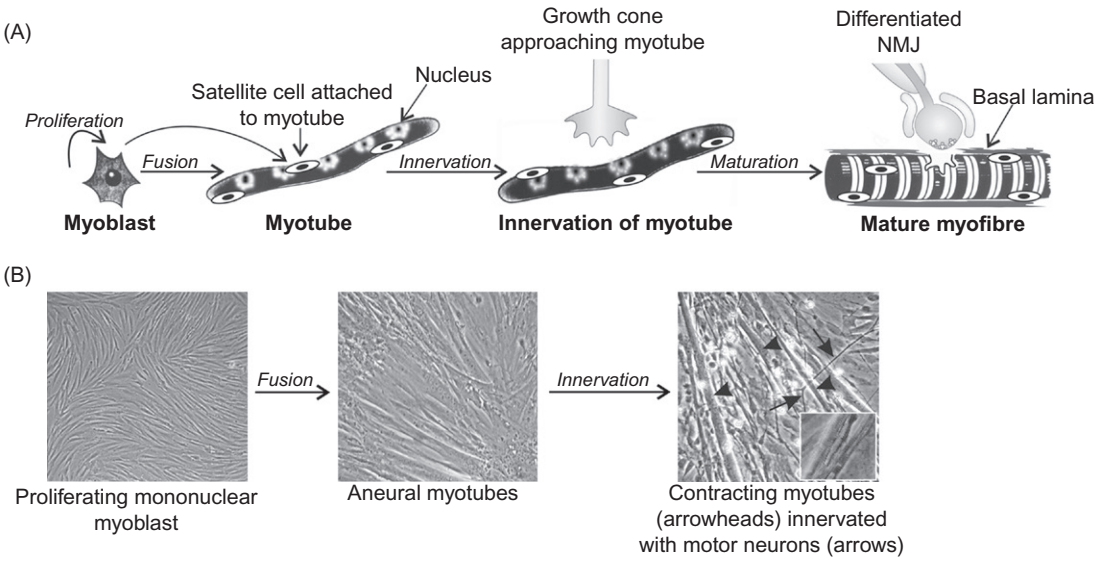

FIGURE 51.1 (A) Schematic presentation of the stages of muscle development. (B) Reproduction of these stages under *in vitro* conditions. (A) Stages of muscle development from mononuclear myoblast to the mature innervated myofiber. Nuclei, which are still centrally located in myotubes, move to the periphery in the mature fiber as a result of the synthesis of contractile elements in the sarcoplasm. Mature fibers contract and exhibit cross-striations. Only mononuclear myoblasts still have the capacity to divide and proliferate. Some of them do not fuse and become entrapped as mononuclear satellite cells under the basal lamina of the mature fiber. (B) Reproduction of this process in the experimental model of the *in vitro* innervated human muscle; axons are labeled by arrows and functionally innervated myotubes by arrowheads. Cross-striations could be seen in the innervated myotubes at higher magnification (insert).

All stages of muscle regeneration can be reproduced in the experimental model of the *in vitro* innervated human muscle (Figure 51.1B). This system was first described in the 1980s (Kobayashi and Askanas, 1985; Askanas et al., 1987) and then was further characterized in the studies in various laboratories, including ours. In this experimental model, satellite cells are released by trypsinization from small pieces of adult human muscle routinely removed at orthopedic operations. The experiments using human muscle cells were approved by the National Medical Ethics Committee of the Ministry of Health of the Republic of Slovenia (permit numbers 63/01/99 and 71/05/12) and were conducted in accordance with the Declaration of Helsinki and Good Laboratory Practice regulations. Satellite cells released by trypsinization are the source of mononucleated myoblasts that then proliferate and, at certain density, start to fuse and form multinucleated myotubes. To establish innervation, an explant of the rat embryonic spinal cord is placed on a monolayer of muscle cells. After 6–10 days of co-culture, motor neurons functionally innervate myotubes, which then start contracting. Such co-cultures are long-lived and contract for up to 6 months. Morphology of the NMJs closely resembles the NMJ *in vivo* (Askanas et al., 1987). Also, glial cells in the embryonic spinal cord explant recapitulate temporally regulated developmental steps observed *in vivo* (Mars et al., 2001). For details of preparation and various applications of this model, see the works by Grubic et al. (1995), Mars et al. (2003), Jevsek et al. (2004), Pirkmajer et al. (2010), Pegan et al. (2010), and Mis et al. (2013).

NONCHOLINERGIC EFFECTS OF DFP IN REGENERATING HUMAN SKELETAL MUSCLE

The Effect of DFP on IL-6 Secretion from the Mononuclear Myoblasts and Myotubes

Cultured human myoblasts fusing myoblasts and myotubes used in our experiments constitutively secrete interleukin-6 (IL-6). IL-6 is a major cytokine released from the skeletal muscle under various conditions (Febbraio and Pedersen, 2005). We found that IL-6 secretion from cultured muscle cells is stimulated by the major proinflammatory agents like tumor necrosis factor (TNF-α) and endotoxin lipopolysaccharide (Prelovsek et al., 2006). Because IL-6 is a potent stimulator of myoblast proliferation (Austin and Burgess, 1991; Austin et al., 1992; Cantini et al., 1995; de Letter et al., 2001; Baeza-Raja and Munoz-Canoves, 2004; Serrano et al., 2008), it is assumed that the physiological meaning of myoblast IL-6 secretion under such conditions is promotion of muscle regeneration, so that the myopathy attributable to septic conditions is, at least to some extent, compensated by muscle regeneration.

As mentioned, OP poisoning resulting in severe hypoxia often causes critical illness that is often accompanied by myopathy diagnosed as critical illness myopathy (CIM). Development of CIM is even more likely in the setting of OP poisoning because OPs directly lead to development of myopathy (Dettbarn et al., 2006). It is therefore important to find out whether the OP intoxication affects IL-6 signaling and, consequently, muscle regeneration. The effects of OP on IL-6 secretion from human myoblasts can be expected because it has already been reported that OPs drastically interfere with cytokine signaling in the mouse immune system (Alluwaimi and Hussein, 2007).

We show that IL-6 secretion from human myoblasts is significantly inhibited by DFP (Figure 51.2). IL-6 secretion was decreased by 50% in myoblasts, which might significantly reduce the efficiency of muscle regeneration process in damaged muscle. The mechanism underlying this effect of DFP remains to be established and is probably complex. In our culture medium, DFP is hydrolyzed quickly and becomes practically inactive in less than 1 h (Worek et al., 2004). Despite rapid hydrolysis, DFP-induced intracellular alterations may persist beyond the period during which DFP is still active. In our recent study, we observed 53% decrease in IL-6 secretion from human myoblasts after treatment with 10^{-5} M tabun (Katalinic et al., 2013). Our experiments are in accordance with the study of Zabrodskii et al. (2012), who demonstrated reduced concentrations of blood IL-6 in rats exposed to 30 days of sub-lethal doses of sarin and methylparathion.

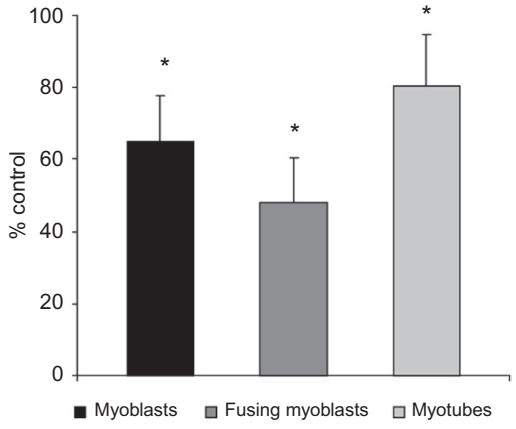

FIGURE 51.2 IL-6 secretion from myoblasts, fusing myoblasts, and myotubes. Cultured human myoblasts, fusing myoblasts, and myotubes were exposed to DFP (10^{-5}M). Concentration of IL-6 (expressed per 100,000 nuclei) was determined 24 h later with ELISA (Endogen, Rockford, IL). *Significant difference (Student *t* test, $P < 0.05$; $n = 3$) between control and treated cultures was observed at all stages studied, but was most prominent in the myoblasts.

Heat Shock Proteins in Human Myoblasts and Myotubes After Treatment with DFP

One of the most prominent cellular responses to stress is a rapid upregulation of heat shock proteins (HSPs), a family of highly conserved proteins that exert cytoprotective effects attributable to their chaperone functions in protein folding and protein degradation (Welch, 1992; Morimoto, 1993; Kiang and Tsokos, 1998; Kregel, 2002). Various physical, chemical, and biological environmental stress factors including xenobiotics can induce this response (Wu and Tanguay, 2006). Because OP pesticides are among inducers of HSP response *in vivo* and *in vitro* (Bagchi et al., 1996), we tested whether DFP actions include HSP-mediated stress response in myogenic precursors. We measured expression of HSP 27 and HSP 70, which are typically induced by various stress factors. The expression of stress factor HSP 27, but not of HSP 70, was slightly but significantly increased in DFP-treated human myoblasts (Figure 51.3), which is consistent with the reported selectivity of HSP induction in various tissues (Wu and Tanguay, 2006). We observed the DFP-stimulated increase in HSP 70 expression in myotubes, which indicates that HSP response to DFP becomes more prominent during the myotube stage (Figure 51.3). This pattern of effects is just the opposite to the observed decrease in IL-6 secretion (Figure 51.2), indicating that stress response at the HSP level does not result in increased IL-6 secretion. Increase in HSP70 was also demonstrated in several organs of common carp exposed to chlorpyrifos (Xing et al., 2013), suggesting a conservative mechanism unrelated to specific species, tissue, or OP. From the standpoint of muscle regeneration, it remains to be investigated whether the observed HSP response to DFP in any way protects proliferative potential of myoblasts that may be hampered by DFP-induced decrease in IL-6 secretion. These results are again indicative of the wide spectrum of effects that OPs induce by direct action on muscle tissue.

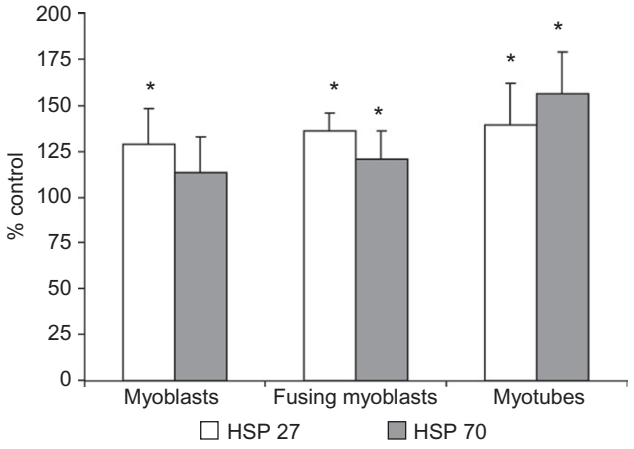

FIGURE 51.3 The effects of DFP on the HSP 27 and HSP 70 levels in myoblast, myoblasts in fusion, and myotube cultures. Levels of HSP 27 and HSP 70 were determined 24 h after addition of DFP (10^{-5} M). They were quantitated by Western blot with Chemi Genius BioImaging System (Syngen, Cambridge, UK). *Significant difference (Student *t* test, $P < 0.05$; $n = 4$) between control and DFP treated cultures was observed in all determinations except for HSP 70 in myoblasts.

Response of Human Myoblasts to Hypoxia

Severe OP poisoning can result in respiratory failure with arterial oxygen partial pressures less than 50–60 mmHg (6.65–7.99 kPa) (Tsao et al., 1990). In such circumstances, peripheral tissues, including skeletal muscle, become extremely hypoxic, which might have important effects on the myogenic precursors during regeneration. Central to the cellular response to hypoxia is hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor consisting of oxygen-regulated α-subunit (HIF-1α) and oxygen-independent β-subunit (HIF-1β). HIF-1α, which is normally almost undetectable because of its continuous degradation in the ubiquitin–proteosome pathway, rapidly accumulates in hypoxia and translocates to the nucleus, where it dimerizes with HIF-1β to form a functional transcription factor that controls expression of hundreds of genes related to cellular adaptation to hypoxia (Manalo et al., 2005; Semenza, 2007a). Through its effects on gene expression, HIF-1α not only increases oxygen delivery to hypoxic tissues by stimulating erythropoiesis and angiogenesis, but also promotes cell survival by redirecting cellular energy metabolism toward glycolysis (Pasteur effect) (Seagroves et al., 2001; Semenza, 2007b). Because of its central role in hypoxic response, we tested whether such adaptation also takes place in human myoblasts *in vitro*. We exposed human myoblasts to acute hypoxia (1% O_2 for 4 h) and found markedly increased HIF-1α levels compared with normoxic control (Figure 51.4). This result suggests that human myoblasts respond to hypoxia in a fashion similar to that of other cell types (Pirkmajer et al., 2010).

Myoblast proliferation is accompanied by apoptosis, which reduces the number of myogenic precursors and therefore affects the final outcome of the regeneration process. To determine if hypoxia affects muscle regeneration by altering the extent of myoblast apoptosis, the apoptotic markers were followed in human myoblasts under hypoxic conditions. We found that the activity of effector caspases in myoblasts remained unaltered during 48 h exposure to 1% oxygen, suggesting low susceptibility to hypoxia-induced apoptosis (unpublished results).

The Effects of DFP on the NRE in Human Myoblasts

NRE, a trans-membrane serine esterase linked to the endoplasmic reticulum, has been identified as a

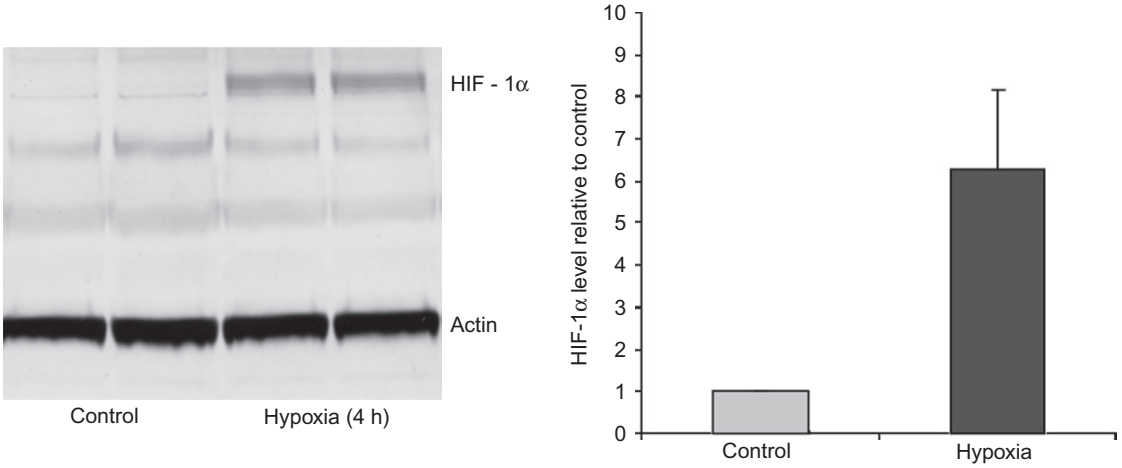

FIGURE 51.4 The effect of acute hypoxia on HIF-1α expression in human myoblasts. Human myoblasts were exposed to 1% O_2 for 4 h. A representative Western blot is shown on the left. Relative expression level of HIF-1α (three independent experiments) is shown on the right (arbitrary units, control = 1) (Student *t* test, $P = 0.004$). Quantification was performed with Chemi Genius BioImaging System (Syngen, Cambridge, UK).

member of the patatin domain–containing enzymes family (Kienesberger et al., 2008). It has high homology to neuronal NTE. The interactions of NTE with highly toxic OPs have been reported as a cause of delayed paralyzing syndrome observed in OPs intoxications (reviewed in Richardson et al., 2013). Three major isoforms of NRE, 102, 150, and 225 kDa, were detected in different stages of skeletal muscle regeneration *in vitro* (Figure 51.5A). Notably, the 150 kDa isoform was markedly upregulated on fusion of myoblasts into myotubes, suggesting NRE might have a role in differentiation of myogenic precursors. However, NRE activity was decreased in human myoblasts exposed to DFP (Figure 51.5B). Taken together, these results indicate that OPs may impair myogenesis via NRE inhibition. Inhibition of NRE may represent a novel mechanism underlying OP poisoning-related myopathy (paper in preparation).

EXPRESSION AND ROLE OF ACHE IN HUMAN MYOBLASTS

Most of the contractile and synaptic muscle proteins cannot be detected before formation of myotubes. Conversely, AChE is already expressed in the myoblast stage (Tennyson et al., 1971; Grubic et al., 1995). Although its function in this earliest stage of muscle ontogenesis is not known, it can only be noncholinergic or noncatalytic because other components of the cholinergic system are not present in myoblasts (Meshorer and Soreq, 2006).

To further explore the role of AChE in myoblasts, we followed various functional parameters in human myoblasts after selective elimination of AChE expression by siRNA.

Recovery of AChE mRNA Expression and AChE Activity After Gene Silencing of AChE and After Exposure to DFP

The *de novo* AChE synthesis importantly contributes to the recovery of AChE activity after OP poisoning (Grubic et al., 1981). Thus, we explored recovery of AChE mRNA and activity after gene silencing of AChE or DFP exposure. Although AChE level (determined on the basis of its catalytic activity) decreased to approximately 50% of control after siRNA treatment (Figure 51.6), we could not detect any functional or morphological changes in myoblast cultures in comparison with control. Addition of DFP to siRNA-treated myoblasts, which blocked practically all AChE catalytic activity, also resulted in no visible changes in myoblast cultures. This observation suggests that complete loss of AChE catalytic activity is not conducive to alterations that could be observed at the morphological level.

We then followed and compared the levels of AChE mRNA and AChE activity in siRNA-treated myoblasts and estimated the relationship between AChE expression at the mRNA and mature protein level. Because AChE is polymorphic and alternative splicing of primary transcript gives rise to three AChE mRNA species, tailed (T), hydrophobic (H), and read-through (R), which encode differently targeted AChE catalytic subunits (Massoulie, 2002), we followed these mRNA species separately in these experiments.

Three stages could be distinguished during a 170 h (approximately 7 day) period, during which we followed AChE activity:AChE mRNA ratio in the siRNA-treated myoblasts. During the first 10 h after siRNA treatment, AChE mRNA already decreased to approximately 60%

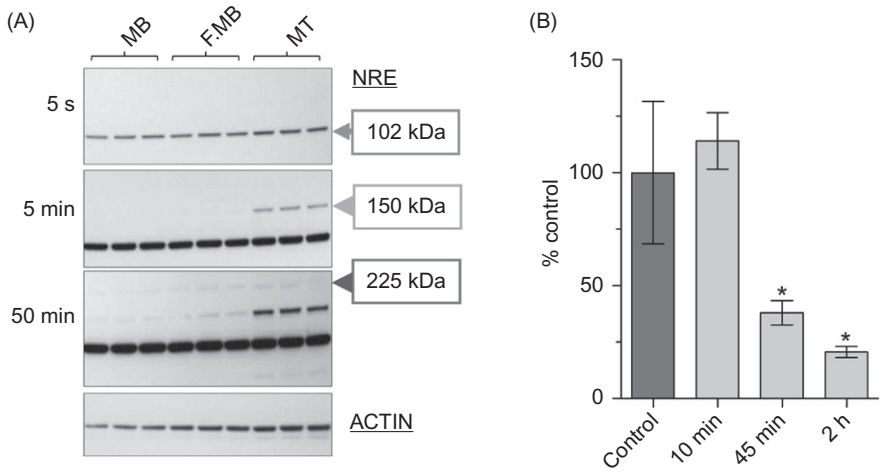

FIGURE 51.5 (A) Expression of NRE in cultured human muscle. (B) Inhibition of NRE by DFP. (A) Expression of NRE in cultured human myoblasts (MB), fusing myoblasts (F.MB), and myotubes (MT). NRE-specific bands were detected at 102, 150, and 225 kDa (note different times of film exposure) with a significant increase of 150 kDa isoform between fusing myoblasts and myotubes. (B) Time-dependent inhibition of NRE-specific esterase activity by DFP. Myoblasts were exposed to 10^{-5} M DFP for 10, 45, and 120 min. NRE-specific esterase activity was measured in myoblast homogenates using *p*-nitrophenyl valerate as a substrate. Data shown represent means ± SE (n = 4–6). *$P \leq 0.05$ versus control.

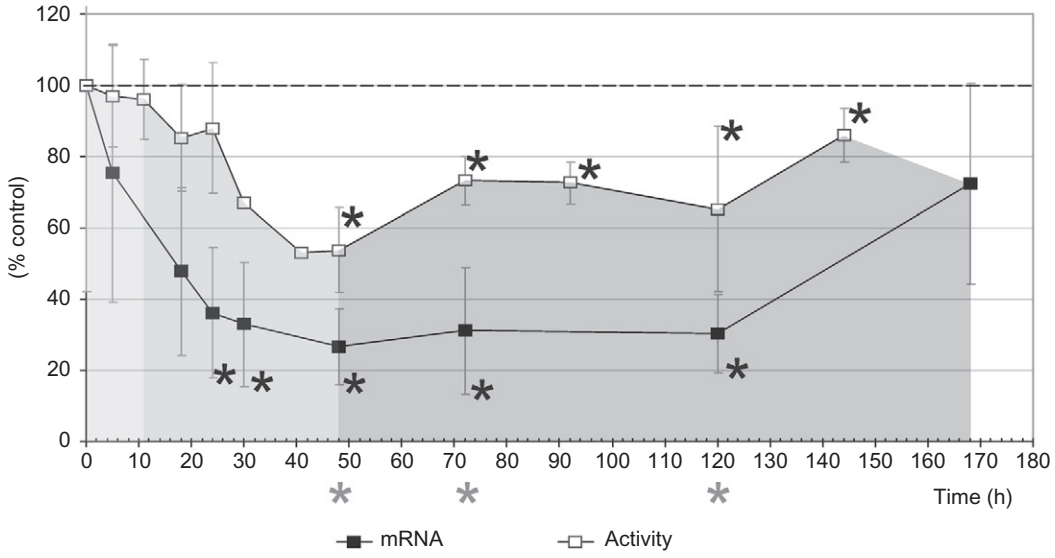

FIGURE 51.6 Relative changes of AChE-T mRNA and AChE activity in lysates of human myoblasts during the first week after siRNA application. Cultures of control and treated myoblasts were prepared from the satellite cells of the same donor and were processed in parallel. Gene silencing was achieved by lipofection of siRNA (Dharmacon); for details see Mis et al. (2006). Q-PCR with TaqMan chemistry was used for mRNA measurements. AChE mRNA levels were standardized to GAPDH mRNA. AChE activity levels determined by Ellman technique were expressed per 10,000 cells, the number of which was determined on the basis of Hoechst 33258 nuclear staining. Each point represents mean of three to seven measurements, and in each of which we determined the AChE-T mRNA or AChE activity ratio between control and siRNA-treated levels (% control). *The statistically significant (Student t test, $P < 0.05$) differences relative to the starting point. The statistically significant (Student t test, $P < 0.05$) percent differences between AChE activity levels determined by Ellman technique and mRNA levels at the same time point are indicated by * under the x-axis. Three shades of grey correspond to three stages discussed in the text.

of control level, whereas AChE activity remained practically unchanged. During the second stage (hours 10–50), AChE mRNA decreased to approximately 30% of control. AChE activity followed this decline and reached its lowest level of approximately 50% of control 50 h after siRNA treatment. From hour 50 onward, a new (more or less) constant relationship between AChE and its mRNA is established (Figure 51.6).

Of the three mRNA species, AChE-H and AChE-T mRNA followed practically the same pattern of

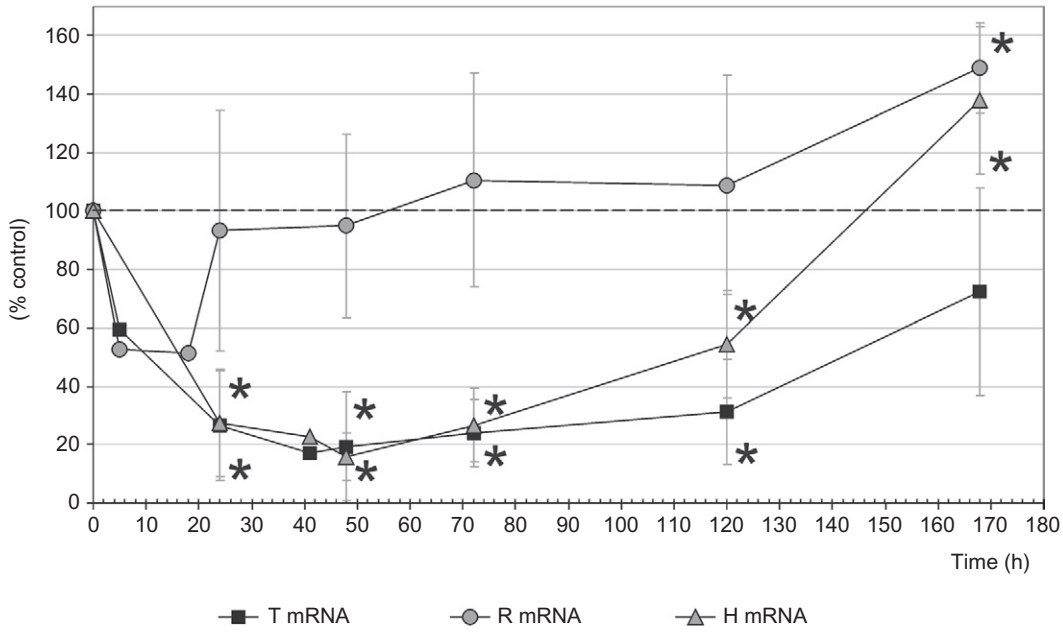

FIGURE 51.7 Relative changes of AChE-H, AChE-R, and AChE-T mRNAs in human myoblasts during the first week after siRNA application. Q-PCR with TaqMan chemistry was used (standardized to GAPDH mRNA). Each point represents mean of five separate siRNA experiments. In absolute terms, the levels of AChE-T mRNA were approximately 50-times higher than the levels of H and R. The differences regarding starting point (*) were statistically significant (Student *t* test, $P < 0.05$).

expression after siRNA treatment of human myoblasts, whereas AChE-R mRNA behaved in a completely different and less reproducible manner (Figure 51.7). In comparison with the other two mRNA species, which decreased to approximately 20% of control after siRNA treatment, AChE-R mRNA never decreased to less than 50% of control. It quickly recovered and then increased so that 7 days after siRNA treatment, its level was almost 1.5-times higher than that in controls. After approximately 150 h, but not earlier, we also observed similar overshoot for AChE-H, but never for AChE-T. All three AChE mRNA species could be detected at all developmental stages. Patterns of their expression were developmental stage–dependent, but AChE-T was the predominat species in all stages.

At present, we have no explanation for the different expression pattern of R-mRNA species in comparison with the other two. Increased AChE-R mRNA levels have been reported in mouse brain after exposure to stress (Nijholt et al., 2004). However, under *in vivo* conditions, external factors not present *in vitro* could be responsible for changed expression of this mRNA. There have been several reports from the Soreq group in which AChE-R variant was specifically induced by various stressors (Cohen et al., 2003; Grisaru et al., 2006; Ofek et al., 2007; Evron et al., 2007; Shapira-Lichter et al., 2008; Shaltiel et al., 2013). However, we do not know whether our results obtained in an artificial environment in which systemic stress response is absent are related to these findings. In any case, R-species represent only a very small fraction of AChE mRNA, and it is not known whether it is translated.

No significant changes in the levels of any of the three AChE mRNA species could be observed at any of the developmental stages of human muscle after treatment with DFP, suggesting that OPs have no direct influence on AChE expression in human muscle during the regeneration process.

The Role of AChE in Myoblast Apoptosis

One of the frequently reported noncholinergic AChE functions is its participation in apoptosis. There is evidence that reduced expression of AChE decreases apoptotic markers in hematopoietic stem cells (Soreq et al., 1994), human neuroblastoma cells (Yang et al., 2002), and various cell lines (Zhang et al., 2002). In addition, it has also been demonstrated that AChE overexpression results in increased apoptosis (Yang et al., 2002) and that cells that otherwise do not express AChE do so when entering apoptosis (Zhang et al., 2002). As for the molecular mechanism underlying these effects, there is evidence that AChE participates in apoptosome formation (Park et al., 2004). To investigate if early AChE expression in myoblasts reflects its role in the development of the apoptotic apparatus, we followed the effect of siRNA-mediated AChE silencing on the apoptotic markers after staurosporine-induced apoptosis in cultured human

myoblasts. Decreased apoptosis in siRNA-transfected myoblasts and increased AChE expression in staurosporine-treated myoblasts confirmed AChE involvement in apoptosis (Pegan et al., 2010).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Myopathies are one typical consequence of OP poisoning (Dettbarn et al., 2001, 2006). They might be additionally complicated by reduced efficiency of muscle regeneration. Our findings demonstrate that various intracellular mechanisms are influenced by exposure of precursors of muscle regeneration to DFP. Our experiments revealed significantly reduced IL-6 secretion after DFP treatment, which might hamper myoblast proliferation and the efficiency of muscle regeneration. Increased levels of HSPs in DFP-treated myoblasts suggest that cellular response to stress is induced after DFP treatment. The increased level of HIF-1α after exposure to hypoxia shows that adaptation mechanisms against hypoxia are also organized during the early stages of muscle regeneration.

Acknowledgments

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CHAPTER

52

Cholinesterase Inhibitors: From Molecular Mechanisms of Action to Current and Future Prospects

Nir Waiskopf and Hermona Soreq

INTRODUCTION

The classical functions of the cholinesterase enzymes, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE), involve regulation of cholinergic signals by acetylcholine (ACh) hydrolysis. However, their presence in locations where no ACh is released suggests that cholinesterases (ChEs) have additional, context-dependent nonclassical functions. Our understanding of the essential roles of these enzymes in the central nervous system (CNS), neuromuscular junctions (NMJ), and the autonomic nervous system predicts severe consequences following interference with their functions (Taylor, 1996; Soreq and Seidman, 2001; Darvesh et al., 2003; Silman and Sussman, 2005). The acute and life-threatening manifestations of anti-ChE toxicity were observed following the use and misuse of nerve agents, pesticides, and therapeutic anti-ChEs. These span dysregulated molecular interactions, as well as severe and potentially fatal clinical presentations. The immediate negative physiological consequences associated with critical deficiency in ACh hydrolysis are readily explained by the well-characterized role of cholinesterases in terminating the cholinergic signal. Yet the long-term outcomes of sublethal exposure to anti-ChEs may reflect complex alterations in cholinergic functions, including modified cholinesterase concentrations and composition, that are also associated with diverse disease states. Studies of the structure, functions, and natural regulation of cholinesterases allow better understanding of the molecular mechanisms and consequences of anti-ChEs poisoning and can lead to development of improved therapeutic agents. Hence, this chapter starts with an overview on human cholinesterases.

HUMAN CHOLINESTERASES

Decades of research led to a widely accepted view of cholinesterases as a family of enzymes that hydrolyze choline esters, including ACh, thereby terminating neurotransmission in the CNS, NMJ, and the autonomic nervous system. The ChE family members share more than 50% sequence homology and have basically similar folding patterns. However, their substrate and inhibitor specificities divide them into two distinct entities: AChE, which mainly hydrolyzes ACh (hence also called the *specific cholinesterase*) and BChE which is 20-fold less efficient than AChE in ACh hydrolysis but can hydrolyze a considerably wider range of substrates (hence called *pseudocholinesterase* or *nonspecific cholinesterase*). AChE is the primary ChE in the nervous system, whereas BChE is found in higher concentrations in peripheral systems. For example, AChE concentration is 10-fold higher than BChE in the brain, whereas in the circulation, BChE concentration is 100-fold higher than AChE. Hence, the total capacity to hydrolyze ACh, also termed *cholinergic status*, depends on the cumulative sum of AChE and BChE activities, which balance each other to maintain homeostasis (Figure 52.1A; Ben Assayag et al., 2010; Shenhar-Tsarfaty et al., 2014).

Human Cholinesterases: The Common Core

The existence of the two distinct cholinesterases and their functional definition as specific cholinesterase and pseudocholinesterase was first introduced by Mendel and Rudney in the early 1950s. By the early 1990s, these definitions were well complemented by theoretical

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and experimental structural-molecular observations. Cloning of cholinesterase genes from plants, invertebrates, and vertebrates (including humans) and the consequent elucidation of the three-dimensional structure of cholinesterases (Figure 52.1B and C; Nicolet et al., 2003; Ngamelue et al., 2007; Dvir et al., 2010; Nachon et al., 2011; Nachon et al., 2013) allowed a better understanding of the structure-function relationship in these enzymes, while opening new and yet-unsolved questions.

The cholinesterases were found to be serine hydrolases, belonging to the α/β-fold superfamily. Their three-dimensional crystal structures revealed a globular core, penetrated by a narrow groove (the "gorge") lined by aromatic residues, at the bottom of which lies the active site. Several functional subsites in the active site gorge were identified. These include: (i) the catalytic triad, composed of glutamic acid, histidine, and serine residues, which catalyze substrate hydrolysis by a charge relay mechanism; (ii) the acyl pocket, which interacts with the substrates' acyl group; and (iii) the anionic choline binding site, which interacts with the substrates' cationic groups. Another important component is the oxyanion hole, which stabilizes the transition state by accommodation of the negatively charged carbonyl oxygen.

At the functional level, the structural differences between the enzymes in the active site gorge can explain

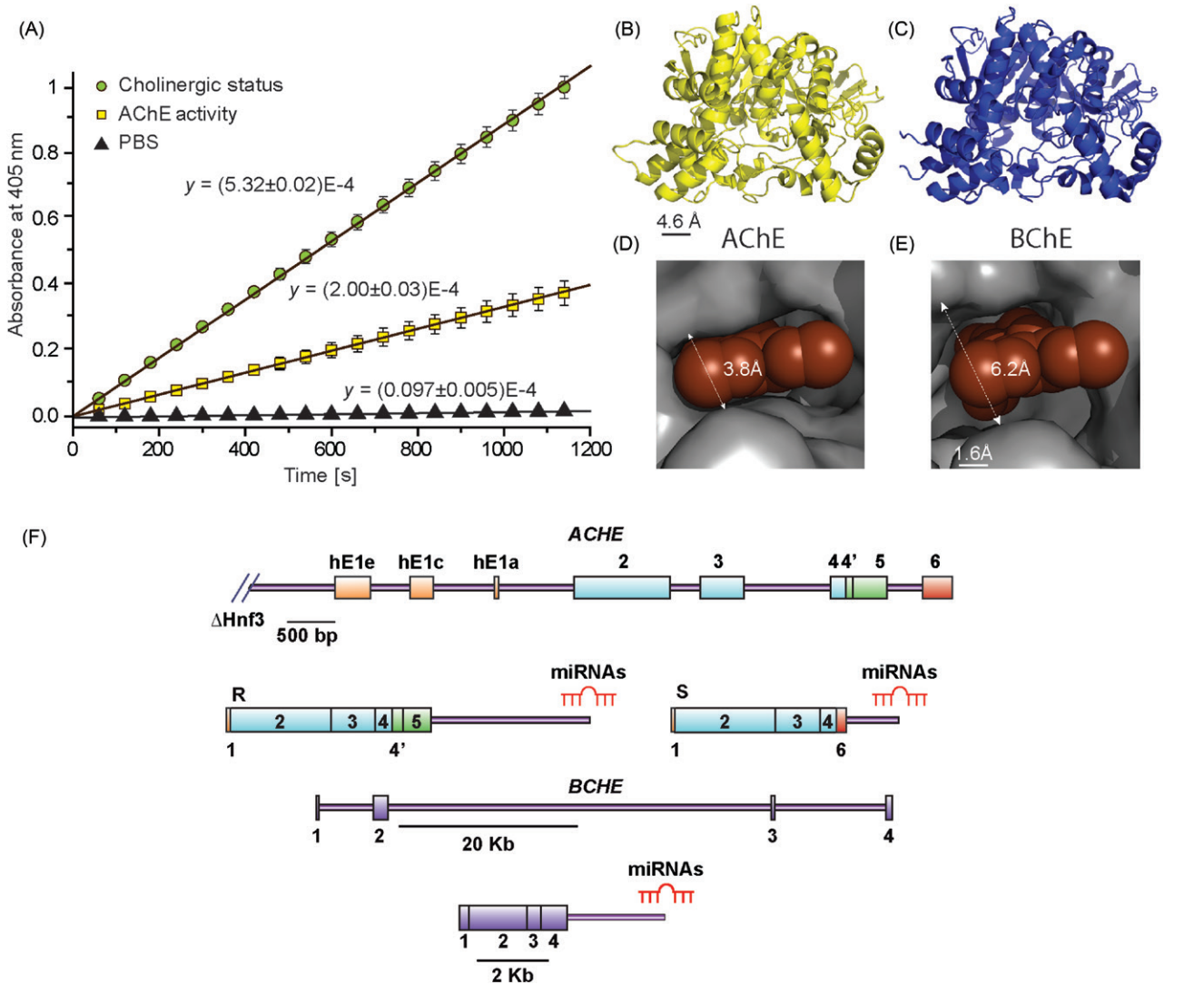

FIGURE 52.1 Cholinesterase enzymes. (A) Measurement of cholinesterase activity in human serum. The quantification of substrate hydrolysis as absorption changes over time shows that both AChE and BChE contribute to the total capacity to hydrolyze ACh, termed *cholinergic status*. (B) and (C) Cholinesterase homology, as observed in their crystallographic structures. (D) and (E) Modeling cholinesterases with the inhibitor donepezil demonstrates the differences in the volume of the active site gorge and explains the differences in substrate specificity and catalytic efficiency between AChE and BChE. (F) *ACHE* and *BCHE* genes and mRNAs. Alternative promoter usage of the 5′ of the *ACHE* gene and alternative splicing contribute to the diversity in AChE mRNA and protein isoforms. *Source: (A)–(E) and (F) are based on Arbel et al. (2014) and Nadorp and Soreq (2014), respectively.*

the differences in substrate specificity and hydrolysis efficiency. For example, BChE has less aromatic residue lining at the gorge, resulting in enlargement of the active site gorge volume by approximately 200 $Å^3$ compared to AChE; this affects the accessibility and accommodation of different substrates and inhibitors to the active site (Figure 52.1D and E; Nicolet et al., 2003; Ngamelue et al., 2007). Moreover, replacement of two phenylalanines in the entrance to the AChE's acyl pocket by two smaller amino acids, leucine and valine, in the BChE's acyl pocket, allows the accommodation of larger acyl groups and hence larger substrates (Darvesh et al., 2003). These differences explain why BChE has a broader catalytic spectrum with preference for longer-chain substrates, whereas AChE is characterized by its substrate specificity, which is essentially limited to ACh, but with 20-fold greater efficiency in ACh hydrolysis compared to BChE (Soreq and Seidman, 2001; Darvesh et al., 2003).

In addition to the catalytic active site, there exists a secondary substrate-binding site, referred to as the *peripheral anionic site (PAS)*, residing in proximity to the active site gorge. This site contributes to the first step of the catalytic pathway, to allosteric modulation of the catalytic activity, to mediation of many inhibitor interactions, and to some of the nonhydrolytic functions of AChE (Soreq and Seidman, 2001; Dvir et al., 2010). However, BChE's PAS lacks three out of the four aromatic residues that compose the AChE's PAS, resulting in different reactions toward ligand binding. For example, in the presence of excess substrate, ACh binding to AChE's PAS results in substrate inhibition, whereas its binding to BChE's PAS inversely results in substrate activation (Darvesh et al., 2003).

Additional differences between the cholinesterases and their isoforms results from variable posttranslational modifications which depend on (among other factors) cell type and physiological state (Silveyra et al., 2006). These include glycosylation, oligomerization, proteolytic processing, and (for one of the AChE variants) attachment to a glycosylphosphatidylinositol (GPI) anchor. For example, with human AChE, all three potential glycosylation sites are occupied, and with human BChE, at least eight of the nine potential sites are occupied (Darvesh et al., 2003).

Human Cholinesterases: Myriad Molecular Forms

The classic view of two cholinesterases whose sole function is to terminate cholinergic signals at the synapse has been repeatedly challenged by observations that were incongruent with this view and suggested a far more complex picture of multiple molecular forms with distinct temporal and spatial expression patterns. This complexity mainly results from alternative splicing in the case of AChE, but largely reflects frequent single nucleotide polymorphisms (SNPs) in the *BCHE* gene.

Humans possess two cholinesterase genes at two separate loci: *BCHE* (3q26) with four exons and three introns (Darvesh et al., 2003); and *ACHE* (7q22) with six exons and four introns (Soreq and Seidman, 2001). In the *BCHE* gene, more than 40 SNPs were identified. Some of these were found to produce isoforms with modified catalytic activity, sensitivity to inhibitors, and protein interaction (Podoly et al., 2009; Howard et al., 2010). For example, the common (wild type/WT) variant of BChE shows greater catalytic activity than the atypical BChE (D70G), K variant (A539T), J variant (E497V), and H variant (V142M) (Darvesh et al., 2003). The different BChE isoforms can construct distinct molecular forms, such as hydrophilic monomers and disulfide-bonded dimers, which may further form dimer-dimer soluble globular tetramers. BChE multimers can also possess distinct amphiphilic features and form asymmetric multimers of membrane-bound forms composed of tetramers anchored to the membrane by proline-rich domains. For example, the collagen Q (ColQ) in NMJ, the proline-rich membrane anchor (PRiMA) in the CNS or the proline-rich peptides derived from lamellipodin in serum (Li et al., 2008), all of which interact with the C-terminal domain of BChE.

In the human *ACHE* gene, only 19 SNPs were identified to date. As with BChE, these SNPs can affect AChE activity and interactions (Valle et al., 2011). However, the plethora of AChE protein isoforms results mainly from the translation of several messenger RNA (mRNA) variants arising through highly regulated epigenetic, transcriptional, and posttranscriptional processes (Figure 52.1F). Alternative splicing at the 3′-end of the *ACHE* pre-mRNA yields three different mRNA transcripts, which encode proteins with distinct carboxyl termini (Soreq and Seidman, 2001). The synaptic form, AChE-S (also known as *T*, for "tailed"), is generated by splicing out exon 5. This is the dominant transcript in most tissues. AChE-S mRNA translation gives rise to a 40-amino-acid C-terminal extension of the core AChE of 534 amino acids. This C-terminal peptide contains a key cysteine residue, which allows the formation of dimers through disulfide bonds. These in turn are able to form, similar to BChE, tetramers stabilized by hydrophobic interactions between tryptophan amphiphilic tetramerization (WAT) domains, which can later bind ColQ at the NMJ or the structural subunit PRiMA in the CNS, forming membrane-bound multimers (Noureddine et al., 2008). Impaired ColQ binding to AChE due to ColQ mutations leads to congenital myasthenia (Engel, 2012), highlighting the importance of this interaction.

A second form, the readthrough AChE-R, is encoded by a transcript containing pseudointron 4′ and exon 5 as an alternative 3′ terminal exon. This variant accumulates

under various stressful insults through a feedback response that activates ACH transcription and shifts splicing in the brain, muscle, spermatocytes, hematopoietic cells, intestine, and other parts of the body (Kaufer et al., 1998; Lev-Lehman et al., 2000; Meshorer et al., 2002; Grisaru et al., 2006; Meshorer and Soreq, 2006; Pick et al., 2006; Gilboa-Geffen et al., 2007; Mor et al., 2008). AChE-R has a shorter C-terminal peptide than AChE-S, which lacks the cysteine residue responsible for dimerization; hence, AChE-R remains monomeric and soluble (Soreq and Seidman, 2001; Zimmermann, 2013).

The third form, AChE-E (also known as H, for "hydrophobic"), is incoded by a transcript with exon 5 as the 3′ terminal exon but its C-terminal peptide is subsequently cleaved after amino acid 14 of E5 (557 from the N-terminus). The newly generated C-terminus is GPI-anchored to the erythrocytic membrane and includes cysteine residue which allows AChE-E dimerization (Soreq and Seidman, 2001).

Although the protein products of these mRNAs differ in their C-terminal peptide sequence and capacity to oligomerize, all of the molecular forms of AChE carry an identical active site and display similar catalytic properties. Importantly, the distinct C-termini dictate dissimilar subcellular localizations, biological roles, and noncatalytic properties.

The 5′-end of the AChE pre-mRNA is also subject to alternative promoter usage, together yielding five and three alternative transcripts in mice and humans, respectively (Figure 52.1F; Meshorer et al., 2004). The alternative 5′- and 3′-end significantly increase the combinatorial complexity of AChE. Most of the identified 5′ exons produce alternative untranslated regions. However, at least one of those exons in humans is translated to a protein, with an extended N-terminus (N-AChE; Meshorer et al., 2004; Toiber et al., 2008). Based on the combinations of the 3′ and 5′ alternative transcripts, N-AChE-E, N-AChE-R, and N-AChE-S might all exist (Meshorer and Soreq, 2006). Since N-AChE was shown to associate with the plasma membrane of blood cells (Meshorer et al., 2004), it might have the capacity to directly dock the soluble AChE-R to synaptic membranes, without ColQ or PRiMA.

Regulation of Cholinesterase Gene Expression

The regulation of cholinesterase enzymes primarily occurs at the epigenetic, transcriptional, and posttranscriptional levels (Soreq and Seidman, 2001; Shaked et al., 2009; Sailaja et al., 2012; Shaltiel et al., 2013). *ACHE* transcription is subject to epigenetics regulation via modulation of histone methylation events in the *ACHE* promoter (Sailaja et al., 2012). This explains the long-lasting increases in AChE levels following exposure to inhibitors (Friedman et al., 1996) or stressful events (Kaufer et al., 1998; Meshorer et al., 2005). The transcriptional control of the *ACHE* gene is principally regulated by two proximal promoters, one that is clearly dominant and another alternative promoter (Soreq and Seidman, 2001). Additionally, enhancers are present distally and within the first intron (Camp et al., 2008), as well as proximally to the four alternative first exons (Meshorer and Soreq, 2006). The upstream region of the *ACHE* locus contains numerous putative transcription factor binding sites, including those that potentially bind NF-κB and AP-1, as well as a glucocorticoid response element binding site (Soreq and Seidman, 2001). An increase in *ACHE* transcription is noted as a consequence of various stimuli as diverse as differentiation, anti-ChE exposure, and transiently reduced levels of AChE (Friedman et al., 1996; Kaufer et al., 1998; Evron et al., 2007). Inversely, decreases in *ACHE* transcription are seen in mice deficient in the neural growth factor TrkA (Soreq and Seidman, 2001).

One example is the transcriptional regulation of the collagen-tailed AChE-S variant, which is primarily detectable under normal conditions, in vertebrate skeletal muscle. Muscle-specific expression of AChE is believed to be controlled by the transcription factors MyoD and myocyte enhancer factor-2 acting on the regulatory region located within the first intron (Camp et al., 2008). This region is exceptionally well conserved between higher vertebrates, suggesting a generalization of this phenomenon. Negative regulation of *ACHE* transcription has been shown via a calcitonin gene-related peptide (CGRP)–dependent mechanism. CGRP is secreted from motor neurons and binds to the calcitonin receptor located in the NMJ (Rossi et al., 2003). Receptor activation results in increased intracellular cyclic adenosine monophosphate (cAMP), and subsequent attenuation of *ACHE* transcription through a cAMP-response element in the *ACHE* promoter. It has been shown that CGRP also reduces synthesis of PRiMA, which is required for the assembly and localization of skeletal muscle AChE, and that overexpression of PRiMA itself can drive *ACHE* transcription (Xie et al., 2007).

Yet more recently, AChE has been shown to be subject to microRNA (miRNA) regulation at the posttranscriptional level. MiRNAs are small molecules (20–25 nucleotides long) that can target and bind many mRNA transcripts, each leading to their posttranscriptional silencing (Bartel, 2009). *In silico* screening identified 244 miRNAs that can target the 3′-untranslated regions of different cholinesterase transcripts: 116 for BChE, 47 for the AChE-S splice variant, and 81 for the normally rare splice variant AChE-R. Of these, 11 and 6 miRNAs target both AChE-S and AChE-R, and AChE-R and BChE transcripts, respectively (Hanin and Soreq, 2011). To date, the evolutionarily conserved miRNA-132 and the primate-specific miRNA-608 have both been experimentally

validated as targeting AChE, with consequences for both inflammatory responses (Shaked et al., 2009; Maharshak et al., 2013) and cognition (Shaltiel et al., 2013; Hanin et al., 2014). Importantly, carriers of a common SNP in the *ACHE* gene that disrupts miRNA-608 suppression show a 60% elevation in brain AChE activity (Hanin et al., 2014). Other cholinesterase-targeting miRNAs are currently being investigated and show promising results.

In addition to the regulation on cholinesterase production, other regulation pathways keep the cholinergic system in balance. For example, acute exposure to anti-ChEs induces brain increases in AChE transcripts accompanied by massive decreases in the vesicular ACh transporter, which together compensate for at least part of the exposure consequences (Kaufer et al., 1998). Also, increased hydrolysis of ACh in AChE-overexpressing transgenic mice was at least partially counterbalanced by a parallel daytime increase in ACh synthesis (Farchi et al., 2003).

Cholinesterases Primarily Function as Acetylcholine-Hydrolyzing Enzymes

The cholinesterases are mainly known for their classical role—catalyzing the hydrolysis of choline esters such as ACh, thus terminating neurotransmission in the NMJ, CNS, and autonomic nervous system. NMJ cholinesterases are anchored to the cell membrane and organized in a spatially related manner close to the nicotinic ACh receptors (nAChRs), a superfamily of ligand-gated ion channels. Within the muscle, axons branch to innervate multiple muscle fibers, each fiber receiving a single synaptic input. The postsynaptic membrane of the skeletal muscle forms deep invaginations called *junctional folds*. Nicotinic AChR are clustered at the peaks of the folds, close to the neuronal presynaptic active zone enriched in ACh-releasing vesicles. The synaptic cleft is filled by the basal lamina of the NMJ, highly enriched in AChE, whereas BChE is concentrated in subsynaptic folds (Blondet et al., 2010). Depolarization of the nerve terminal due to a neuronal impulse is followed by an influx of calcium ions into the terminal via voltage-gated calcium channels. This influx results in the exocytosis of many synaptic vesicles and the release of a corresponding number of ACh quanta into the synaptic space. Binding of two ACh molecules to the nAChR multisubunit complex opens the AChR channel, allowing the inward flux of cations along their electrochemical gradient and producing endplate potential. If this depolarization exceeds the threshold required to open the voltage-gated sodium channels at the depths of the folds, an action potential is generated, leading to muscle contraction. The evoked responses that can be recorded from the surface of the muscle following nerve stimulation are called *compound muscle action potential*, which represents the cumulative action potentials of all muscle fibers activated. The cholinesterases contribute to the regulation of this signal transduction by ACh hydrolysis. Part of the resulting choline pool is recycled with the aid of a high affinity transporter in the pre-synaptic nerve terminal, and is being used as substrate by choline acetyltransferase (ChAT) to generate new ACh molecules (Sarter and Parikh, 2005).

The catalytic activity of cholinesterases is important not solely for regulating neurotransmission, but also for modulating the cholinergic anti-inflammatory reflex. This term was coined after recognition of the ability of excessive stimulation of nAChRs to prevent the secretion by macrophages of the pro-inflammatory cytokines TNFα, interleukin (IL)-1β, IL-6, and IL-18, but not the anti-inflammatory cytokine IL-10 (Tracey, 2010). More specifically, upon inflammatory stimuli, the α7 nicotinic ACh receptor (α7nAChR) is activated by ACh, which results in the inhibition of NF-κB nuclear translocation and suppression of cytokine release by monocytes and macrophages (Tracey, 2010).

The importance of AChE to this process is evident in that low-dose lipopolysaccharide induces a 10-fold increase in miRNA-132 levels, which in turn reduces AChE protein levels and activity in the human serum, potentiating the anti-inflammatory reflex (Shaked et al., 2009; Waiskopf et al., 2014). Thus, ACh hydrolysis by ChEs interferes with the ACh anti-inflammatory effect, and their regulation can rebalance it.

Cholinergic Hyperexcitation and Induction of AChE-R Production

Acute psychological stress induces cholinergic stimulation, yielding neuronal hyperexcitability. Immediate reactions to such cholinergic stimulation include a dramatic increase in neuronal c-fos mRNA (Kaufer et al., 1998; Lev-Lehman et al., 2000). The c-fos protein further binds and regulates the promoters of the *CHAT*, *VCHAT*, and *ACHE* genes. A consequent decrease in ChAT and VChAT then suppresses ACh synthesis and packaging, while enhanced AChE mRNA synthesis and translation facilitates ACh hydrolysis, together yielding an effective restraint on the hypercholinergic response. The larger AChE activity observed in both brain and NMJ following stress further reflects a change in alternative splicing and the induction of a soluble AChE variant, AChE-R (Kaufer et al., 1998). This profound feedback response serves well in the short term to quickly reduce the cholinergic hyperactivity following an acute insult. However, repeated cholinergic stimulation may induce long-term hypersensitization, with potentially damaging consequences. Supporting this notion, transgenic mice overexpressing AChE in the CNS present delayed

cognitive and neuroanatomical pathologies (Soreq and Seidman, 2001; Cohen et al., 2002; Farchi et al., 2003). In humans, traumatic stress with a severe stress response is often followed by long-term pathological changes (McEwen and Morrison, 2013). Under extreme conditions, such changes are clinically referred to as the anxiety-associated *posttraumatic stress disorder.* Physiological relevance of AChE-R to these increases was reported in healthy human volunteers, where higher blood AChE-R levels occurred in association with the subjects' state of anxiety (Sklan et al., 2004). It is still unknown whether the stress-induced alternative splicing of AChE occurs as a cause or an outcome of anxiety, or both. However, in either case, this phenotype may reflect physiological relevance of the overexpressed AChE-R in reaction to stress. Apart from this apparent association, AChE-R may mediate at least some of the adverse cellular changes associated with delayed stress responses (e.g., neuronal spine loss or glial hyperactivation; Soreq and Seidman, 2001).

Under normal conditions, AChE-S is an essential component of functional NMJ, but AChE-R is not. Compatible with this notion, the loss of the ColQ collagen-like structural subunit linking AChE-S to the NMJ leads to congenital myasthenia (Engel, 2012), and modified AChE levels were associated with muscle dystrophy in both chickens and mice (Comim et al., 2011; Vidal et al., 2013).

Similar to its regulation in the brain, AChE-R accumulates in neuromuscular tissues under conditions of disease and stress. AChE-R levels rise both in the serum and muscles of experimental autoimmune myasthenia gravis (EAMG) rats and in the serum of myasthenia gravis (MG) patients (Angelini et al., 2013). In the primate spinal cord, AChE-R accumulated under handling stress in a cell size–dependent manner, suggesting that the characteristic response of shift in the alternative splicing of AChE pre-mRNA spans different cell types and is pivotal for controlling motor functions. Cholinergic spinal cord interneurons innervate large spinal cord neurons, but not the motor neurons themselves, and they respond to stress conditions by elevating AChE-R and by reducing the translocation of AChE-S mRNA into neurites. This is compatible with the shift in alternative splicing toward AChE-R and its transport to neural extensions, where it replaces the normally expressed transcript, AChE-S (Meshorer et al., 2002). A rapid and transient elevation in serum AChE-R following stress responses further suggests a mechanism for suppressing ACh-mediated excitation under stress. However, prolonged elevation in AChE-R (for example, under chronic stress) may damage cholinergic signaling and exert noncholinergic effects. A direct role for AChE-R in MG symptoms likely involves several functional levels. The first is the clear cholinergic function of AChE-R in controlling ACh levels at the NMJ. The second is interference with the anti-inflammatory role of ACh, exacerbating the outcome of the autoimmune origin of the disease. The third may involve changes in the levels of AChE-targeting miRNAs, which can secondarily modify the levels of other targets of such miRNAs (Hanin et al., 2014; Tay et al., 2014).

Nonclassical Cholinesterase Functions

In addition to the traditional roles of ChEs as regulators of cholinergic transmission, many nonclassical roles have been proposed for them (Soreq and Seidman, 2001; Birikh et al., 2003; Darvesh et al., 2003). These include protein–protein interactions and unusual hydrolytic activities. BChE was thought to have additional roles ever since the first evidence of its ability to catalyze the hydrolysis of noncholine esters and the discovery of silent BChE variants that do not possess hydrolytic capacity (Darvesh et al., 2003). For example, BChE was found to have aryl acylamidase activity and was proposed to attenuate amyloid plaque formation and contribute to neurogenesis. In this respect, the K variant of BChE shows weakened protein interactions and lower stability and hydrolytic activity, in spite of an intact active site (Podoly et al., 2009; Shenhar-Tsarfaty et al., 2011).

The first discovered noncatalytic role of AChE-S was the enhancement of embryonic neurite extension, both in cell cultures and in *Xenopus* motor neurons that express human AChE-S (Soreq and Seidman, 2001). Since then, the most significant advances in understanding the nontraditional roles of ChEs emerged through AChE-R studies (Meshorer et al., 2002). AChE-R was first recognized as a stress-induced, soluble AChE variant, but researchers quickly noticed that it contributed to numerous important processes, from fear to hematopoiesis and inflammation. Specifically, both AChE-S and AChE-R were found to interact with the scaffold protein RACK1 (Birikh et al., 2003; Waiskopf et al., 2014). These interactions are important in the regulation of inflammation and fear memory and long-term fear potentiation (Birikh et al., 2003; Nijholt et al., 2004; Waiskopf et al., 2014). Moreover, a proliferation function of AChE-R has been recognized in hematopoietic cellular processes and osteogenesis (Soreq and Seidman, 2001; Pick et al., 2006), whereas excess of AChE-S was inversely shown to interfere with lymphopoiesis (Perry et al., 2007).

Another potential function is based on AChE's sequence homology to cell surface adhesion molecules such as neuroligin (Bourne and Marchot, 2014), suggesting that it may be involved in synaptogenesis and synaptic maintenance (Silman and Sussman, 2005). In brain synapses, AChE-R might compete with neuroligin upon interaction with its partners (i.e., neurexin), interfering with and even modifying the postsynaptic signaling pathway. Such interference may serve as a potential mediator

of cytoarchitectural changes in CNS synapses (Soreq and Seidman, 2001). Both neuroligin and neurexin are also expressed and function at the NMJ, suggesting parallel relevance to the NMJ architecture. Within the NMJ, synaptic differentiation is induced by axon-derived agrin and muscle-derived laminin. Laminin 1 was suggested as a potential partner for AChE (Paraoanu and Layer, 2004) and for the cleavable C-terminal AChE-R peptide, ARP (Johnson and Moore, 2007).

AChE-S was also suggested to play a role in accentuating amyloid plaque formation (Soreq and Seidman, 2001). On the other hand, AChE-R was found to be drastically up-regulated in amyloid beta-treated cell culture (Li et al., 2013) and to inversely attenuate plaque formation through its C-terminal domain (Berson et al., 2008). Importantly, AChE-R elevation in the serum of stressed mice was associated with an apparent cleavage of ARP. This peptide contributes to hematopoietic proliferation and pro-inflammatory cytokine production (Grisaru et al., 2006; Pick et al., 2006; Gilboa-Geffen et al., 2007), contextual fear and long-term potentiation enhancement (Nijholt et al., 2004; Farchi et al., 2007), neuronal development, plasticity, and inflammation-associated neuropathies (Dori et al., 2007).

ANTI-ChEs AND THE CHOLINERGIC SYSTEM

The vital role of the acetylcholine-hydrolyzing enzyme AChE in terminating cholinergic neurotransmission has been recognized for almost as many years as ACh has been recognized as a neurotransmitter. For nearly the same amount of time, AChE research was intimately linked to the study of its inhibitors. The vulnerability of cholinesterases to the natural toxins of many organisms, including fungi, plants, and animals, promoted the discovery and synthesis of new anti-ChEs for use as pesticides, therapeutics, and, unfortunately, as chemical warfare agents (CWAs; Gupta, 2006). These inhibitors helped to elucidate the enzymatic mechanism of action and this knowledge, in turn, was used to design even more potent or more specific anti-ChEs.

Molecular Mechanisms of Cholinesterase Inhibition

The therapeutic/toxic action of cholinesterase inhibitors is targeted at well-known domains of cholinesterases, such as the catalytic site at the bottom of the gorge (Nachon et al., 2013), the anionic peripheral site (Silman and Sussman, 2005), or both (Cheung et al., 2012). Given the structural differences between cholinesterases, their inhibitors can be classified by their selectivity to particular ChEs. For example, phenserine, huperzine A, and BW284c51 are selective AChE inhibitors and tetra(monoisopropyl)pyrophosphoramide (Iso-OMPA) and ethopropazine are selective BChE inhibitors, whereas paraoxon and rivastigmine inhibit both. Alternative classifications may be based on the origin of the inhibitors (whether natural (e.g., galantamine, onchidal, and coumarins) or synthetic compounds (e.g., tacrine, donepezil)), reversibility, or according to their chemical nature. The last divides most of them into two main groups: carbamates and organophosphates (OPs).

Carbamates are used as pesticides (e.g., aldicarb, carbofuran, carbaryl, ethienocarb, and fenobucarb); medications to elevate ACh levels in diverse pathologies such as Alzheimer's disease, MG, glaucoma (e.g., neostigmine, rivastigmine, physostigmine, and pyridostigmine); and in veterinary medications. Some of the members in this group, similar to ACh, can undergo acylation (carbamylation) to form a carbamoyl-enzyme intermediate. The subsequent hydrolysis rate of this hemi-substrate is very slow, occupying the enzyme carbamoylated for a long time and competing with the natural substrate, hence often regarded as pseudo-irreversible inhibition (Figure 52.2A). Interestingly, atypical BChE was found to be far less sensitive than the WT BChE to inhibition by pyridostigmine and several other carbamate anti-ChEs (Loewenstein-Lichtenstein et al., 1995), thus some of the carbamates are variant-specific inhibitors.

The second major group of ChE inhibitors, the OP compounds, was introduced as CWAs in World War II (e.g., tabun, sarin, and soman). Since then, they were developed as CWAs (e.g., nerve agents such as VX, VE, VG, and VM), but also as agricultural pesticides (e.g., malathion, parathion, diazinon, fenthion, dichlorvos, trichlorfon, chlorpyrifos, dimethoate, triazophos, and ethion), flame retardants (e.g., triphenyl phosphate), therapeutics to elevate ACh levels (e.g., echothiophate and diisopropyl fluorophosphate, DFP), and veterinary medications. Similar to carbamates, OPs react with the active site of ChEs and other serine esterases due to the structural resemblance to their substrates (Nachon et al., 2011). The phosphorylated enzyme can be reactivated at a considerably slower rate than the carbamylated enzyme, and certain OPs can further undergo dealkylation following their interaction with the enzyme, which completely blocks its reactivation (known as *aging*; Taylor, 1996; Wandhammer et al., 2013) (Figure 52.2A).

Importantly, whereas most OPs pass the blood–brain barrier (BBB), carbamate anti-ChEs can be divided into those that penetrate the BBB, such as physostigmine and rivastigmine, and those that are unable to cross it under normal conditions, such as pyridostigmine, neostigmine, and ambenonium. Hence, CNS toxicity symptoms are less common with carbamate poisoning. For anti-ChE toxicity and BBB dysfunction and damage, see Chapter 49.

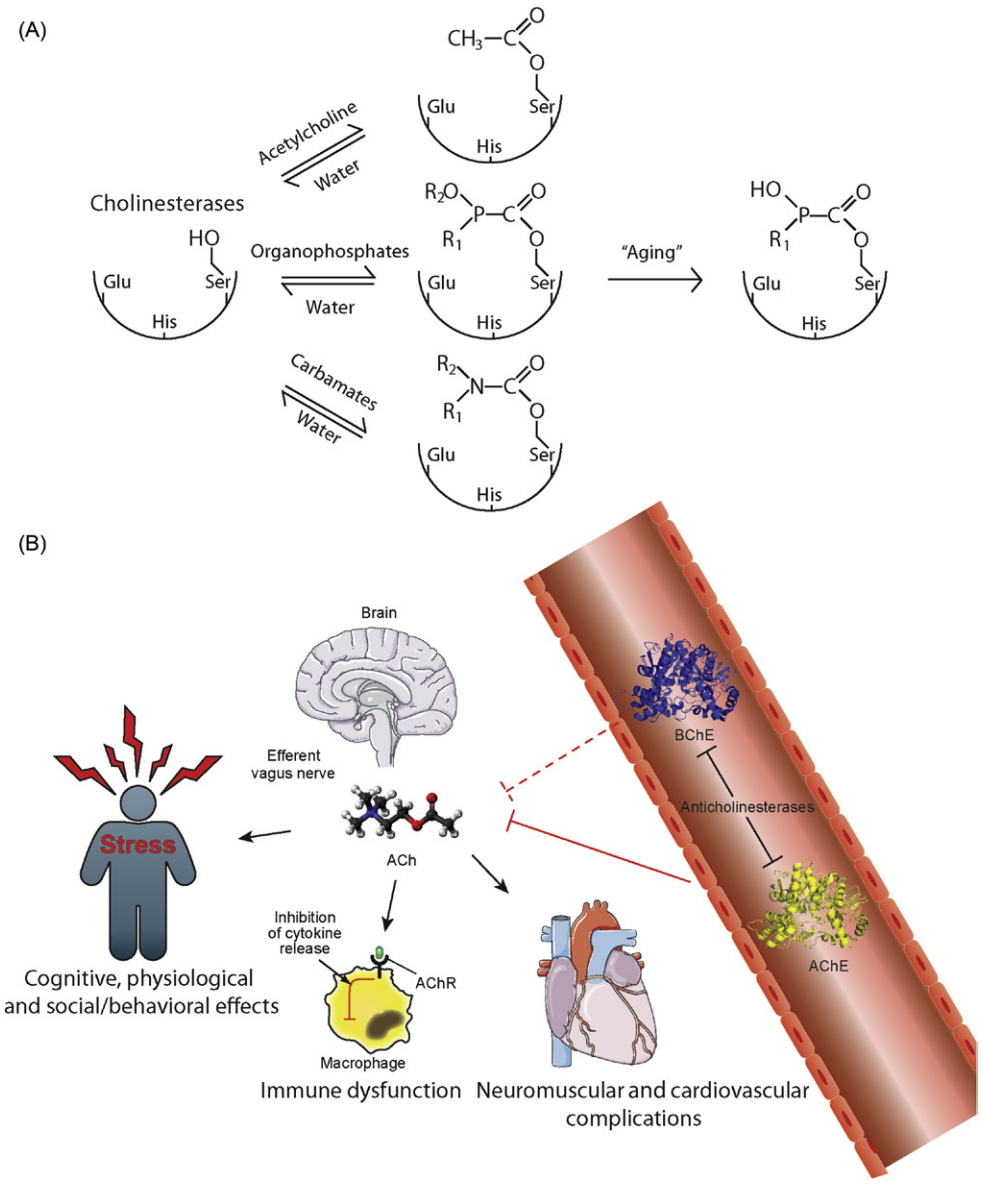

FIGURE 52.2 Anti-ChEs and the cholinergic system. (A) Cholinesterase catalytic triad interactions with acetylcholine and anti-ChEs. (B) Schematic representation of the long-term effects of exposure to anti-ChEs. *Source: (B) is based on Arbel et al. (2014).*

Short-Term Effects of Anti-ChE Exposure

ACh accumulation under exposure to ChE inhibitors and the subsequent nonregulated overstimulation of nicotinic and muscarinic ACh receptors initiates a variety of detrimental processes, including cholinergic hyperexcitation and neuromuscular malfunctioning, which can culminate in severe consequences for the exposed patient (Yanagisawa et al., 2006). In the peripheral nervous system (PNS), the short-term effects of anti-ChE exposure commonly involve autonomic dysfunction, including excessive stimulation of secretory organs, resulting in salivation, lacrimation, urination, and defecation, and muscle fasciculations, and can even include complete paralysis of different muscles. CNS exposure is usually accompanied by symptoms such as CNS depression, anxiety, restlessness, and confusion. Moreover, severe exposure can cause seizures, coma with decreased respiration, and even death, commonly due to cardiac effects or respiratory insufficiency due to muscle weakness even a few days after the exposure (Taylor, 1996).

Anti-ChEs also exert deleterious effects on immune functions (Corsini et al., 2013). OP-mediated suppression of TNF-α, IL-1β, and iNOS has been shown, as well as a confirmation of decreased humoral immunity through suppression of IL-4 (Singh and Jiang, 2003).

This, in turn, has led to investigation of OP-mediated suppression of the hypothalamic-pituitary-adrenal axis (Pena-Philippides et al., 2007). However, anti-ChEs were also found to initiate acute immune responses. For example, the nerve agent soman induces an increase in the pro-inflammatory cytokine TNFα, IL-1β, and IL-6 in rats, including in the brain, where IL-1β is thought to contribute to irreversible brain damage (Banks and Lein, 2012). This bidirectional impairment of immune functions may reflect systemic responses that affect more than the cholinergic system alone.

Therapeutic anti-ChEs have also been shown to exert anti-inflammatory properties (Tyagi et al., 2007), presumably through their role in increasing ACh levels. Specifically, the cholinergic anti-inflammatory pathway has profound implications for excessive nAChR stimulation in the context of an infected host by increasing the susceptibility to initial infection or therapeutically reducing the host overreaction seen in sepsis. Therefore, it is important to consider also treating those anti-ChEs-exposed patients who do not exhibit acute cholinergic symptoms in order to reduce the likelihood of opportunistic infection following survivable anti-ChEs intoxication.

Long-Term Effects of Anti-ChEs

Intoxication, when surviving an acute exposure, also induces long-term consequences, including OP-induced cardiac complications, delayed neuropathy, cognitive, social and behavioral effects, and delayed muscle weakness (Figure 52.2B). The effects of anti-ChEs, like many other stressful insults, are mediated by the accumulation of the early immediate protein c-fos, followed by up-regulation of *ACHE* gene expression (Friedman et al., 1996) and a 3′ alternative splicing shift from AChE-S to AChE-R (Kaufer et al., 1998; Meshorer et al., 2002). This prolonged overexpression of AChE-R due to the intoxication causes dramatic deleterious consequences, parallel to those observed after acute psychological stress; those also span degenerated synaptic folds in the NMJ, enlarged motor endplates, disorganized muscle fibers, and branded terminal nerves, accompanied by physiological malfunctioning (Lev-Lehman et al., 2000; Farchi et al., 2003).

AChE-R mRNA levels remain elevated for several weeks following a daylong exposure, even to exceedingly low doses of the AChE inhibitor, DFP (Lev-Lehman et al., 2000; Meshorer et al., 2002). In the striatum, exposure to AChE inhibitors predictably increased AChE-R mRNA by threefold, but it did not affect AChE activity or total AChE mRNA, possibly suggesting the arrested translation or production of inactive AChE-R (Perrier et al., 2005), most likely due to miRNA effects (Shaked et al., 2009; Shaltiel et al., 2013; Hanin et al., 2014). In the hippocampus, exposure to AChE inhibitors could not be rebalanced by subsequent cholinergic stimulation due to a limited ability for additional AChE-R induction (Meshorer et al., 2002), which may reflect epigenetic modulation of *ACHE* transcription (Sailaja et al., 2012). Intriguingly, AChE-R mRNA was found in the apical dendrites of neurons from all cortical layers following exposure to cholinesterase inhibitors (Kaufer et al., 1998; Meshorer et al., 2002), suggesting possible local regulation and translation of AChE in the synapse and in noncholinergic neurons. Engineered mice overexpressing AChE-R model these changes and display significant deficits in both normal and social behavior and memory (Cohen et al., 2002; Nijholt et al., 2004; Farchi et al., 2007), compatible with the assumption that excess of AChE-R is causally involved with the long-term effects of anti-ChE exposure.

Two weeks following DFP exposure for 4 days, mouse tongue muscles show significant increase in AChE catalytic activity (Lev-Lehman et al., 2000). The punctuated expression pattern of NMJ-related AChE-S mRNA remains unchanged, but AChE-R mRNA is significantly induced and exhibits a diffuse, extrajunctional distribution. AChE-S overexpressing TgS mice, which also show host AChE-R mRNA overexpression, presented a similar expression pattern (Lev-Lehman et al., 2000). Furthermore, in control animals, histochemical activity staining localized the signal to motor endplates, whereas in TgS mice, the soluble AChE-R presented dispersed staining in muscle fibers, which is not restricted to the endplate region, parallel to its pattern of distribution in muscles of myasthenic rats.

In both TgS mice and strain-matched controls chronically treated with DFP, AChE-R overexpression was accompanied by neuromuscular pathology. This neuropathology was exhibited as a chaotic fiber disorder with severe atrophy and vacuolization, compared to control mice where fiber organization was conspicuous (Lev-Lehman et al., 2000). A significant increase was observed by silver staining in small unbundled neurites, but not in large nerve bundle fibers, both in TgS mice overexpressing the synaptic AChE variant and following chronic DFP treatment, compared to strain-matched controls. Compatible with the neurite-promoting activity of AChE, these findings indicate axon branching under the influence of AChE-R overexpression and suggest that the affected muscles were subjected to denervation-reinnervation processes. Importantly, nontransgenic mice treated with DFP and untreated TgS mice exhibited a significant increase in the number of endplates in their diaphragm muscle compared to FVB/N controls (Lev-Lehman et al., 2000). These endplates were smaller in diameter, supporting the notion that reinnervation occurred. Intact TgS muscles stimulated directly via the phrenic nerve examined *ex vivo* demonstrated rapid

fatigue following initial stimulation, accompanied by enlarged decrements and delayed recovery compared to strain-matched controls; this finding was attributed to both neuronal and muscle impairments (Farchi et al., 2003).

The persistent hypercholinergic transmission due to cholinesterase inhibition also results in rapid internalization of the AChR, with homeostasis being restored through the simultaneous process of AChE replacement (Krejci et al., 2006). OP exposure has been shown to induce nAChR desensitization, validating the concept of receptor-level compensation (Katz et al., 1997). In an elegant experiment, a one-time blockade of AChE resulted in a 54% internalization of the AChR at the NMJ within 3 days, presumably in an attempt to mitigate the increased synaptic ACh. Hence, the long-term effects of anti-ChE exposure result from interference with the regular functioning of different cholinergic components.

Anti-ChEs as Therapeutic Agents

Plant-originated anti-ChEs, such as huperzine A, have been used for thousands of years in the treatment of aging-induced memory impairment (Haviv et al., 2007). In 1877, prior to the discovery of ACh as a neurotransmitter in the brain, physostigmine (eserine)—a carbamate extracted from the seeds of *Physostigma venenosum*—became the first cholinesterase inhibitor used therapeutically to control increased ocular pressure caused by wide-angle glaucoma (Taylor, 1996).

Anti-ChEs, such as neostigmine and physostigmine, were also the first effective treatments for MG already in common use in 1935 (Engel, 2012). However, since physostigmine is capable of crossing the BBB, neostigmine was the preferred therapeutic agent. Pyridostigmine bromide (Mestinon) became available in the 1950s as a longer-lasting agent, active for 3–6 h with fewer side effects and the ease of oral administration. Medical forces in Western armies also have used it as a prophylaxis in anticipation of anti-ChE exposure (Friedman et al., 1996). Longer-lasting agents, including OP inhibitors, were not used since they accumulated to overdose levels. Subsequently increased interest in anti-ChE for Alzheimer's disease therapeutics led to the development of tacrine, as the first ChE inhibitor approved in 1993 by the US Food and Drug Administration (Giacobini and Gold, 2013). More recently, second-generation anti-ChEs, such as rivastigmine, donepezil, and eptastigmine, have shown higher efficacy, lower toxicity, and easier administration (Ringman and Cummings, 2006).

Importantly, anti-ChE therapy can also cause a variety of side effects, including gastrointestinal problems, salivation, sweating, and cardiac and hypotension effects. Hence, further research and development of anti-ChEs for therapeutics is required.

DETECTION AND PREVENTION OF ANTI-ChE TOXICITY

The toxicity of anti-ChEs led to their development as CWAs, with the most recent example being the Syrian use of nerve agents to oppress in-land citizens (Sample, 2013). This and the effects of daily life exposure to anti-ChEs in agriculture and industry led to the development of detection methods and therapeutic agents to prevent the lethal effects of anti-ChEs (Periasamy et al., 2009; Masson, 2011; Worek and Thiermann, 2013).

Detection and Use of Nanoparticles and Other Technologies

Different technologies and methods based on cholinesterases have been developed for low cost and fast detection of anti-ChEs with high sensitivity, accuracy and storage stability (Periasamy et al., 2009). These include a novel development of cholinesterases conjugated to nanomaterial-based sensors. In such systems, the cholinesterases are conjugated to nanomaterials such as carbon nanotubes (CNTs), metallic nanoparticles (NPs), or semiconductor NPs, which enable the use of their unique properties, in the nano regime, to achieve the requirements from satisfactory sensors.

Various strategies were examined for the preparation and modification of multiwall CNT (MWCNT) for applications such as electrochemical sensors, solar cells, and photovoltaic devices with advantages such as high electrical conductivity, mechanical strength, and stability (Zhang et al., 2013). This was utilized for the development of anti-ChE sensors based on cholinesterases deposited on electrodes (Periasamy et al., 2009; Cevik et al., 2013). In most of these systems, thiocholine, the hydrolysis product of substrates such as acetylthiocholine or butyrylthiocholine, participates in a redox reaction that allows amperometric and cyclic voltammetry measurements. The comparison of the response with and without anti-ChEs allows the detection and quantification of their concentrations. For example, using such systems, the impressively low sensitivity limits of 4×10^{-13} M for paraoxon and 5×10^{-9} M for triazophos were measured with glassy carbon electrode (GCE)–MWCNT-AChE (Periasamy et al., 2009). In parallel, developments in the synthesis, surface engineering, and conjugation of metallic and semiconductor NPs to proteins for imaging, sensing, and delivery opened this platform for anti-ChE sensors as well.

Cholinesterases were conjugated by different bioconjugation techniques to colloidal NPs of different compositions, dimensions, and surface coating allowing fine tuning of the NPs to the desired application (Figure 52.3A; Waiskopf et al., 2011, 2013). Cholinesterases were also conjugated to NPs deposited on electrodes,

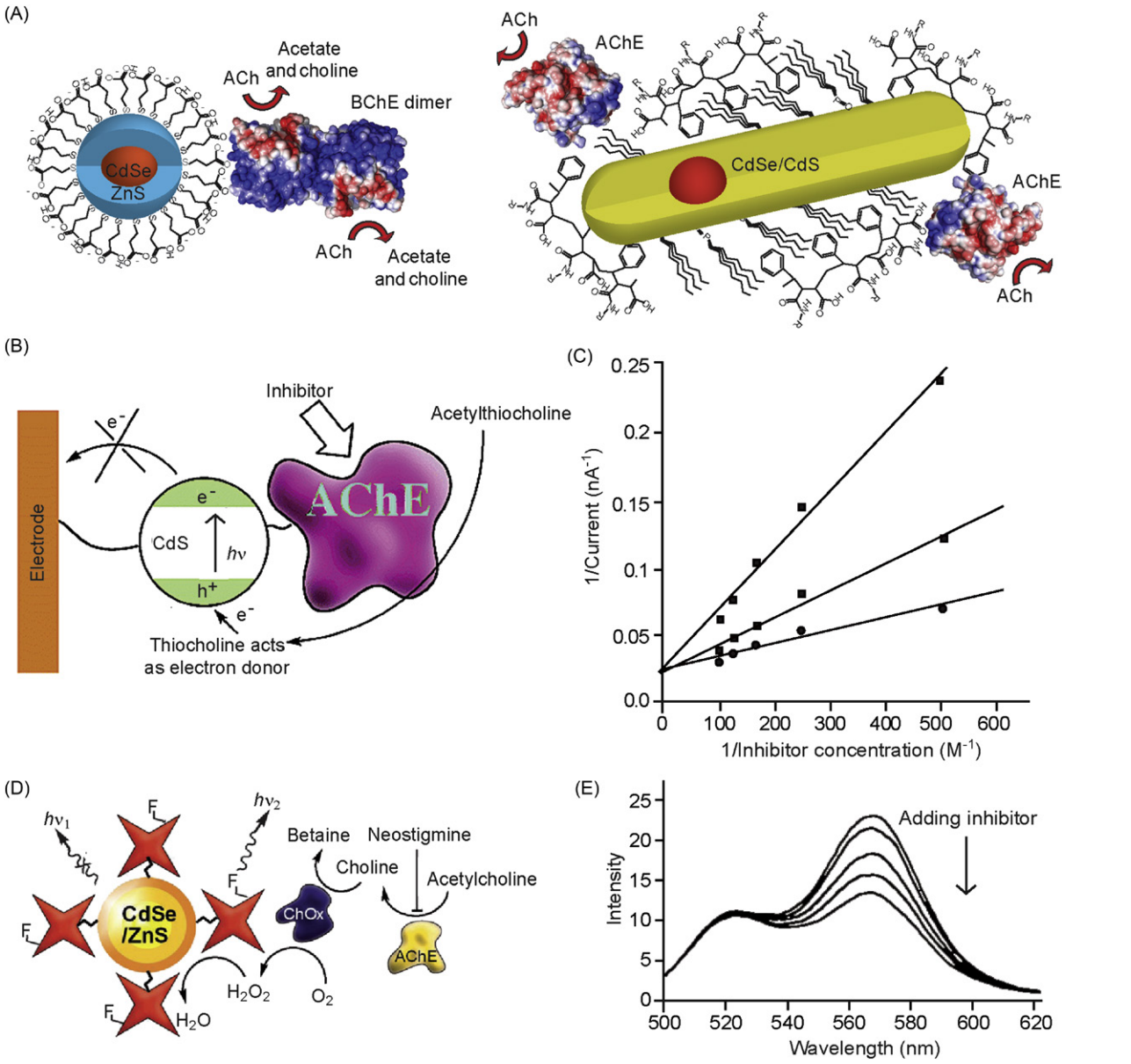

FIGURE 52.3 Cholinesterase-based sensors. (A) Cholinesterases conjugated to NPs of different composition, dimensions, and surface coating. (B) Illustration of a photoelectrochemical sensor of anti-ChEs using cholinesterases conjugated to NPs on an electrode. (C) Experimental data with different concentrations of ACh, which show the reduction in current with the increase in anti-ChE concentration. (D) Illustration of the anti-ChE sensing method based on interference with a chain reaction that starts with cholinesterase-driven hydrolysis of acetylcholine molecules and ends with emission from semiconductor NPs. (E) Experimental data showing the reduction in semiconductor NPs emission intensity with the increase in anti-ChE concentration. *Source: (A) was reprinted and adapted from Waiskopf et al. (2013) with kind permission from Springer Science and Business Media. (B) and (C); (D) and (E) reprinted and adapted with permission from Pardo-Yissar et al. (2003) and Gill et al. (2008), respectively. Copyrights (2003) American Chemical Society and (2008) John Wiley & Sons, respectively.*

increasing the number of conjugated enzyme molecules due to the large surface area/volume ratio. This, in addition to the tailoring of the NPs and the conjugation techniques, allows better charge transfer efficiency upon oxidation of the hydrolysis product thiocholine. This led to increased sensitivity to anti-ChEs in electrochemical sensors as compared with cholinesterase deposition on bare electrodes. For example, AChE conjugated to CdS or gold NPs showed sensitivity to 10^{-5} M BW284c51 and 5×10^{-10} M of trichlorfon or 7.6×10^{-6} M of methyl-parathion, respectively (Figure 52.3B and C; Pardo-Yissar et al., 2003; Periasamy et al., 2009). More complex systems combining several types of NPs on the electrode were also examined. For example, CdTe-AuNPs-chitosan microspheres-GCEs showed sensitivity to 1.34×10^{-9} M of monocrotophos (Periasamy et al., 2009).

In a different approach, the reduction properties of thiocholine were used to grow gold NPs on an AChE-chitosan-gold electrode using $HAuCl_4$ as a precursor. The growth of gold NPs upon substrate hydrolysis and its inhibition by anti-ChEs resulted in changes in cyclic voltammetry showing sensitivity to 6×10^{-11} M of malathion (Periasamy et al., 2009).

It is noteworthy that the high sensitivity of anti-ChEs detection in methods utilizing thiocholine also has some problems. For example, the thiol group has high affinity to metals such as gold and cadmium, which can affect the measurements. One suggested way to overcome this issue is to quantify anti-ChE concentrations without the use of thiolated substrates. This was achieved in a three-step procedure. First, phosphorylated cholinesterases were bound to ZrO_2 NPs deposited on an electrode due to specific interaction of the ZrO_2 with the phosphoric group. Second, cadmium-based NPs conjugated to antibodies for cholinesterases were added to the solution for binding the cholinesterases. Third, electrochemical stripping analysis of the cadmium after acid dissolution allowed the quantification of the OPs. Using this method, sensitivity levels of 8×10^{-12} M for phosphorylated AChE and 3×10^{-11} M for phosphorylated BuChE was achieved (Periasamy et al., 2009; Lu et al., 2011). In a similar method, phosphorylated cholinesterases were first mixed with magnetic-particle-antiphosphoserine polyclonal antibodies for magnetic separation, and then cadmium-based NPs conjugated to antibodies against cholinesterases were used. Using this approach, sensitivity to phosphorylated AChE and BChE of the order of 10^{-11} M was achieved (Zhang et al., 2013).

These conjugated cholinesterase systems are stable for long periods of time since the bound materials stabilize the cholinesterases and protect them against proteases and harsh environmental conditions. However, the conjugation procedure might damage the enzymatic activity (Waiskopf et al., 2013) and irreversible inhibition of cholinesterases activity upon use can prevent reuse of these sensors. Hence, different NP-based assays with nonconjugated cholinesterases were also developed. For example, colorimetric assays based on growth or aggregation of gold NPs upon substrate hydrolysis showed better detection limits than 10^{-10} M of anti-ChE concentrations (Periasamy et al., 2009; Wang et al., 2009; Sun et al., 2011; Wang et al., 2013).

Fluorometric assays emerge as an additional approach, in which different chain reactions starting from substrate hydrolysis and ending with change in semiconductor NP fluorescence may be used to detect anti-ChEs (Gill et al., 2008; Chen et al., 2013; Meng et al., 2013). An example for such a chain reaction can be seen in Figures 52.3D and E. The chain reaction starts with choline, the product of acetylcholine and butyrylcholine hydrolysis, which is used by choline oxidase (ChOx) as a substrate. ChOx oxidizes choline to betaine while generating hydrogen peroxide (H_2O_2). Then H_2O_2 quenches the fluorescence of quantum dots, allowing the detection of cholinesterase activities with sensitivity limits of 4.49×10^{-9} M to dichlorvos (Gill et al., 2008; Chen et al., 2013; Meng et al., 2013). This chain reaction has also been used in an electrochemical sensor sensitive to H_2O_2 formation for the detection of anti-ChEs. For example, screen-printed graphite electrodes modified with γ-MnO_2 nanostructures that show electrocatalytic activity toward the oxidation of H_2O_2 presented sensitivity up to 5×10^{-11} M of chlorpyrifos (Dontsova et al., 2011). More recently, this screen-printed graphite electrode-MnO_2 NPs system was also found to be sensitive to thiocholine, achieving sensitivity limits of 6×10^{-10} M to diazinon (Eremenko et al., 2012).

An additional novel approach to detect cholinesterase inhibitors is based on field effect transistor technology, with sensitivity to change in local pH or in protein structure upon ACh hydrolysis. An ion-sensitive field effect transistor with AChE bound to its gate showed sensitivity of up to 10^{-5} M of ACh and the ability to detect physostigmine concentrations in the range of 10^{-4} to 10^{-7} M, based on the decrease in the recorded current in comparison to the response without the inhibitor (Hai et al., 2006). In another study, AChE was bound to a floating gate-derived field effect transistor, with a response range of 10^{-2} to 10^{-9} M ACh and achieved a sensitivity to 10^{-8} M physostigmine (Goykhman et al., 2009).

Generally, few limitations for these systems have been explored in the literature, including damaging of the sensor by any factor that will affect the enzymatic activity or other reactions used in the method and the inability to recognize the inhibitor using such cholinesterase-based sensor. Therefore, in addition to cholinesterase-based sensors, many other sensors and methods have been developed and used to detect anti-ChEs in the past, each with its own advantages and disadvantages (reviewed by Kim et al., 2011). Anti-ChE detection methods thus should be tailored to the specific anti-ChEs and the requirements from the sensor.

Recombinant Cholinesterases as Therapeutic Agents

Current medical intervention in the case of acute exposure to anti-ChE agents includes the use of the muscarinic receptor antagonist atropine to block overstimulation, and oximes to reactivate OP-modified cholinesterases (Worek and Thiermann, 2013). However, these conventional treatments have limited effectiveness and may involve serious short- and long-term side effects (Worek and Thiermann, 2013). In fact, the routine treatments, while successfully decreasing anti-ChE-induced lethality, rarely alleviate post-exposure toxicity

and result in significant performance deficits and even permanent brain damage (Masson, 2011).

The shortcomings of the pharmacological approach to the problem prompted the development of an alternative approach (namely, the use of cholinesterases as anti-ChE-binding stoichiometric bioscavengers (Masson, 2011)). This approach is considered one of the inherent protection layers in which BChE acts as a natural scavenger of OP nerve agents and pesticides (Rosenberg et al., 2013). Several strategies for production of cholinesterases have recently been evaluated, including purification from outdated blood-banked human plasma (Lenz et al., 2005), from mammalian cell cultures, from the milk of transgenic goats (Podoly et al., 2009) or from transgenic plants (Evron et al., 2007; Geyer et al., 2010).

Administration of exogenous cholinesterases can provide protection against nerve agents, as was demonstrated in a variety of rodents (Lenz et al., 2005) and in primates (Rosenberg et al., 2013). Considerable success was achieved with polyethylene-glycol-conjugated recombinant BChE as an OP bioscavenger (Rosenberg et al., 2013). Nonetheless, this therapeutic approach depends on the availability of large amounts of recombinant human enzymes, which are required in stoichiometric rather than catalytic quantities. Indeed, the 1:1 stoichiometry necessary for anti-ChE bioscavenging by ChEs, stemming from the virtual irreversibility of the interaction of the enzymes with the inhibitors, is one of the major limitations of this approach. Co-administration of oximes to enhance regeneration of the bioscavengers after OP exposure is only partially effective (and it is not effective at all against some nerve agents, such as tabun and soman) and the chemistry involved in the process results in highly reactive and toxic phosphyloximes (Worek and Thiermann, 2013).

The effects of treating mice with plant-derived AChE-R in conjunction with their exposure to OPs were also tested (Evron et al., 2007). Interestingly, by 10 days post-exposure, AChE-R prophylaxis ameliorated the chronic effects of OP toxicity by mitigating the long-term up-regulation of *ACHE* gene expression; this treatment reduced plasma murine AChE-R levels and limited the NMJ dysmorphology. However, in addition to its capacity to restore the cholinergic balance immediately after acute intoxication, prolonged overproduction of AChE-R is associated with the overproduction of pro-inflammatory cytokines (Grisaru et al., 2006), behavioral impairments (Birikh et al., 2003), declarative memory loss (Nijholt et al., 2004; Farchi et al., 2007), muscle malfunctioning (Brenner et al., 2003), and excessive myelopoiesis (Pick et al., 2006; Gilboa-Geffen et al., 2007). Hence, pretreatment with the enzyme would predictably cause a transient elevation of inflammation markers. Nevertheless, the relatively rapid clearance of the plant-derived AChE-R_{ER}, which makes it transient, would limit the duration of such effects. Moreover, interference with the overproduction of endogenous AChE-R in the circulation should further limit the duration of such symptoms, avoiding much of the delayed post-poisoning phenotype, which is due to exposure-induced AChE-R accumulation. These findings highlight the advantages of plant-produced AChE-R_{ER} for both short- and long-term protection.

By utilizing AChE-R, a known positive modulator of inflammation, we have also demonstrated a means of counteracting the immunosuppressive actions of nicotinic AChR stimulation. AChE-R has been shown to induce a pro-inflammatory cytokine response, as well as an enhanced cellular response. We demonstrated that the effects of OP-induced immunosuppression in skeletal muscle are reversed following treatment with AChE-R.

Thus, the cholinesterases can be used not only as stoichiometric bioscavengers, but also as therapeutic proteins that are capable of preventing both the acute presentation and chronic dysmorphology and immunosuppression of OP toxicity.

Catalytic Bioscavengers

An ideal bioscavenger should be broad-acting, avoid aging, and present satisfactory reactivation kinetics; i.e., it should be catalytically active. Several enzymes from diverse organisms, ranging from bacteria to invertebrates and vertebrates, are known to hydrolyze anti-ChE compounds and their utility as catalytic bioscavengers of nerve agents and pesticides does not go unnoticed. The genetics and structure of these enzymes are diverse, but despite this, they are classed all together as OP hydrolases or as phosphotriesterases (Wales and Reeves, 2012). The most broadly active members of this artificial grouping are the enzymes from *Pseudomonas diminuta* and *Flavobacterium* sp.; their study yielded valuable structural and functional insights, but their utility for treating humans is questionable because of their bacterial origin (Wales and Reeves, 2012). On the other hand, mammals have several serum enzymes that reveal low, but significant, hydrolyzing activity toward OPs. These include not only paraoxonase (PON), but also members of the α/β-fold hydrolase family, particularly BChE and carboxylesterase (CarbE; Li et al., 2005).

Of the three members of the PON family, the most studied, and hence best known, is PON1. The genes encoding for the various PONs are more than 60% identical, and all three are mapped to nearby loci on the long arm of chromosome 7 (Bourquard et al., 2008), and, interestingly, only 5.5 Mb away from the *ACHE* locus (Sklan et al., 2004). Of the three, PON1 and PON3 are serum enzymes associated with high-density lipoprotein, whereas PON2 is expressed in many tissues (Bourquard et al., 2008). The three PONs can hydrolyze a variety of

esters and lactones, but despite their name, weak OP hydrolyzing activity is associated only with PON1 (Harel et al., 2004; Bourquard et al., 2008). Although the precise physiological roles of PONs remain elusive, epidemiogenomic studies, *in vitro* biochemical experiments, and *in vivo* experiments with knockout mice, build a compelling case for the involvement of at least PON1 in protection from atherosclerosis (Aviram and Vaya, 2013; Shenhar-Tsarfaty et al., 2013). At the same time, similar lines of evidence point to a much broader role in protection, especially neuroprotection. PON1, true to its name, is an OP hydrolase, but its hydrolytic capacity is quite promiscuous. Levels of serum PON activity, on the one hand, correlate well with polymorphisms in the *PON1* locus and, on the other hand, correlate with sensitivity to anti-ChE pesticides (Costa et al., 2013).

Based on biochemical data but mainly on the crystal structure of recombinant PON1 (Harel et al., 2004), Tawfik and coworkers offered a model for the architecture and function of the lactonase active sites of the PON enzymes (Khersonsky and Tawfik, 2006). PON has a six-bladed, β-propeller structure, with two calcium ions located in the central tunnel. One is buried and acts as a structural Ca^{2+}, whereas the second is catalytic Ca^{2+}, which is solvent-exposed and functions as a stabilizer of negatively charged intermediate products. The PON enzymes also have two His residues, the so-called His^{115}-His^{134} dyad, which mediates the lactonase and esterase activities of the enzymes. His^{115} acts as a general base to activate a water molecule attacking the carbonyl oxygen of the substrate and His^{134} activates His^{115} by a proton relay mechanism (Khersonsky and Tawfik, 2006). While the His dyad model is consistent with other data and nicely explains both lactonase activity and PON1's anti-atherosclerotic function, it fails to describe the phosphotriesterase activity of the enzyme. Nonetheless, molecular evolution experiments from the Tawfik laboratory, together with site-directed mutagenesis by that group and others, indicate that while the lactonase and OP hydrolase active sites are partially overlapping, substrates are oriented differently so that they face different chemically active residues (Khersonsky and Tawfik, 2006). Several mutations specifically increase the OP hydrolase activity. For example, H134Q increases it threefold to sixfold relative to WT, while significantly reducing the lactonase activity (Harel et al., 2004; Khersonsky and Tawfik, 2006). Even more dramatic, using directed evolution of PON1, Tawfik and coworkers achieved 340-fold increased catalytic efficiency in the hydrolysis of G-type nerve agents in comparison to WT-like recombinant PON1 (Gupta et al., 2011; Goldsmith et al., 2012).

It is noteworthy that the two enzymes that have thus far have been shown to provide the best protection against OP poisoning, AChE and BChE, belong to the α/β-fold hydrolase family. They bind OP anti-ChEs very efficiently, but the phosphorylated enzymes fail to reactivate. However, certain mutants of human BChE were shown to reactivate considerably faster, effectively making them OP hydrolases (Geyer et al., 2010). Unfortunately, although significant improvements were achieved, the process is still very inefficient, especially with the soman-inhibited enzyme, which is rapidly undergoing aging. In this context, it is interesting to note that murine BChE, but not its human counterpart, can spontaneously reactivate (Li et al., 2005). CarbE is another member of the same family, with broader catalytic activities and sensitive to OPs. CarbE can self-activate, and it is speculated that His residue within the sequence WI**H**GGGL plays a role in this process. The corresponding sequence of BChE (and AChE) is WI**Y**GGGF. One of the OP-hydrolase-enhancing mutations, G117H of BChE, is in the same region (WIYGG**H**F). (Note: the bold letters refer to changes in the amino acid sequence.) But unlike PON1 and the ChE enzymes, CarbE is not normally found in human serum (Li et al., 2005). The OP-hydrolyzing activities associated with native CarbEs, murine BChE, and recombinant human BChE raise the possibility of evolving these enzymes into more efficient phosphotriesterases.

Selective RNA-Targeted Suppression of AChE-R Overexpression Effects

The idea that anti-ChEs intoxication leads to prolonged overexpression of AChE-R, resulting in deleterious consequences, called for in-depth research. The effects of excess AChE-R in neuromuscular pathologies could be alleviated by small molecule inhibitors, as in MG; however, their effects were short-lived. This could be due to indiscriminate inhibition of both AChE-S and AChE-R, which would induce general increases in ACh and subsequent feedback overproduction of yet more AChE-R, exacerbating muscle weakness. Supporting this notion, MG patients, with elevated AChE-R levels, typically require oral drug administration every 4–6 h, and up to every 3 h in more refractory cases (Ropper and Brown, 2005). Antisense oligonucleotide suppression of nascent AChE-R mRNA transcripts emerged as an effective means for challenging the hypothesis that AChE-R was causally involved with these symptoms, as well as a promising therapeutic alternative. Known by the names *AS3*, *EN101*, *Monarsen*, and *BL-7040*, the AChE mRNA-targeted agent is a 20-residue-long antisense molecule, 2′-oxymethylated at its 3′-terminal position (Angelini et al., 2013). It is targeted at exon 2 of AChE mRNA, which is common to the different AChE mRNA splice variants (Soreq and Seidman, 2001; Meshorer and Soreq, 2006). Nevertheless, the nascent, relatively unstable AChE-R transcripts

demonstrated particular sensitivity to the antisense treatment compared to AChE-S mRNA. This was the case in cultured primary brain cells (Meshorer et al., 2002), live mice (Cohen et al., 2002; Birikh et al., 2003; Nijholt et al., 2004), and human-originated cell lines (Perry et al., 2004). Antisense suppression of AChE-R was also shown in EAMG rat muscles and in monkey spinal cord neurons, leaving AChE-S almost unaffected (Brenner et al., 2003; Angelini et al., 2013). Moreover, anti-ChEs induce a feedback response and up-regulation of the AChE-R variant, suggesting that they would actually exacerbate AChE-R overexpression in myasthenic muscles, perhaps explaining their short-lived effect. In contrast, antisense treatment should not activate this feedback loop, providing yet another added value to its use.

In muscle, AChE's catalytic activity increased 2 weeks after repeated DFP exposure for 4 consecutive days. This increase reflected the overexpressed AChE-R and could be significantly reduced by systemic antisense treatment. Antisense treatment brought down AChE's catalytic activity to the range of control animals (Lev-Lehman et al., 2000). Importantly, this treatment further prevented the increase in NMJ endplate density, supporting the notion that these plastic changes were also attributed to AChE-R. The muscle weakness, characteristic of AChE-R excess, resembles the reported weakness syndrome that follows continuous or repeated exposure to agricultural anti-ChE pesticides (Ross et al., 2013). Thus, excess AChE-R may be pivotal for rapid regaining of homeostasis following OP exposure, but its long-term accumulation entails multiple delayed damages.

The successful antisense treatment in animal models led to clinical trials in human MG patients. The agent is provided in a single daily oral dose, followed by measurable improvement in muscle functioning (Angelini et al., 2013). The reported effect lasts over 24 h, with a 100-fold lower required molar dose than that of pyridostigmine (Sussman et al., 2012). Thus, the antisense treatment seems promising for intoxication-induced effects of AChE-R overexpression.

Antagomir-Mediated Suppression of the AChE Targeting miRNA-132

Of note, OP poisoning victims show prolonged suppression of serum AChE activity that was attributed until recently to persistent exposure of the blood enzyme to the OP poison. Recently, we discovered that the post-exposure decline in AChE activity reflects drastic increases in the levels of miRNA-132, a natural regulator of AChE, and that conditional increase in miRNA-132 levels in engineered mice causes multiple brain and body pathologies. These findings offer a new explanation for the post-exposure decline in AChE activity and call for developing a new treatment strategy. We tested the feasibility of prophylactic protection against OP poisoning by injecting a chemically protected antisense oligonucleotide (AM132) targeted against miRNA-132 to mice exposed to increasingly lethal doses of the anti-ChE OP insecticide metabolite paraoxon. Muscle miRNA-132 levels were drastically reduced post-treatment; miRNA-132 increases were avoided, and treated mice exhibited fewer poisoning symptoms, faster recovery, and extended survival time compared to controls. Furthermore, 1 week after poisoning, treated mice demonstrated reduced anxiety reactions (in an elevated plus maze) and better navigation memory performance (in a Morris water maze). Taken together, these findings suggest that the post-poisoning decline in AChE activity, and possibly many of the delayed pathologies, are due to increases in miRNA-132, further suggesting that post-exposure treatment with AM132 may avoid such detrimental consequences (Ofek et al., 2011).

CONCLUDING REMARKS

Throughout this chapter, we have explored the current thinking and experimental data in support of the principal roles that cholinesterases play in maintaining synaptic and systemic cholinergic homeostasis. This view puts the various protein members of the cholinesterase family, especially the readthrough variant of AChE, at a crucial (and therefore vulnerable) intersection between the CNS and PNS and other body networks, such as the hematopoietic and immune systems. We presented an intricately balanced picture consisting of epigenetic, transcriptional, posttranscriptional, and posttranslational regulation. Aiming at mitigating the short- and long-term consequences of exposure to chemical stressors that interfere with cholinergic neurotransmission indeed requires systemwide actions. Through gain and loss of function experimental approaches and the use of various *in vivo*, *ex vivo*, and *in vitro* systems, a more coherent picture is beginning to emerge. This allows the development of both novel cholinesterase-based sensors to detect anti-ChE, and novel therapeutic strategies relying on enzyme supplementation in combination with RNAi agents to restore a healthy cholinergic status by carefully manipulating AChE levels to ameliorate the chronic effects of anti-ChE toxicity.

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SECTION V

RISKS TO ANIMALS AND WILDLIFE

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CHAPTER

53

Potential Agents That Can Cause Contamination of Animal Feedstuffs and Terror

Robert.W. Coppock and Margitta Dziwenka

INTRODUCTION

Terror Objectives

Foodstuffs and feedstuffs (intended for human and animal consumption, respectively) are vulnerable to terrorist attacks and acts of crime. A terrorist attack is intended to cause injury, death, and economic loss to civilian populations (WHO, 2002). The organizations and individuals that commit acts of terror generally have multiple objectives (Carus, 2001). These motives include revenge, creation of panic and disorder, intimidation of the target population and governments, alteration of the form of governance, influence over government policy, toppling political leaders, and challenging economic stability. Criminal acts generally are committed for monetary gain, revenge, or a personal social–psychological agenda (Carus, 2001). Agri-terrorism and agri-crimes can target specific agricultural industries. Defense against agri-terrorism and agri-crimes requires diversion of resources that could otherwise be used for economic growth. Emergency preparedness must include infrastructure and professional personnel for all events and hazards inclusive of terrorism and criminal acts against the livestock, poultry, and companion animals and the animal-to-human food web. Timely decision-making is essential in determining if disease in livestock is attributable to exposure to chemical–physical agents or infectious agents (Neher, 1999; Kosal and Anderson, 2004). Most preparedness plans give disproportionate priority to infectious agents (WHO, 2002; Kosal and Anderson, 2004). Toxicologic focus of the investigation often lags until the likelihood of infectious etiology is considered low. Laboratory information that provides timely holistic scope and etiology of the incident is essential for timely decision-making (Crutchley et al., 2007). Laboratory capacity and expertise are essential for identification of chemical and physical agents. The required infrastructure for thorough emergency preparedness includes full-service government-subsidized diagnostic laboratories for animals. Acts committed by terrorist and criminal perpetrators can overwhelm laboratory, regulatory, public health, and other resources (WHO, 2002; Kosal and Anderson, 2004). Well-rehearsed multiple-hazard protocols to deal holistically with an attack on food-animal production and supply chain are essential for emergency preparedness and the coordinated prompt response. National defense organizations are keen to identify and separate terrorists with political and devout agendas from criminal lawbreakers (Carus, 2001).

A terrorist attack targeted at feedstuffs could have a remarkable impact on the feed and livestock industries and human health. Large handling and manufacturing facilities provide opportunities for point source act-of-terror–linked adulteration of feedstuffs that result in wide distribution of chemical and physical agents (Kosal and Anderson, 2004). The World Health Organization (WHO, 2002) believes that a local terrorist attack on food could have global impacts. Specific industries and livestock sectors can be targeted with significant economic losses (Crutchley et al., 2007). Terrorist attacks can also indirectly affect feedstuffs with collateral damage. For example, fallout from a dirty bomb can contaminate forages, cereals, and other feedstuffs, and emissions from a fire can be deposited over a large area. Consuming contaminated edible animal products exposes humans to acute, subacute, and chromic effects of a toxic substance. Persistent organic pollutants (POPs) can be

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bioconcentrated and likely have multigenerational effects in humans (Blanck et al., 2000). Companion animals can be exposed through contaminated animal products being incorporated into pet foods (Brown et al., 2007). Attacks on companion animal feedstuffs resulting in illness and deaths in pets can have considerable emotional impacts on a community. The melamine–cyanuric acid contamination of pet food showed that a combination of adulterants caused sickness (Puschner et al., 2007).

Agricultural Food Ecosystem and Terror

A brief review of the agri-food ecosystem is important in the discussion of terrorist-linked toxicology of animal feedstuffs. Generally, the activities and technologies in the agro-ecosystem are maximized for human control and economic gain. All aspects of food production in the agro-ecosystem, including input supplements, chemicals, and fertilizers, can be the targets for terrorists. Harmful agents and organisms can be added directly or indirectly to feedstuffs. The vulnerability of animal feeds can be illustrated by the large-scale toxicological incidents inclusive of chemical residues in animal products that have been reported. Feed contamination incidents have important economic and human health consequences (Carter, 1976; Lok and Powell, 2000; Kosal and Anderson, 2004). There is likely an under-reporting of occurrences of feedstuffs-linked animal intoxications and there is generally fragmentary governmental infrastructure to collect, process, and use veterinary toxicoepidemiological data (Guitart et al., 2010).

Upsetting the Margins Between Safe and Unsafe Practices

The production of cereal grains, oilseeds, other commodities, and forage for the human food web essentially occurs in a manmade agroecosystem (Coppock and Jacobsen, 2009). Activities in the agroecosystem include seeding, growing, harvesting, storing, and transportation of feedstuffs and foodstuffs. The agronomic ecosystem is driven by economic decisions that generally maximize profitability and minimize labor and input costs. This ecosystem is dependent on management decisions that ensure all activities are performed in a timely manner, input costs are reduced, and all operations are conducted in a manner that generally ensures the quality and safety of foodstuffs and feedstuffs. The margin between safe and unsafe practices is generally close. The time-sensitive activities include seeding, watering, fertilization, weed and other pest control, harvest timing and methods, preservation, storage, and shipping. All aspects of the system require that chemical contamination of feedstuffs does not occur.

The agroecosystem is essentially unprotected and is vulnerable to attacks by terrorists. These attacks may not be directly focused on the agroecosystem, but they can indirectly narrow the margins between safe and unsafe practices. For example, the tactics used by terrorists can have huge impacts on foodstuffs and feedstuffs and can be as simple as, for example, disrupting the distribution of electrical and fossil fuel energy, availability of water, or targeting industries and infrastructure serving the agricultural sectors. A remarkable reduction in available and affordable energy can immediately alter practices traditionally used to harvest and preserve feedstuffs and increase the risk of growth of toxigenic fungi. Disruption of water availability can delay all of the input processes and increase the susceptibility of a crop to infection with mycotoxigenic fungi. The agroecosystem food web also includes manufacturing of feedstuffs and foodstuffs. Food-producing animals in the agroecosystem are vulnerable to heat-stable toxins and persistent chemicals that are bioconcentrated and relayed to humans in edible animal products. Animal by-products containing persistent chemicals can be used in food-animal feedstuffs and petfoods.

Examples exist when large incidents of animal intoxication occurred because of upsets in the agroecosystem and because of human error wherein chemical contamination not linked to terror had economic impacts (Kojima and Fujita, 1973; Kay, 1977; Fries, 1985; Coppock et al., 1988; Kosal and Anderson, 2004; Dorea, 2006; Burns, 2007). These incidents have long-term impacts on human health (Kojima and Fujita, 1973; Henderson et al., 1995; Blanck et al., 2000; Kosal and Anderson, 2004). Chemical-induced illness at the local level can have a significant impact on the health system, challenge the ability of government agencies to understand the scope of the incident, and strain intragovernmental relations (Kojima and Fujita, 1973; Green et al., 1987; Lok and Powell, 2000; Kosal and Anderson, 2004).

MYCOTOXINS AND TOXIGENIC FUNGI

Background

The alleged use of mycotoxins as a chemical weapon is a controversial subject (Tucker, 2001; Katz and Singer, 2007). Mycotoxins and toxigenic fungi can be used for terrorist attacks on both feedstuffs and foodstuffs (Scholthof, 2003; Tucker, 2003; Shannon, 2004; Klassen-Fischer, 2006). Veterinary and human medical professionals in North America do not commonly consider mycotoxicoses as disease entities in a differential diagnosis. In addition to low consideration as the etiology of disease, the clinical presentation caused by mycotoxins can easily be diagnosed as being caused by other

etiologies (Paterson, 2006). Thus, the delay in the identification of mycotoxins as the causal agent(s) could occur over a considerable period of time. Delay in recognition can increase the numbers of animals and humans intoxicated. There is an example of large-scale human and animal exposure to mycotoxins because the agroecosystem was disrupted by war (Joffe, 1978). There are endemic exposures to mycotoxins in Africa because of drought, economic hardships, wars, and agricultural practices (Marasas, 2001; Gnonlonfin, et al., 2013). Mycotoxin contamination of foodstuffs and feedstuffs can be an indirect effect of terrorism and war because of disruption of agricultural practices. In addition to adverse effects on human and animal health, substantial additional economic hardship would likely occur concurrently because of trade issues.

Applications of Biotechnology

It should be assumed that the effective use of mycotoxins as terrorist weapons can be increased with the use of biotechnology. Development of fungi for bioterrorism has been reported to share technologies with the military development of fungi for use as weapons (Paterson, 2006). The genetics of mycotoxin production are being elucidated and this knowledge is assumed to result in reduction of health risks. It is well-known that the conditions for mycotoxin production are the presence of toxigenic fungi having favorable growing conditions that include temperature, substrate, and moisture content (Woloshuk and Shim, 2013). It is possible for aggressive strains of toxigenic fungi to be isolated or developed that produce mycotoxins at lower temperatures and moisture levels than would be considered the usual limits for a particular fungal species (Moretti et al., 2013; Subramaniam and Rampitsch, 2013). Also, fungal varieties can be genetically modified (GM) in a manner that would enhance toxin production by removing the environmentally activated "restrictors" of toxin production. The adaptation of mycotoxigenic fungi and mycotoxins in terrorist activities likely follows the developments in fungal genetic research and biotechnology.

Potential Use of Fungal Biocontrol Agents

Fungal biocontrol agents (FBCAs) could be used in terrorist activities. Some FBCAs have been developed using bioselection and some have been developed using GM technologies (Paterson, 2006). A number of the FBCAs produce mycotoxins, and known toxigenic fungi have been used to control unwanted vegetation. Studies have been performed using FBCAs in plants used to produce illicit drugs (Charudattan et al., 2011). The same technologies can be used to target crops used as feedstuffs in livestock and poultry production.

Economic Losses from Use of Mycotoxins as Weapons

The use of toxigenic fungi and mycotoxins for bioterrorism can have economic consequences. Increasing the environmental prevalence of aggressive strains of toxigenic fungi could increase the losses due to mycotoxins from $2.5 billion to some substantially higher number (CAST, 2003). These losses may be underestimated because Robens and Cardwell (2003, 2005) projected the loss attributable to aflatoxins alone at $1.5 billion per year without including secondary industry and trade losses. International commodity trading of dry cereal grains could be used as a method of disseminating the spores of GM aggressive toxigenic strains of a particular fungus. Once released into the environment, the GM fungi would be difficult to control.

Use of Mycotoxin Contaminated Feedstuffs

Mycotoxin-contaminated feedstuffs could be purchased at a bargain price and used as a terrorist tactic in the manufacture of a completed feed. For example, horse or swine feeds could be formulated with a high level of grain by-products that are known to contain mycotoxins. Field incidences of mycotoxicoses not linked to terrorist activities have been reported from the use of mycotoxin-contaminated by-products in formulating ruminant, horse, and swine feeds (Wilson et al., 1990; Osweiler et al., 1992; Riet-Correa et al., 2013). Pet foods have been contaminated with aflatoxins and they caused illness and deaths of companion animals in regional areas (Arnot et al., 2012; Wouters et al., 2013).

Residues in Edible Tissues

Food-producing animals can ingest mycotoxin-contaminated feedstuffs and mycotoxin residues can be present in edible animal products (Coppock and Dziwenka, 2014). The concern for residues of mycotoxins in edible animal products has primarily been focused on residues of aflatoxins (Coppock et al., 2012). Residues of some mycotoxins in animal products can be regulated. Incidents of aflatoxin-contaminated feedstuffs being fed to milk-producing animals and subsequent contamination of foodstuffs are a public concern (Prandini et al., 2009). In North America there has been a consolidation of the dairy herds producing large volumes of milk and fewer and larger feed suppliers. Bioterrorism using aflatoxins to contaminate feedstuffs could contaminate a large volume of dairy products with aflatoxin M_1.

Mycotoxicology

A discussion of mycotoxicology is beyond the scope of this chapter. For further details on mycotoxicology, the reader is referred to other recent publications (CAST, 2003; Gupta, 2012).

MICROBIAL TOXINS

Botulism Toxin

Background

Botulism neurotoxins (BoNTs) are potential terrorist weapons in foodstuffs and feedstuffs chains and are given priority because of the extreme potency of BoNT (Wein and Liu, 2005; Woudstra et al., 2013). BoNT can easily be produced at low cost as a preformed toxin. Botulism can occur by *Clostridium botulinum* growing *in vivo* in the gut and wounds causing toxicoinfection (examples are infant botulism and visceral botulism in domestic animals) (Anniballi et al., 2013). *C. botulinum* produces environmentally resistant spores and exposures to BoNT and *C. botulinum* spores are spread through oral and cutaneous routes. Preformed BoNT can be added directly to foodstuffs and feedstuffs. The *C. botulinum* spores or substances with high spore counts can be added to foodstuffs and feedstuffs and, if favorable conditions exist, then the BoNT can be preformed in foodstuffs and feedstuffs before consumption occurs. The *C. botulinum* spores in foodstuffs and feedstuffs can, under favorable conditions, trigger *in vivo* formation of BoNT by toxicoinfection. Clinical reports have shown that botulism occurs in livestock and some outbreaks can be associated with feedstuff containing animal carcasses (Bienvenu et al., 1990; Kinde et al., 1991; Braun et al., 2005; Otter et al., 2006). Type D botulism has been identified in cattle fed chicken litter, type C has been associated with ensiled chicken litter, and type B has been associated with forages (Long and Tauscher, 2006).

Mechanism of Action

BoNT, after uptake, undergoes biotransformation within the neuron and subsequently binds with synaptic vesicular proteins and blocks the release of acetylcholine from the presynaptic membrane (Osborne et al., 2007). Clinical signs of botulism are weakness, tremors, recumbency, laryngeal paresis, and other signs of nervous system dysfunction (Braun et al., 2005). Botulism toxins do not appear to be excreted in milk (Galey et al., 2000).

Potential Production and Use

Botulism toxins can be produced and incorporated into ingredients used in feedstuffs. The use of dead rodents or other animals can serve as a substrate for *C. botulinum*, providing anaerobic conditions exist (Galey et al., 2000). Contamination of feedstuffs, especially silage, with the carcasses of dead mice or other small animals could be a method of producing and disseminating botulism toxins. There is some evidence that insect larva take-up BoNT and are unaffected. Insect larvae in carrion contain BoNT (Hubalek and Halouzka, 1991). The growth and use of insect larvae for use in animal and poultry feeds could be a terror tactic for botulism. For further details on toxicity of botulism toxins, readers are referred to Chapter 28.

PLANT TOXINS

Background

Poisonous plants and their seeds could be used to adulterate feedstuffs (Keremidis et al., 2013). Seeds of poisonous plants have been incorporated into animal rations and have resulted in animal intoxication (Burrows and Tyrl, 2001). For example, grain screenings that contain high levels of toxic plant seeds could be distributed as potential feed ingredients. Toxic seeds can be directly introduced into animal rations.

Castor Beans (Ricin)

Background

Castor beans (*Ricinus communis* L.) are grown commercially for castor oil, which is sought-after for cosmetics, coatings, industrial, and automotive applications (Millard and LeClaire, 2008). There is interest in its use in biodiesel. There is approximately 1 tonne of residual cake produced for every 0.5–0.6 tonne of oil produced. Castor bean cake is sold as fertilizer, soil enhancers, and other uses; in some countries, it is used as a feed supplement. The castor bean contains a cocktail of toxins (Worbs et al., 2011). Ricin, a potent toxin in castor bean, is water-soluble and is easy to extract from castor bean meal. Ricin is soluble in water over a wide range of pH values. The ricin content of castor bean varies with the growing conditions and genetics of the plant (Millard and LeClaire, 2008; Worbs et al., 2011). A general guide is 1–2 mg of ricin per gram of ground castor seed, and levels can be as high as 20 mg of ricin per gram of seeds. Ricin can be 5% of the total protein in castor beans and is present in the waste mash after the oil has been extracted. The ricin content of the waste mash generally is 1–10% (w/w). Ricin should be considered relatively heat-stable at temperatures used for food processing (Audi et al., 2005; Jackson and Halbert, 2006). The seed husks of *R. communis* are also poisonous and the recognized tolerable level is 10 mg of seed husks per kilogram of feed, and a fatal dose in cattle is estimated

to be 0.5 mg/kg body mass (Alexander et al., 2008). The ricinine piperidine alkaloid, a toxin of lesser potency, is found in all parts of the castor plant and is heat-stable. Ricinine stimulates the central nervous system.

Ricin as a Weapon

Ricin has been used to commit murder and kill animals. It captures the interest of both terrorist organizations and those involved in protecting populations from terrorists. It is available in tonne quantities by using inexpensive technologies to extract it in crude form from castor seed meal, a by-product that has a low sale value (Shea and Gottron, 2013).

Toxicity and Mechanism of Action

The seed coat of the castor bean needs to be fractured for the ricin to be released (Burrows and Tyrl, 2001). Ricin in waste mash is biologically available. Most animal species including birds are sensitive to castor bean poisoning (Jensen and Allen, 1981; Mouser et al., 2007). The degree of toxicity depends on the dose (number of seeds) ingested and liberation of ricin by degradation of the seed coat by some form of mastication. The liberated ricin is absorbed from the gastrointestinal tract. The estimated lethal dose of castor bean seeds by species is given in Table 53.1; however, it is likely that the lethal dose for some species is lower than the doses found in the literature. Ruminants are considered to be more resistant to ricin (Alexander et al., 2008) than are horses and dogs. The most likely reason is degradation of ricin by microbes in the rumen. This phenomenon also suggests that the dose in neonatal ruminants would likely be substantially lower than for mature ruminants.

Ricin is a lectin-type globular glycoprotein and has A and B chains (Doan, 2004; Audi et al., 2005). Ricin is considered a type 2 ribosome-inactivating protein (RIP). The ricin B chain attaches to the surface of the plasma membrane at the galactose-containing glycoproteins, pits are formed, and ricin is internalized by endocytosis and forms an intracellular vesicle. The vesicles containing ricin coalesce and form endosomes. The cellular uptake of ricin is a slow process and this can explain the lag time between exposure and onset of clinical signs. The internalized ricin can dissociate from the endosomes and can be transported to lysosomes for degradation or the vesicles can be removed from the cell by exocytosis. Ricin can bleb off the endosome and return to the cell surface by vesicular and tubular structures, or it is transported to the Golgi apparatus. In the Golgi apparatus, the A chain undergoes retrograde transport to the endoplasmic reticulum (Audi et al., 2005). Cleavage of the A and B chains occurs in the endoplasmic reticulum. From the endoplasmic reticulum, the ricin A chain is translocated to the cytosol. Here, the ricin A chain catalytically inactivates ribosomes. The A chain is an enzymic polypeptide that catalyzes the N-glycosidic cleavage of adenine from 28S rRNA loupe in the 60S ribosomal subunit. The removal of adenine from 28S rRNA in the ribosome destroys its

TABLE 53.1 Sensitivity of Different Species to Castor Bean Intoxication[a]

Species	Lethal Castor Bean Mass per kg of Body Mass (mg Ricin/kg Body Mass)	Comment
Horse	7–240 mg (7 mg)	Considered the most sensitive species.
Human	225 mg (20)	Dose altered by the degree of mastication.
Duck	0.7–1.2 g	1/8 duck died at 16 days after treatment. Grinding of the seeds by the proventriculus can be a variable in toxicity.
Chicken	10–14 g	Grinding of the seeds by the proventriculus can be a variable in toxicity and values could be considerably lower for macerated seeds.
Geese	400 mg	Grinding of the seeds by the proventriculus can be variable in toxicity.
Rabbit	1.2 g	Mastication considered to be required.
Pig	1.2 g	Mastication is required. 0.5 g husks per kg body mass is also fatal.
Goat	5 g	Age of ruminant can be a factor as the rumen may inactivate ricin. Inactivation by the rumen and mastication of seeds are factors in toxicity.
Sheep	1–2 g	Age of ruminant can be a factor as the rumen may inactivate ricin. 1.4–2.8 mg ricin containing cake fed for 8 months and caused intestinal pathology.
Cattle	1–2 g	Age of ruminant can be a factor as the rumen likely inactivates ricin. Considered to be more resistant to intoxication than sheep.

Source: Burrows and Tyrl (2001).

[a]*It is likely that the lethal dose for some species is lower than the doses found in the literature.*

functionality and thereby blocks protein synthesis. One A chain can enzymatically inactivate 1,500 ribosomes/min. Using other mechanisms, ricin causes the release of inflammatory mediators. Ricinine is a central nervous system stimulant and is theorized to inhibit the postsynaptic ϒ-aminobutyric acid receptor subtype A and to alter the release of glutamate.

Analytical Methods

Analytical methods used to identify ricin include immune methods (ELISA) and liquid chromatography/mass spectrometry (LC/MS). Gastric contents can be assayed and ricin can be detected in blood and body fluids by radioimmunoassay and LC/MS (Darby et al., 2001; Mouser et al., 2007). Ricinine levels can be assayed in serum and urine using LC/MS (Røen et al., 2013). Polymerase chain reaction methods are available to detect the DNA in castor beans.

Clinical and Pathological Findings

Clinical signs of ricin intoxication are high morbidity, abdominal pain, emesis, diarrhea, and lethargy. Hematemesis and melena may be observed. Clinicopathology includes elevated hepatic enzymes in serum, hyperphosphatemia, and sometimes hypoglycemia. Electrolyte imbalance can occur because of diarrhea and emesis. Pathological findings in a dog were hepatocellular necrosis, fibrosis of central veins and sinusoids, and lymphocytic infiltrate (Mouser et al., 2007). Jejunal mucosa can be eroded and infiltrated with leukocytes, and the tunica muscularis can also be affected. Hemorrhage can be observed in the mucosal and muscular layers. Necrosis can be seen in the spleen. Renal pathology has also been reported.

Other Plant Source Type 2 RIPs

Abrin is a plant source type 2 RIP. It is found in *Abrus precatorius* (rosary pea, Indian licorice, jequirity bean). The toxicology of abrin is considered to be very similar to ricin. A similar *Abrus* toxin is pulchellin, produced by *Abrus pulchellus* (Millard and LeClaire, 2008). The rosary pea has been reported to be more toxic than castor beans (Griffiths et al., 1994). Like castor beans, the rosary pea must be masticated to release the toxins (Burrows and Tyrl, 2001). Species sensitivity is variable and horses are considered to be the most sensitive. The mature goat is considered to be a more resistant species, and seed in the form of 2 g/kg body weight is reported as a lethal dose. The reported lethal dose of seeds for the horse is approximately 100 mg/kg body mass and for cattle it is reported to be 600 mg/kg body weight. It is likely that abrin is denatured in the rumen.

There is limited commercial production of the jequirity bean. The rosary pea is grown as an ornamental plant and has escaped into the wild in the warmer climate in the United States. *Abrus* sp. is not grown as an oilseed or for other large-scale commercial use.

For further details about ricin and abrin, readers are referred to Chapter 27.

RAPIDLY ACTING AND EASILY AVAILABLE SUBSTANCES

Cyanide

Cyanide (CN^-) is considered a terrorist weapon (Cameron and Pate, 2001; Ballantyne and Salem, 2008; Wismer, 2012). Cyanide has a history of use in controlling problem wildlife and unwanted feral animals, and it has alleged use as an agricultural terror agent in livestock drinking water (Wiemeyer et al., 1986; Cameron and Pate, 2001). Two common forms of cyanide salts are sodium cyanide and potassium cyanide. Cyanide can be used to adulterate feedstuffs and water.

Mechanism of Action

The toxicology of CN^- is complex (Ballantyne and Salem, 2008; Wismer, 2012). The lethal effect of cyanide is blockage of cytochrome C oxidase, which results in loss of electron transfer, and molecular oxygen is not utilized. In addition to cytochrome C, other cytochromes are adversely affected by forming complexes with the cytochrome iron. The oxidized cytochrome–CN complexes are stable, but the complex is relatively unstable in a reductive environment. The instability of the cytochrome–CN^- complex in a reductive environment is the focus of the majority of treatment regimes.

Plant Sources—Ruminants

Certain plants contain cyanogenic glycosides (Nicholson, 2012). Cyanogenic plants and trees are common in some geographical regions; for local criminal action, these could be placed in pastures. Plant parts containing cyanogenic glycosides, precursors that form hydrogen cyanide (HCN) in the rumen, could be placed in ruminant rations. Birds can be poisoned by consuming seeds and berries from cyanogenic plants (Woldemeskel and Styer, 2010).

Treatment of Cyanide Poisoning

Treatment of cyanide poisoning has been discussed in detail by Wismer (2012) and Nicholson (2012), and in Chapter 23.

Insecticides

Large numbers of cattle and other domestic livestock have been contaminated and poisoned with insecticides that were inadvertently incorporated and incorporated

by criminal intent into feedstuffs (Neher, 1999; Guitart et al., 2010). Coordinated effort is required to diagnose, contain, and eliminate insecticide poisoned and contaminated animals from the food web. There have been instances in which the same transport vessels have been used to transport/store insecticides, seeding crops, and silage, resulting in cross-contamination. Terrorists, to adulterate animal feedstuffs, could use insecticides. The use of insecticides that are persistent organic compounds (POCs) for terror actions may also present a residue problem in the surviving animals. The toxicology and treatment of insecticides have been discussed in detail by Gupta (2006, 2012).

PERSISTENT ORGANIC COMPOUNDS

Background

POCs, also known as POPs, could be candidates for a terrorist attack on livestock. Most POCs are biomagnified in the food web. The economic and political impact from their use in a terrorist attack would likely be huge based on the impacts from reported inadvertent contaminations of feedstuffs and feed ingredients. The majority of the POCs are bioconcentrated in body lipids. Most of these compounds require high doses to cause acute illness in livestock, poultry, and fish. At low doses the resultant illness is generally less evident, and there are residues in edible animal products and rendered products used in animal feedstuffs. The occurrences of POCs contaminating feedstuffs and feed ingredients are underappreciated (Kim et al., 2007). The lack of specific clinical signs and lesions increases the risk of elusive diagnosis, animals passing slaughter inspections, and their products being consumed by humans (Carter, 1976; Fries, 1985; Hayward et al., 1999; Bernard et al., 2002; Dorea, 2006). For the POCs, the safety of foodstuffs and feedstuffs are reliant on chemical analyses (Kim et al., 2007). The occurrences of POCs being relayed from contaminated food-producing animals and from animal-source foods to humans are known (Kay, 1977; Reich, 1983; Fries, 1985; Bernard et al., 2002; Huwe and Smith, 2005; Dorea, 2006; Kim et al., 2007). A historical incident occurred in Michigan when a flame retardant consisting of polybrominated biphenyls (PBBs) was mixed into livestock feeds (Carter, 1976; Reich, 1983). The flame retardant was manufactured for use in thermoplastics and was shipped to the feed manufacturer in place of magnesium oxide. Environmental sources of POCs are also important in the agroecosystem (Stevens and Gerbec, 1988). Ball clay contaminated with polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs) in animal mineral mixes and other incidents clearly show that these events can occur over a substantial period of time before low-level contamination is recognized and foodstuffs and feedstuffs are protected (Reich, 1983; Bernard et al., 2002; Kim et al., 2007). Proactive analytical surveillance for POPs requires substantial resources consisting of highly trained personnel, sophisticated laboratory procedures, and infrastructure to meet laboratory safety requirements. The analytical and personnel support for investigations into POCs contaminating livestock feedstuffs can challenge budgets, and there can be bureaucratic pressure to restrict or delay investigations because of budgetary targets and political approvals (Reich, 1983; Bernard et al., 2002). Some of the highest levels of POPs in consumed foodstuffs have occurred because of feedstuffs being contaminated. The health impacts of POPs in the human diet are likely underappreciated and there is evidence to show the effects can be multigenerational (Blanck et al., 2000).

Potential Economics of Terror Attack Using POCs

The history of incidents clearly shows that feedstuffs contaminated with POCs have caused remarkable economic loss (Reich, 1983; Bernard et al., 2002). Most POCs are biomagnified in the food web. If feedstuffs have a wide distribution, then the measurable economic impacts can be immense. For example, with the ball clay incident from two soybean meal manufactures, it was estimated that 1.7 million eggs per day (at that time approximately 1% of the US egg production) and 35% of the farm-reared catfish were contaminated with PCDDs/DFs (Hayward et al., 1999). These animal-source foodstuffs, contaminated with PCDDs/DFs, were consumed by humans (Fiedler et al., 1997; Rappe et al., 1998). Michigan suffered one the largest animal feed contamination incidents in the history of the United States. Feedstuffs were contaminated with PBBs and more than 500 farms were under quarantine (Carter, 1976). More than 865 tons of feedstuffs, 29,800 head of cattle, 5,920 hogs, 1,470 sheep, 1.5 million chickens, 34,500 kg dairy products, and 5 million eggs were destroyed (Carter, 1976). The total cost was estimated in 1983 currency value at more than $215 million (Reich, 1983). In the United States, more than 400 upright silos were coated with a polychlorinated biphenyl (PCB) containing resin and livestock and their edible products were contaminated with PCBs (Willett et al., 1987). In Belgium, a tank of animal fat (animal feed ingredient) feed was contaminated in January 1999 with a PCB (congener closely matched Aroclor 1260/1254) containing PCDDs/DFs and caused an unprecedented feed and food crisis (Lok and Powell, 2000; Bernard et al., 2002). It is estimated that 150 kg of PCBs with PCDDS/DFs contaminated the recycled animal fat. Nine feed manufactures used the contaminated oil to formulate poultry and livestock feeds. There was a considerable lag time

for the government to deal with this issue inclusive of political elections. Several countries banned imports of animal source foodstuffs and products containing animal source ingredients. Estimates losses from this disaster are US $1.5 billion (Lok and Powell, 2000).

Human Exposures

Human populations have been exposed to POPs by consuming contaminated animal-source foodstuffs. The health effects from exposure of the human population to POPs are likely underappreciated. The POPs are passed *in utero* from mother to fetus and postnatally to the neonate via breast milk. There is some evidence that adverse multigenerational effects occur (Blanck et al., 2000). The timing of exposure in life stages, from embryo to senility, can be important in the expression of adverse effects.

HEAVY METALS AND METALLOIDS

Heavy metals and metalloids have been used to maliciously poison livestock.

Lead

Lead is readily available in the form of spent lead acid batteries and shotgun shot. Because lead has been removed from paint, incidents with lead-acid batteries, gunshot, and environmental sources account for the majority of cattle poisonings in North America. Lead is excreted in milk, deposited in bones, liver, and kidney, and is considered a food safety issue (Bischoff et al. 2014; Coppock et al., 1991). Lead poisoning is likely underdiagnosed and can be confused with thiamin-responsive polioencephalomalacia in cattle. The favorable clinical response to thiamine therapy could result in failure to diagnose plumbism. When clinical signs of lead poisoning are observed, appropriate specimens should be taken and submitted to the laboratory for lead analyses.

Lead in Feedstuffs

A small amount of lead can poison a large number of livestock. Lead is solubilized by silage fluids and migrates in the liquid phase of silage (Coppock et al., 1988; Galey et al., 1990; Bischoff et al., 2014). Lead shot could be maliciously added to livestock feedstuffs.

Treatment of Lead Poisoning

Lead poisoning is responsive to thiamine and chelation therapy (Coppock et al., 1991).

Arsenic

Arsenic is used to maliciously poison livestock. Arsenic is readily available and can be mixed with feedstuffs and water. Arsenic can be present in production water from oil and gas field activities. Arsenic can be used for crimes of revenge. In acute arsenic poisoning, survival is generally low.

Toxicology

Readers are referred to other books for discussions of the toxicology of heavy metals (Gupta, 2012); and chapter 15 in this book.

CONTAMINATED TRANSPORT VESSELS

Historically, occurrences of feedstuffs becoming contaminated in transport vessels were primarily because of the failure to clean the vessels after use. Failure to clean transport vessels or leaving substances to be commingled with animal feedstuffs could be an act of terrorism or criminal action that could cause significant contamination of animal feed ingredients that could have broad dissemination in animal feedstuffs and contaminate human foods.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The agro-ecosystem is essentially unprotected and vulnerable to terrorist criminal activity using chemical substances. Emphasis has been placed on identification of infectious agents as the cause of disease in livestock and poultry. Few intoxicating substances produce pathognomonic signs, clinicopathologic profiles, and lesions. A holistic approach is required for prompt identification of the cause of disease in livestock and prompt identification of the etiology of animal disease is dependent on a full-service diagnostic laboratory infrastructure. Farm gate biosecurity protocols are becoming more common, but the majority of these programs do not include protection of the agroecosystem from chemical threats. The use of chemicals that are acutely toxic can kill a large number of livestock. When sick and dead livestock have been observed, actions are taken to divert the animals from the food web. Animals poisoned with chemicals that cause less overt and subtle signs of intoxication can enter the human food web. Most of the POCs are biomagnified in the food web and generally cause chronic toxicity. Review of published incidents shows that considerable time is generally required for the etiology of chronic intoxication with POCs to be identified. During this interval, animal products have passed inspections and entered the human food web, and animal by-products have been incorporated into animal and poultry feeds. Economic losses occurring from POPs

contaminating livestock feedstuffs, when they occur, are generally huge. The impact of POPs on human health is likely underappreciated and there is mounting evidence these effects can be multigenerational. The load demand on laboratories and personnel can be substantial when widespread contamination of feedstuffs/foodstuffs has occurred and the toxic substance is known. There is a neglected need to include the use of chemicals as terror agents in infrastructure development and response protocols. Clever use of chemical mixtures for a terrorist attack on food could delay identification of the etiology and reproducing the syndrome; melamine–cyanuric acid in pet foods is a good example. A delay in definitively defining the syndrome would help the terrorists to achieve their objective of maximizing psychological, sociological, economic, and political impacts by preventing governments and corporations from ensuring the public that food is safe.

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CHAPTER

54

Chemical Warfare Agents and Risks to Animal Health

Tina Wismer

INTRODUCTION

Animals are susceptible to all four basic types of military agents: choking (such as chlorine gas and phosgene), blister (such as mustard, lewisite, and phosgene oxime), blood [such as cyanide and hydrogen cyanide (HCN)], and nerve agents [such as tabun, sarin, soman, and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX)]. They can also be affected by incapacitating agents such as 3-quinuclidinyl benzylate (BZ), riot control agents (RCAs), ricin, and abrin. Since chemical warfare agents (CWAs) can be deployed by a variety of inexact methods (including bombs, spray tanks, rockets, missiles, land mines, and artillery projectiles), domestic and wild animals living in proximity to human populations can be affected (USACHPPM, 2001a). Americans own 83.3 million dogs and 95.6 million cats and more than 62% of American households own at least one pet (American Pet Products Association, 2013–2014). Due to the expansion of suburbia, human–wildlife interactions are also increasing. Livestock are very important potential targets of attack as they have secondary ramifications for human health and disruption of the food chain.

The Centers for Disease Control and Prevention (CDC) Strategic Planning Working Group, as part of their preparedness plan for possible terrorist attacks using biological or chemical weapons, has called for "prompt diagnosis of unusual or suspicious health problems in animals" (Anonymous, 2000a). The CDC recommended establishing "criteria for investigation and evaluation of suspicious clusters of human and animal disease or injury and triggers for notifying law enforcement of suspected acts of chemical terrorism." With many of the military agents, there are few initial indicators of a chemical attack. It has been proposed that animals could serve as sentinels for chemical terrorism. These animals would be similar to the canaries used by coal miners in the United Kingdom and the United States to provide early warning of deadly mine gases.

Throughout history, it has been noted that during chemical warfare attacks, animals may also be affected. A newspaper article from World War I gives details of the effects on animals during an unspecified type of gas attack (Anonymous, 1918):

> Results show that horses suffer much from the noxious fumes, and are subsequently thrown into a state of nervous terror on again scenting them. Mules are more inclined to stand their ground, and appear as if trying not to breathe … cats quickly scent the gas, and run about howling. Guinea pigs are first to succumb … Rats and mice emerge from their holds, and are found dead in quantities, which as the soldiers say, is the only advantage of a gas attack by the enemy.

The same account discusses how much these animals meant to the soldiers, as they even managed to fashion gas masks for several of these species to protect them during gas attacks (Anonymous, 1918). The soldiers believed that different species of animals may have been more sensitive to certain types of gas attacks than humans:

> … Poultry of all kinds are useful for giving warning, ducks and fowl becoming agitated 10 min or so before the oncoming gas clouds. Many kinds of wild birds are greatly excited, and the usually unruffled owl becomes, as it were, half demented. Only the sparrow seems to disregard the poisonous vapor, and sparrows chirp on where horses are asphyxiated, and bees, butterflies, caterpillars, ants and beetles die off in great numbers. The gas at once kills snakes, and earthworms are found dead in their holes many inches below the ground.

Handbook of Toxicology of Chemical Warfare Agents.
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Not much has changed in using animals as sentinels over the years. Crates of rabbits were placed on the cargo deck of ships transporting nerve gases during World War II, and the crew were instructed to watch for sudden animal deaths that could signal a gas release (Brankowitz, 1987). Even today, with the development of sophisticated biosensor technology, the use of animals as sentinels continues to be explored (Paddle, 1996). After the Aum Shinrikyo sarin attack in Tokyo, Japanese policemen used canaries to serve as a warning of poison gas release (Biema, 1995). The United States also planned to use chickens during the initial invasion of Iraq in 2003 (Ember, 2003). The chickens were to be used as early warnings of nerve gas agents. It was thought that if caged chickens remained alive following a warning alert, it would be safe for soldiers to remove their gas masks. Using live animals would help avoid possible false alarms with ion-mobility spectrometry biosensors. The plan was never implemented, however, as there were no controlled studies showing that chickens were likely to show effects of nerve agents before humans (Garamone, 2003). The US Environmental Protection Agency (EPA) is now considering evidence regarding the use of animals as sentinels for chemical threats (EPA, 2006).

Some of the CWAs that may pose the greatest risks to animal health are described next. For information on their mechanism of action, readers are referred to Section II of this book.

CWAS

Chlorine Gas

Clinical Signs

Chlorine gas is very irritating, and in concentrated amounts, it can even be corrosive. The eyes, skin, nose, throat, and mucous membranes can all be affected. When inhaled or ingested, chlorine combines with tissue water and forms hydrochloric acid (HCl) and reactive oxygen species (ROS). Oxidation of respiratory epithelium leads to alveolar capillary congestion and accumulation of edematous fluid (Noe, 1963). Death is due to cardiac arrest from hypoxemia secondary to atelectasis, emphysema, and membrane formation (Decker, 1988).

Ocular exposure can result in severe pain and blindness. Erythema and pain are also common after dermal exposure. Both liquid and high concentrations of chlorine gas can cause dermal burns (Raffle et al., 1994). Inhaled chlorine gas causes rhinorrhea, ataxia, syncope, muscle weakness, dyspnea, tachypnea, bronchospasm, and acute lung injury. Inhalation of high concentrations can cause laryngospasm, tachycardia, and hypoxia (Noe, 1963). Cardiovascular collapse and respiratory arrest may develop and lead to rapid death.

Chlorine is teratogenic. When given to pregnant rats, 100 ppm of chlorine caused both biochemical and metabolic effects in the newborns, while 565 mg/kg given prior to mating was embryotoxic (RTECS, 2008). Carcinogenicity can be seen with chronic exposure (RTECS, 2008).

Kinetics

Respiratory, dermal, and ocular irritation starts immediately. The speed of onset and severity of signs is directly related to the concentration (Bingham et al., 2001). The water solubility of chlorine allows it to have a greater effect on the lower respiratory tract, as a large percentage bypasses the upper airways. Acute lung injury peaks in 12–24 h. Death usually occurs within 48 h (Decker, 1988).

With mild exposure, signs disappear within 6 h, but can continue for more than 24 h with severe exposures. Exposure to moderate or severe concentrations can result in chronic respiratory dysfunction (Decker, 1988). This can be career- or life-ending if the affected animal is an equine athlete or a working dog.

Decontamination and Treatment

Move animals into fresh air and onto higher ground. Monitor respiratory rates and oxygenation status (SpO_2). If coughing or dyspnea develops, provide supplemental oxygen and ventilation. Bronchodilators should be used to counteract bronchospasm (Wang et al., 2004). Sedation and pain control may be needed so that the animal can be handled safely.

Corticosteroid use is controversial. Animal models have shown positive results in hastening recovery from severe chlorine gas poisoning; however, administration to humans has not been shown to provide any significant change (Traub et al., 2002). Wang et al. (2004) demonstrated that pigs exposed to chlorine gas responded better to a combination of aerosolized bronchodilators and corticosteroids (terbutaline, budesonide) than to either therapy alone. Sheep nebulized with 4% sodium bicarbonate had decreased mortality and improved oxygenation after inhalation of chlorine gas (Chisholm et al., 1989).

For ocular exposure, the eyes should be flushed with generous amounts of tepid 0.9% saline or tap water for at least 15 min. After flushing, fluorescein should be used to stain the eyes and check for corneal ulcers (Grant and Schuman, 1993). Bathing with dish soap and water will remove chlorine from the skin and fur or feathers.

Chlorine does not leave an environmental residue, so animals may be returned to affected pastures within hours to days, and more quickly in warm environments (Munro et al., 1999). When entering the area contaminated with chlorine gas, rescuers should wear self-contained breathing apparatus (SCBA) and protective

clothing (i.e., gloves, gowns, and masks) until the gas dissipates. The risk for secondary contamination of rescuers is low. Chlorine gas does not bind to leather or fabrics.

Species Susceptibility

Chlorine gas is heavier than air and will settle in low areas. Pets and smaller livestock may be more at risk than humans due to their proximity to the ground. There are no controlled studies showing that animals are more sensitive to chlorine gas or that they will develop signs sooner than humans.

Phosgene

Clinical Signs

Most animal exposures to phosgene are from inhalation, but there can also be dermal exposures to the liquefied material. The severity of pulmonary injury correlates with the concentration and length of exposure, and initial symptoms are not always a good indicator of prognosis (Diller, 1985; Bingham et al., 2001). Due to low water solubility, phosgene is able to penetrate deeply into the lungs, where it irritates the lower respiratory tract (Franch and Hatch, 1986). When phosgene gas contacts water in the lungs, it forms HCl, causing cellular injury (Murdoch, 1993). Phosgene also causes protein and lipid denaturation and changes in membrane structure, leading to increased pulmonary vascular permeability (Borak and Diller, 2001). This fluid accumulation in the lung interstitium and alveoli results in gas diffusion abnormalities and pulmonary edema (Diller, 1985). Pulmonary injury is exacerbated by elevated levels of leukotrienes and neutrophil chemotactic agents, including intercellular adhesion molecule-1 (Zhang et al., 2010). The neutrophils release cytokines and other reactive mediators, worsening pulmonary injury (Sciuto et al., 1995). Localized emphysema and partial atelectasis occur. Death is from anoxia secondary to pulmonary edema (Borak and Diller, 2001; Proctor and Hughes, 2004).

Dyspnea, cough, cyanosis, and hemoptysis can progress to hypoxemia and hypoventilation (Borak and Diller, 2001). Animals may develop secondary organ damage from anoxia. Dogs experience bradycardia, followed by tachycardia and progressive hypotension with severe phosgene poisoning (Patt et al., 1946). With concentrations greater than 200 ppm, phosgene can enter the blood and cause hemolysis and coagulopathies (Sciuto et al., 2001). Direct contact with liquid phosgene can cause dermal burns (Proctor and Hughes, 2004). Ocular exposure to both liquid phosgene and highly concentrated phosgene gas can cause severe eye irritation and corneal opacification (Grant and Schuman, 1993; Proctor and Hughes, 2004).

Prognosis is directly related to the severity of pulmonary injury. Animals that survive the first 24–48 h still have a guarded prognosis. These animals are more susceptible to infectious agents, as they have suppressed natural killer cell activity. Infections may become evident 3–5 days after exposure. Animals may develop chronic exercise intolerance and abnormal pulmonary function (Borak and Diller, 2001). Working dogs and horses may no longer be able to fulfill their functions.

Kinetics

Respiratory signs develop 2–6 h postexposure in most patients, but can be delayed up to 15 h with exposures to lower concentrations (<3 ppm) (Borak and Diller, 2001). Concentrations of 3–5 ppm cause immediate conjunctivitis, rhinitis, pharyngitis, bronchitis, lacrimation, blepharospasm, and upper respiratory tract irritation. Extended (170-min) exposure was fatal (Diller 1985; Proctor and Hughes, 2004). Exposure to 50 ppm for 5 min or longer will cause pulmonary edema and rapid death (Chemstar, 1996; Borak and Diller, 2001; RTECS, 2008). If the animal survives, pulmonary edema begins to resolve in 2–3 days.

Decontamination and Treatment

Move animals to fresh air and higher ground. Bathe animals with soap and water and flush eyes for 15 min with tepid water or 0.9% saline. Animals should be monitored for 24 h for the development of pulmonary edema (Borak and Diller, 2001). If animals are coughing or dyspneic, administer 100% oxygen. Animals may need to be intubated and ventilated. They can be nebulized with beta-adrenergic agonists to combat bronchospasm. Patients with pulmonary edema should be managed the same as an acute respiratory distress syndrome (ARDS) patient (i.e., mechanical ventilation with oxygen and positive end-expiratory pressure).

Rabbits exposed to toxic levels of inhaled phosgene did not develop noncardiogenic pulmonary edema if given intravenous (IV) aminophylline and subcutaneous terbutaline within 10 min of exposure (Kennedy et al., 1989). Intertracheal *N*-acetylcysteine (Mucomyst) administered to rabbits 45–60 min after inhalation of phosgene (1500 ppm/min) decreased pulmonary edema, production of leukotrienes, and lipid peroxidation, and it also maintained normal glutathione levels compared to rabbits exposed only to phosgene (Sciuto et al., 1995). Rat and rabbit studies have shown that ibuprofen also protects against acute lung injury from phosgene (Guo et al., 1990). Oxygen, sodium bicarbonate, and aerosolized surfactant have all been shown to be beneficial in dog experiments (Mautone et al., 1985). Corticosteroids, prostaglandin E1, and atropine may be helpful in reducing phosgene-induced pulmonary edema (Chemstar, 1996). Pentoxifylline has been shown

to inhibit intercellular adhesion molecule-1, which not only decreases migration, accumulation, and activation of neutrophils, but also decreases the synthesis of other pro-inflammatory cytokines (Zhang et al., 2010). Intravenous fluids should be used for cardiovascular support, but monitor for overhydration. Colloids are preferred over crystalloids, as they will remain in the vascular space for a longer period of time. Oxygen supplementation will resolve most of the arrhythmia.

Phosgene is nonpersistent in the environment. Moisture reduces air concentrations (Borak and Diller, 2001). The potential for secondary contamination of rescue personnel is low, but rescuers should wear proper protective clothing. Phosgene gas does not persist in fabric or leather.

Species Susceptibility

Phosgene is heavier than air and will settle close to the ground. This can affect species that are low to the ground or that are pastured in low-lying areas. There is no indication that animals are affected before or at lower levels than their human counterparts.

Mustard Gas

Clinical Signs

Mustard gas is a vesicant that is toxic by all routes of exposure (oral, inhalation, dermal, and ocular) (Sidell et al., 1997; Pohanish, 2002). Mustard causes both localized and systemic cellular damage, and tissues with high cell turnover are the most affected (NATO, 1973). Mustard gas can produce erythema, severe pruritus, blistering, ulceration, and necrosis of exposed skin (Borak and Sidell, 1992; Dacre and Goldman, 1996; Budavari, 2000; Pohanish, 2002). Skin lesions are most severe at warm and moist sites (genitalia, perineal regions, groin, skin folds, and axillae), due to the high number of sweat glands in these areas. Amounts as low as 0.02 mg of mustard will cause blisters (Smith et al., 1997).

Ocular exposure can cause pain, lacrimation, corneal ulceration, swelling, blepharospasm, and blindness, as the eyes are very sensitive to mustard gas (NATO, 1973; Borak and Sidell, 1992; Dacre and Goldman, 1996; Garigan, 1996). Pathognomonic signs of mustard gas poisoning include porcelain-white areas in the episcleral tissues and the formation of large, varicose veins (Grant and Schuman, 1993). Conjunctivitis and keratopathy can be seen chronically after exposure (Grant and Schuman, 1993).

Inhalation of small amounts of mustard gas produces nasal discharge, sneezing, epistaxis, and coughing within 12–24 h of exposure. Higher concentrations or longer exposures can cause pulmonary damage, hypoxia, and respiratory acidosis. Seizures may be seen with extremely acute high doses (Sidell et al., 1997).

Mustard gas is also a radiomimetic (Sidell et al., 1997). It destroys precursor cells in the bone marrow, leading to leukopenia, thrombocytopenia, pancytopenia, and anemia (Borak and Sidell, 1992; Dacre and Goldman, 1996). Infection can be seen secondary to bone marrow damage (Sidell et al., 1997). Bone marrow aplasia and death can be seen in severe cases.

Mustard gas is a possible animal teratogen. Some rat studies show abnormalities of the musculoskeletal system in the offspring of rats orally dosed with mustard gas, but only at doses high enough to be toxic to the dams, while other rat and rabbit studies showed no correlation (Dacre and Goldman, 1996; RTECS, 2008). Injection of mustards, both via IV and intraperitoneally, into male mice caused inhibition of spermatogenesis, but full recovery was seen within 4 weeks (Dacre and Goldman, 1996). Mustard gas is both a human and animal carcinogen (NTP, 2005). It has been linked to carcinomas in the skin, limbs, lungs, thorax, and leukemia in rats and mice after inhalation and IV exposure (RTECS, 2008).

Kinetics

Both liquid and vaporized mustard have rapid skin penetration. The higher the dose, temperature and humidity, the quicker the absorption (NATO, 1973). Mustard is dermally absorbed through hair follicles and sweat glands within minutes. Cellular reactions begin within 1–2 min of mustard making contact with skin or mucous membranes, but clinical effects are delayed between 2 and 24 h (Grant and Schuman, 1993; Sidell et al., 1997).

The skin initially pales and then becomes erythematous within a few hours of exposure (Requena et al., 1988). Erythema, blisters, bulla, and small vesicles form over 2–24 h. The blisters can progress for several more days. Erythema resolves over 3–7 days, while ulcers take 6–8 weeks to heal (Garigan, 1996; Sidell et al., 1997). Discoloration (brown or black hyperpigmentation) commonly occurs after resolution of the burns, especially in areas with thinner skin (Requena et al., 1988).

Ocular absorption happens within minutes. With mild ocular exposures, conjunctivitis and lacrimation begin about 4–12 h after exposure. Moderate ocular exposures produce conjunctivitis, blepharospasm, lid inflammation, corneal damage, and eyelid edema about 3–6 h post-exposure. Severe ocular exposures will lead to marked swelling of lids, corneal ulceration, corneal opacification, severe pain, and miosis in 1–2 h (Requena et al., 1988).

Inhalation produces respiratory signs (rhinorrhea, sneezing, epistaxis, and coughing) within 12–24 h of exposure. A severe exposure produces a productive cough, tachypnea, pulmonary edema (rare), and pulmonary hemorrhage within 2–4 h. Studies in dogs indicate

that equilibrium between blood and tissues was achieved within 5 min of inhalation (IARC, 1975). Ingestion of small amounts can cause hypersalivation and vomiting within 24 h, while larger amounts can cause gastrointestinal bleeding (rare) and bloody diarrhea within 3–5 days.

Mustard preferentially accumulates in fatty tissue (listed in order of decreasing concentration: fat, skin with subcutaneous tissue, brain, kidney, muscle, liver, cerebrospinal fluid, spleen, and lung) (Somani and Babu, 1989). Excretion takes place through the urine in rabbits, mice, and rats (IARC, 1975). Mustard is excreted over 72–96 h after IV administration in rats and mice (Dacre and Goldman, 1996). Complete blood count (CBC) changes are not evident for 3–5 days post-exposure. Leukopenia usually occurs between day 7 and day 10 (Garigan, 1996).

Decontamination and Treatment

Animals should be moved into fresh air. Emesis is not recommended, and activated charcoal administration after oral ingestion is controversial. Activated charcoal appears to have some beneficial effects if administered within 1 h of ingestion. Sodium thiosulfate (2% solution) given orally may help in cases of oral exposure (Borak and Sidell, 1992; Dacre and Goldman, 1996). Perforation and stricture formation can follow esophageal burns.

For ocular exposures, flush eyes with tepid water for at least 15 min. Follow with 2.5% sodium thiosulfate ophthalmic to help neutralize the mustard. The eyes need to be decontaminated quickly, as mustard disappears from the eye very rapidly, and late flushing of the eye generally provides no benefit (Sidell et al., 1997). Topical ophthalmic antibiotics and pain control should be used if corneal lesions are present (Sidell et al., 1997). Corneal transplants may be considered for valuable animals.

Animals need to be bathed with copious amounts of soap and water. If dermal decontamination is not implemented quickly, mustard will react with the skin and cannot be easily removed (Sidell et al., 1997). Sodium thiosulfate (2.5% solution) can be used dermally to neutralize mustard exposures (Garigan, 1996). Animals may also be bathed with dilute (0.5%) hypochlorite solutions (Borak and Sidell, 1992). Monitor for dermal burns. Secondary infection is common. Topical silver sulfadiazine can be applied to all burns, and an Elizabethan collar should be put in place to decrease ingestion of the ointment and self-trauma. Topically applied dexamethasone and diclofenac reduced inflammation in a mouse when applied within 4 h (Dachir et al., 2004). All equine and ovine patients should be inoculated with tetanus toxoid. Vaccination of other species should be determined on a case-by-case basis.

Monitor for respiratory irritation (such as coughing or dyspnea). If seen, monitor arterial blood gases and pulse oximetry. Thoracic radiographs may be taken, but there can be a lag time of up to 2 days before infiltrates are seen radiographically (Smith, 1999). Nebulization with 2.5% sodium thiosulfate or *N*-acetylcysteine may help neutralize the mustard gas. *N*-acetylcysteine may also be given via IV as a potential mustard gas antagonist (Garigan, 1996). Provide oxygen, ventilation, and inhaled beta agonists if needed. Dexamethasone, promethazine, vitamin E, melatonin, nitric oxide synthase inhibitors, protease inhibitors (such as doxycycline), surfactant replacement, and heparin have all shown protective effects against mustard gas poisoning in laboratory animals (Requena et al., 1988; Weinberger et al., 2011).

Serial CBCs with platelets should be monitored for 2 weeks after exposure. Antibiotics should be given if leukopenia develops (Sidell et al., 1997). Mustard can be detected in urine and body tissues for up to 1 week post-exposure using gas chromatography-mass spectrometry (GC-MS; Vycudilik, 1985). This can confirm diagnosis but is not likely to be of value in the management of the patient.

Mustard is persistent in the environment. Mustard may remain in the environment for up to 1 week in temperate areas. It disappears more quickly in hot climates and in desert conditions; persistence is reduced to about 1 day. Since mustard binds to vegetation for days to weeks, grazing animals need to be kept away from these areas (USACHPPM, 2001b).

Rescue personnel must wear protective clothing, eye protection, and a respirator, as the potential for secondary contamination is high (HSDB, 2008). Mustard gas will penetrate wood, leather, rubber, and paints.

Species Susceptibility

Animal studies of skin exposure to nitrogen and sulfur mustard show that these agents cause more severe skin lesions in hairless animals and less in fur-covered species (Smith et al., 1997). Dermal absorption of mustard varies greatly by species. Rats absorb about 75% of a dermal dose through their skin, while only 20% is absorbed through human skin (Smith, 1999). The rat dermal LD_{50} is only 5 mg/kg, while the mouse and human dermal LD_{50}s are 92 and 100 mg/kg, respectively (RTECS, 2008). However, with oral dosing, humans appear to be much more susceptible (oral LD_{50} 0.7 mg/kg for humans, and 17 mg/kg for rats). Due to these variable results, more studies are needed to determine if rats or mice would make good sentinel animals.

Lewisite

Clinical Signs

Lewisite is an arsenical compound that acts locally as a vesicant but also causes systemic effects (Sidell et al., 1997;

HSDB, 2008). Lewisite directly affects enzyme systems, resulting in decreased adenosine triphosphate (ATP) production. Lewisite also causes increased capillary permeability, leading to a significant loss of blood plasma into the extravascular space (known as *lewisite shock*) and hypotension (Sidell et al., 1997). Lung capillaries are affected not only by inhalation, but also by first pass through the lungs following dermal exposure. Pulmonary edema or ARDS can develop (Sidell et al., 1997).

Signs can be seen when lewisite is contacted dermally, orally, ocularly, inhaled, or ingested. Dermal and respiratory exposures are seen most frequently. Lewisite causes dermal, ocular, and respiratory lesions similar to those caused by mustard gas. Lewisite is about 10 times more volatile than mustard gas (Budavari, 2000).

Exposure to lewisite is very painful. Both the vapor and liquid lewisite can penetrate skin. Reddening of the skin is followed by tissue destruction (EPA, 1985a; Goldman and Dacre, 1989; Sidell et al., 1997; Pohanish, 2002). Amounts as small as 0.5 ml may cause severe systemic effects and 2 ml may be lethal. Severe edema develops secondary to increased capillary permeability. Dermal burns are deeper than those seen with mustard gas and are quicker to appear (Goldman and Dacre, 1989; Sidell et al., 1997).

Ocular exposure causes immediate pain, lacrimation, and blepharospasm. Without rapid decontamination, within 1 min, permanent blindness may occur (EPA, 1985a; Pohanish, 2002). In livestock, this is a death sentence, as they cannot survive on the range when blind. Droplets as small as 0.001 ml can cause corneal perforation and blindness (Sidell et al., 1997).

Inhalation of vapor causes irritation to the nasal passages, rhinorrhea, and violent sneezing (HSDB, 2008). Coughing and hemoptysis commonly occur (Sidell et al., 1997; HSDB, 2008). After inhalation, dogs developed necrotizing pseudomembranous laryngotracheobronchitis (Goldman and Dacre, 1989). Death can occur within 10 min after inhalation of high concentrations (EPA, 1985a).

Lewisite does not cause damage to the bone marrow or immunosuppression (Sidell et al., 1997). Arrhythmias and renal dysfunction are due to hypovolemia from fluid loss. Lewisite was fetotoxic to rats and rabbits and is a suspected carcinogen (Goldman and Dacre, 1989; RTECS, 2008).

Kinetics

Immediate pain occurs upon inhalation, dermal, or ocular contact with lewisite. Skin penetration occurs within 3–5 min, especially following liquid exposures (Sidell et al., 1997). The skin becomes red, then gray, within 15–30 min after exposure (EPA, 1985a; Goldman and Dacre, 1989; Sidell et al., 1997; Pohanish, 2002). Severe blisters develop within 12 h. The blisters rupture about 48 h after occurrence, with large amounts of fluid seeping from the site. Healing is generally complete within 4 weeks (much faster than with sulfur mustard-induced lesions).

Lewisite has extensive tissue distribution (HSDB, 2008). The highest concentrations were found in the liver, lungs, and kidneys in rabbits (>7 times blood concentration). Arsenic crosses the placenta and is excreted in the milk. Therefore, nursing animals may be at risk (Barlow and Sullivan, 1982). The elimination half-life of arsenic in rabbits is 55–75 h (HSDB, 2008). For further details on toxicity of arsenic and lewisite, see chapter 15.

Decontamination and Treatment

Remove animals from affected areas. If any coughing or respiratory distress is noticed, monitor blood gases and SpO_2. Provide oxygen and assisted ventilation as needed. Nebulized beta agonists (and possibly corticosteroids) can be used to treat bronchospasm. Monitor electrolytes and packed cell volume (PCV), as there can be fluid shifts out of the vasculature (Goldfrank et al., 2002). Urine arsenic levels may be measured, but they are not clinically useful due to the lag time before results are obtained. Watch for liver and kidney failure.

As lewisite is a vesicant, emesis is not recommended in those species that can vomit (such as dogs, cats, swine, and ferrets). Dilution with milk or water is recommended. Activated charcoal is not recommended, as severe irritation/vesication of the esophagus or gastrointestinal tract is likely to occur. Endoscopy can be performed very carefully to determine the extent of injury. Esophageal perforation, stricture formation, or both may occur.

The eyes should be flushed with copious amounts of tepid water for at least 15 min. If 5% dimercaprol [also known as *British anti-lewisite (BAL)*] ophthalmic ointment can be applied within 2 min, this may prevent a significant reaction. Application up to 30 min after exposure will lessen the ocular reaction but will not prevent all damage (Goldfrank et al., 2002).

Animals should be washed with water and dilute household bleach (5% sodium hypochlorite) as soon as possible. Application of a 5% BAL ointment within 15 min can be effective in diminishing the blistering effects of lewisite (Smith, 1999). Remove BAL ointment with soap and water after 5 min. Leaving the ointment on too long can cause stinging, itching, or urticaria. Burns should be managed with pain control, antibiotics, and debriding as needed.

A chelator should be given if there is dyspnea, pulmonary edema, or skin burns larger than palm size (Goldfrank et al., 2002). BAL is the traditional arsenic chelator, but it has numerous side effects. The deep intramuscular injections are very painful and BAL can cause hypertension, tachycardia, and vomiting.

2,3-Dimercaptosuccinic acid (DMSA, Succimer) can also be used to chelate arsenic (Graziano et al., 1978). 2,3-Dimercapto-1-propanesulfonic acid (DMPS) is used in Europe and has been effective in protecting rabbits from the lethal effects of lewisite (Aposhian et al., 1982).

Lewisite remains in the environment for about 24 h, and it can react with water to form a solid arsenoxide that also has vesicant properties. Affected areas can be treated with strong alkalis to form less harmful substances. Rescue personnel need to wear protective clothing and masks, as the risk for secondary contamination is high. Carcasses should be disposed of properly, either buried deeply (away from water supplies), rendered, or incinerated to ensure safety of the food supply.

Species Susceptibility

Lewisite has the potential to cause skin lesions in any species, but the risk is greatest in hairless animals, such as pigs, and decreases in fur-covered species (Smith et al., 1997). Mice and rats appear to be almost twice as susceptible to dermal lewisite exposures than humans (LD_{50} equals 12, 15, and 30 mg/kg, respectively) (Sidell et al., 1997; DeRosa et al., 2002; RTECS, 2008). More studies are needed to determine if rodents would be good sentinel animals.

Phosgene Oxime

Clinical Signs

In both its liquid and vapor forms, phosgene oxime causes severe pain and local tissue destruction upon contact with skin, eyes, and mucous membranes (Sidell et al., 1997). Signs depend on its route of entry, as phosgene oxime exerts its greatest effects in the first capillary bed it encounters.

Dermal, ocular, and respiratory lesions are similar to those caused by mustard gas. Inhalation and oral absorption may cause respiratory tract irritation, dyspnea, and pulmonary edema. Dermal lesions are erythematous and extremely painful. With ocular exposure to phosgene oxime, very low concentrations can cause lacrimation, inflammation, and temporary blindness, while high concentrations can cause permanent corneal lesions and blindness (Sidell et al., 1997; USACHPPM, 2001c). Death is generally due to respiratory arrest.

Kinetics

Complete absorption occurs in both dermal and inhalational exposures within seconds (Sidell et al., 1997). Dermal lesions begin to form within seconds; grayish tissue damage may be seen within several minutes, and within 1 h, the area becomes edematous. Phosgene will spread in sweat and move to other nonexposed areas of the body (DeRosa et al., 2002). Pulmonary edema can be seen on thoracic radiographs within 2 h of high-dose exposure, 4–6 h of moderate exposure, and approximately 8–24 h after low-dose exposure (Sidell et al., 1997). The skin turns brown, and blistering occurs the next day. It takes about 3 weeks for desquamation, necrosis, crust formation, and purulent exudate to occur (Sidell et al., 1997). Pain can last for several days, and healing of dermal lesions can take from 1 month to over a year.

Decontamination and Treatment

Animals should be moved into fresh air. Emesis is not recommended due to the irritant and corrosive effects of phosgene oxime. Dilution with milk or water is recommended for oral ingestions. Activated charcoal is not recommended as the primary toxicity is a local corrosive injury. Sedation and pain control (opioids) may be needed to allow safe decontamination.

Irrigate eyes with tepid water until pH returns to neutral and remains so for 30 min after irrigation is discontinued (Brodovsky et al., 2000). Time until decontamination after ocular exposure is important since phosgene oxime is absorbed within seconds. Corneal ulcers should be treated with atropine ophthalmics to prevent synechiae formation, and other ophthalmics to aid in re-epithelialization (Grant and Schuman, 1993; Brodovsky et al., 2000).

The animal should be bathed with copious amounts of water and a mild soap. Phosgene oxime reacts quickly with tissue, and decontamination is not expected to be entirely effective after pain has been produced. Sodium hypochlorite (0.5%) or isotonic sodium bicarbonate can help neutralize phosgene oxime that has not yet reacted with tissue. Burns should be managed with topical silver sulfadiazine and systemic antibiotics as needed (Roberts, 1988). Horses and sheep should receive tetanus prophylaxis. Other species should be vaccinated on a case-by-case basis.

Monitor oxygenation and thoracic radiographs in patients following significant exposures. Administer oxygen, perform endotracheal intubation, and provide assisted ventilation if needed. Nebulized beta-adrenergic agonists can help if bronchospasm develops. Administer IV fluids, but monitor for overhydration (Goldfrank et al., 2002).

Phosgene oxime is nonpersistent in the environment, and it hydrolyzes rapidly in aqueous alkaline solutions. Veterinary personnel and rescuers should wear aprons, rubber gloves, and masks when treating patients to avoid secondary contamination.

Species Susceptibility

There are no controlled studies showing that any species of animal would make a good sentinel for phosgene oxime exposure.

Cyanide and HCN

Clinical Signs

Cyanide and HCN are classified as blood agents. They cause toxicity by forming a stable complex with ferric iron (Fe^{3+}) in cytochrome oxidase enzymes. This inhibits cellular respiration, oxygen utilization, and ATP production, causing deprivation of oxygen to the body at the cellular level (Way et al., 1988). Both arterial and venous blood appears cherry red due to the accumulation of oxyhemoglobin (Bingham et al., 2001). Unhaired skin may also appear bright pink due to the high concentration of oxyhemoglobin in the venous return (HSDB, 2008). HCN and cyanogen chloride are volatile and water-soluble, and liquid forms of cyanide and HCN are the most likely to be used for terrorism.

Cyanide exposure can cause transient central nervous system (CNS) stimulation, followed by syncope, ataxia, dyspnea, seizures, paralysis, apnea, and coma (Hall and Rumack, 1986). The odor of bitter almonds may be noted in gastric or ruminal contents and expired breath. Initial tachypnea is followed by respiratory depression. Dyspnea without cyanosis can help with the diagnosis. Chickens develop tachypnea, have rapid eye blinking, hypersalivation, and lethargy (Wiemeyer et al., 1986). Blindness may occur from cyanide-induced damage to the optic nerve and retina (Grant and Schuman, 1993). Hypersalivation, vomiting, and abdominal pain may occur after ingestion (Hall and Rumack, 1986). Metabolic and lactic acidosis is commonly seen. Death can occur within minutes.

Kinetics

Cyanide and HCN can be absorbed by all routes (inhalation, oral, ocular, and dermal) (Hall and Rumack, 1986). There is rapid diffusion into tissues, and cyanide irreversibly binds to its target sites. Cyanide has a wide volume of distribution and will concentrate in red blood cells (RBCs) two to three times greater than in plasma (HSDB, 2008). Cyanide preferentially accumulates in the hypothalamus, with levels about 40% higher compared to other areas of the brain (Borowitz et al., 1994).

Cyanide is metabolized by rhodanese in the liver to thiocyanate (Hall and Rumack, 1986). Thiocyanate is excreted mainly in the urine. Without administration of an antidote, the half-life for the metabolism of cyanide to thiocyanate is 20 min to 1 h (Feldstein and Klendshoj, 1954). Cyanide can be excreted in breast milk (Soto-Blanco and Gorniak, 2003).

Decontamination and Treatment

Remove animals from the affected area. Do not induce vomiting due to the rapid progression of the clinical signs and potential for seizures, coma, or apnea. A gram of activated charcoal will bind 35 mg of cyanide, and activated charcoal may be beneficial if administered immediately after ingestion (Lambert et al., 1988). Irrigate eyes for at least 15–20 min with tepid water. Bathe animals thoroughly with soap and water.

Blood gases and serum electrolytes should be monitored and corrected as needed (Hall and Rumack, 1986). Blood cyanide levels can confirm exposure, but due to the time needed to get the results, they are not clinically useful. Provide supplemental oxygen with assisted ventilation as indicated. Animal study results for hyperbaric oxygen therapy have been questionable (Way et al., 1972). Acidosis ($pH < 7.1$) should be corrected with IV sodium bicarbonate, but acidosis may not resolve until after the administration of antidotes (Hall and Rumack, 1986). Benzodiazepines or barbiturates can be used to control seizures.

Cyanide toxicosis progresses so rapidly that treatment is rarely administered to animals in time. If the animal is still alive but in respiratory distress or a coma, antidotal agents may still be life-saving. The classic treatment for cyanide intoxication includes several steps. Sodium nitrite is given via IV over 15–20 min (fast administration causes hypotension). Sodium nitrite reacts with hemoglobin in the RBCs to form methemoglobin. Methemoglobin combines with free cyanide to form cyanomethemoglobin. Sodium thiosulfate is given after sodium nitrite. Sodium thiosulfate supplies sulfur for the rhodanese reaction (Hall and Rumack, 1987). The sulfur reacts with cyanomethemoglobin to form hydrogen thiocyanate, which is excreted in the urine.

Hydroxocobalamin (Cyanokit) has been the chelator of choice in Europe and Australia, and has been approved for use in the United States since 2007. It works by combining with cyanide to form cyanocobalamin (vitamin B_{12}; Hall and Rumack, 1987). Hydroxocobalamin has been shown to reduce mortality in rats, mice, and beagles and has the advantage of producing neither methemoglobinemia nor hypotension, as sodium nitrite does (Hall and Rumack, 1987; Borron et al., 2006). Dicobalt-EDTA (Kelocyanor) and 4-dimethylaminophenol hydrochloride (4-DMAP) are other chelators available in Europe, Israel, and Australia, but not in the United States (Hillman et al., 1974; Weger, 1990).

Other substances that have been tested in the lab on animals include: stroma-free methemoglobin solutions, alpha-ketoglutaric acid, chlorpromazine, hydroxylamine, phenoxybenzamine, centrophenoxine, naloxone, etomidate, para-aminopropiophenone, and calcium channel blockers (Ashton et al., 1980; Dubinsky et al., 1984; Leung et al., 1984; Ten Eyck et al., 1985; Johnson et al., 1986; Bright and Marrs, 1987; Yamamoto, 1990; Budavari, 2000). The use of these substances has shown positive results, but they have not been tried during actual poisoning situations.

HCN is lighter than air and has a long half-life in air. However, in open spaces, HCN is rapidly dispersed and

is diluted to nontoxic concentrations. Cyanide does not bind to soil or plant material but can mix with water. Contaminated water can be treated with ozone, hydrogen peroxide, or calcium/sodium hypochlorite bleach. Rescue personnel should wear boots, gloves, goggles, full protective clothes, and a self-contained positive pressure breathing apparatus as the potential for secondary contamination is high (NIOSH, 2005).

Species Susceptibility

There are significant interspecies differences in the toxicity of HCN (Sousa et al., 2003). Dogs appear to be more susceptible than humans to cyanide poisoning. This is thought to be due to lower levels of endogenous rhodanese (hepatic enzyme that catalyzes the sulfuration of cyanide to thiocyanate) (Aminlari and Gilanpour, 1991). Barcroft (1931) exposed both a man and a dog simultaneously to HCN gas. The 70-kg man and 12-kg dog were exposed to HCN concentrations between 500 and 625 ppm in an airtight chamber. The dog became ataxic at 50 s, unconscious at 74 s, and began to seizure at 90 s. At 91 s, the man walked out of the exposure chamber with no symptoms, although over the next 10 min, he developed transient nausea and difficulty concentrating (Barcroft, 1931). As HCN is lighter than air, it is probably not that the small dog experienced a higher exposure than the human.

Cats are more resistant to subcutaneous HCN than humans. The LDLo (SQ) for humans is 1 mg/kg, as compared to 11 mg/kg for cats (Sax and Lewis, 1989). The LDLo (oral) for HCN in humans, dogs, and rabbits is comparable (5.7, 4, and 4 mg/kg, respectively) (Sax and Lewis, 1989).

Dogs may be good sentinel animals as they appear to have increased susceptibility relative to humans based on physiological differences. More controlled studies are required, however.

Military Nerve Agents

Clinical Signs

Military nerve agents are probably the most toxic of the known CWAs. Military nerve agents are divided into G (which stands for "Germany") agents (such as sarin, soman, and tabun) and V (which stands for "venomous") agents (such as VX). Nerve agents are extremely dangerous, as they are absorbed without producing any irritation or other sensation on the part of the exposed person or animal (HSDB, 2008).

Nerve agents are organophosphates (OPs). Acute exposure to OPs can cause muscarinic, nicotinic, and CNS signs. Muscarinic effects include salivation, lacrimation, urination, dyspnea, diarrhea, and emesis (SLUDDE), along with miosis, bradycardia, hypotension, and bronchoconstriction. Nicotinic effects include muscle fasciculation and weakness (including the diaphragm), tachycardia, hypertension, and mydriasis. CNS effects include restlessness, anxiety, seizures, and coma (Garigan, 1996). VX has CNS effects that are unrelated to AChE activity and prolonged effects may be seen following convulsive doses (Young et al., 1999). Death is due to paralysis of the diaphragm, airway obstruction from increased bronchial secretions, or depression of the CNS respiratory center (Garigan, 1996).

The G agents present a vapor hazard, as they are very volatile. VX has a high dermal toxicity, even through intact skin, as the liquid does not evaporate quickly (Berkenstadt et al., 1991; Sidell et al., 1997). VX is 300 times more toxic than tabun on skin. A very small drop on the skin may cause sweating and fasciculations at the site. A larger dermal drop may cause loss of consciousness, seizures, apnea, and flaccid paralysis. The toxicity of these substances, listed in descending order on a per-weight basis, is VX, soman, sarin, and tabun (HSDB, 2008).

Delayed peripheral neurotoxicity has been reported in animal studies. Soman produced severe delayed neuropathy in the atropinized hen assay at 1.5 mg/kg (Willems et al., 1984). Sarin, tabun, and VX cause postimplantation mortality and fetotoxicity (HSDB, 2008; RTECS, 2008).

Kinetics

Nerve agents can be absorbed by any route (ocular, oral, inhalation, dermal) (HSDB, 2008; RTECS, 2008). The onset of signs and duration of effects depend on the form of nerve gas (vapor, liquid) and the route of exposure. With a vapor exposure and inhalation, local signs of nasal discharge and respiratory noise begin within 1 min to several minutes and signs can last for a few hours (mild exposure) up to 1–2 days (severe exposure) (Pfaff, 1998). Inhalation of a large amount of the vapor will result in sudden loss of consciousness, apnea, flaccid paralysis, and seizures within seconds to 2–3 min (Sidell et al., 1997). Peak effects are seen within 20–30 min, and death is usually due to respiratory failure (Berkenstadt et al., 1991).

Liquid nerve agents applied dermally cause local sweating and muscular twitching starting 3 min to 2 h after exposure. Signs last for 3–5 days. Following dermal exposure to a large drop, clinical effects start within 30 min but with small drops, a delay of up to 18 h can be seen (Sidell et al., 1997).

Ocular exposure to vapor causes miosis, conjunctival hyperemia, and eye pain within 1 min to several minutes. Signs can last 2–3 days. Liquid tabun penetrates the eye quickly and can result in death nearly as rapidly as an inhalational lethal dose (1–10 min) (EPA, 1985b). Ingestion of the liquid causes muscarinic, nicotinic, and CNS signs about 30 min after mild exposures. The signs

can last several hours up to 2–5 days, depending on the amount of exposure.

Volume of distribution is slightly different for each of the nerve agents. Sarin is distributed to the brain, liver, kidney, and plasma of mice (Little et al., 1986). Soman is distributed throughout the mouse brain, with the highest levels found in the hypothalamus (Wolthuis et al., 1986). Tabun is also found in high concentrations in the hypothalamus after IV administration in mice (Hoskins et al., 1986). Soman is unique in that it has apparent storage in body "depots" and is released over time. This release can result in eventual death in animals who survive the initial dose of soman (Wolthuis et al., 1986).

Most military nerve agents have rapid aging of the OP–enzyme complex. After aging, the inhibitor-enzyme complex becomes resistant to reactivation (Young et al., 1999). VX and tabun are exceptions to this rule, with an aging half-life ($t_{1/2}$) of greater than 40 h (Garigan, 1996). The aging $t_{1/2}$ is only a few minutes for soman and about 5 h for sarin (Garigan, 1996).

Sarin is metabolized to isopropyl methylphosphonic acid (IMPA) and excreted by the kidneys (Little et al., 1986). Approximately 50% of soman is converted to free pinacolyl-methylphosphonic acid within 1 min in mice. The half-life of this metabolite is less than 1 h (Reynolds et al., 1985).

Cholinesterase levels can take weeks to return to normal (Rengstorff, 1985). RBC AChE recovers even more slowly (several days to 4 months), depending on the severity of the depression (Grob, 1956). Pupillary reflexes also remain suppressed for weeks to 1.5 months (Rengstorff, 1985).

Decontamination and Treatment

Administer oxygen and remove the animals from the toxic environment. For ocular exposures, flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Wash all animals three times with either soap and water, dilute bleach solution (1:10 with water), ethanol, or a tincture of green soap (Cancio, 1993). Towelettes impregnated with alkaline chloramine and phenol are used by the military (M291 Skin Decontaminating Kit, Rohm and Haas).

Due to the rapid development of signs, emesis is not recommended after oral ingestion. Activated charcoal can be used in both oral and dermal exposures. Seizures can be controlled with diazepam, methocarbamol, or barbiturates as needed. Assisted ventilation may be necessary if signs progress.

Atropine is an antidotal treatment. It is used to reverse the muscarinic signs, but it will not reverse the nicotinic effects (muscular weakness, diaphragmatic weakness, etc.). Atropine blocks the effects of accumulated acetylcholine (ACh) at the synapse and should be continued until the nerve agent is metabolized (Midtling et al., 1985). Over-atropinization can cause hyperthermia, tachycardia, agitation, mydriasis, and ileus, which can be life-threatening in the horse.

Oximes are used to treat the nicotinic signs. Pralidoxime (2-PAM) is the oxime of choice in the United States, and it is most effective when administered in the first 1–3 h. Due to the quick aging of soman, 2-PAM is rarely given soon enough to be effective (Sidell et al., 1997). 2-PAM can be given up to 48 h after exposure to VX and tabun, due to slow aging (Sidell and Groff, 1974). Other oximes, such as obidoxime dichloride (Toxogonin, LüH-6), are used in other countries (Belgium, Israel, the Netherlands, Scandinavia, Portugal, and Germany). HI-6 is another alternative oxime that has excellent acetylcholinesterase regenerating action with VX, very good action with sarin, good response to soman, but poor or no response following tabun exposures (Hoffman, 1999).

Autoinjectors (AtroPen, Mark I, Combopen MC) are atropine or atropine and 2-PAM combinations available for human use. They are not used in veterinary medicine as they are not adjustable for different-sized patients. Experimental vaccines against nerve agent VX, and monoclonal antibodies which protect against soman, sarin, and tabun toxicity have been produced and are being tested (Dunn and Sidell, 1989).

AChE activity can be tested in plasma, serum, or whole blood. In most animal species, 80% or more of the total blood AChE activity is in the RBCs, as compared to 50% in humans; therefore, whole blood is the preferred sample for most veterinary diagnostic labs. In general, in the context of OP poisoning, whole blood AChE activity that is less than 50% of normal is generally associated with severe symptoms (Midtling et al., 1985). Furthermore, inhibition of RBC AChE is interpreted as follows: (i) 10–20%, no reliable evidence of exposure; (ii) 30–50%, mild poisoning; (iii) 50–70%, medium or moderate; and (iv) 70–90%, severe intoxication.

The G agents are volatile and evaporate over several hours. They are nonpersistent in the environment (Garigan, 1996). Environmental persistence is estimated to be 0.5–1 day for tabun, 1–2 days for soman, and 5 days for sarin. VX is an oily liquid that remains in the environment for weeks or longer after being dispersed (Garigan, 1996; Sidell et al., 1997; Munro et al., 1999; Budavari, 2000). Contaminated soil should be treated with alkaline substances (such as sodium carbonate, sodium bicarbonate, calcium hydroxide, or calcium carbonate) or chlorine compounds (sodium hypochlorite or calcium hypochlorite) (EPA, 1975).

Protective equipment (rubber gowns, aprons, and gloves), along with respiratory protection, must be worn by rescuers and veterinary personnel. Leather and fabrics absorb OPs and are extremely difficult to decontaminate. Collars, muzzles, and other items should be incinerated. For further details on decontamination of nerve agents, see chapter 76.

Species Susceptibility

Nerve agent susceptibility varies widely between species. Animals may be more at risk for greater exposures than nearby human populations. Both the G and the V nerve agents are heavier than air, and animals with a lower breathing zone will be more affected (Rabinowitz et al., 2008). Some species differences can make animals more sensitive than humans, while others will make them more resistant. Rats may be less susceptible than humans, since they possess aliesterases that can reduce the toxicity of certain nerve agents such as soman and tabun (Fonnum and Sterri, 1981; Gupta and Dettbarn, 1987). These enzymes are not present in humans. Nasal breathers, such as rodents, are capable of partially detoxifying nerve agents in the nasal pathways, presumably by hydroxylation and other mechanisms (Garamone, 2003). Carboxylesterase (CarbE) activity in guinea pigs and rabbits may provide protection from soman, when compared to humans (Maxwell et al., 1987).

RBC AChE activity also varies among species. In humans, low RBC AChE activity has been associated with increased susceptibility to nerve agents (Leng and Lewalter, 1999). Pigs, sheep, dogs, rabbits, and cats have less RBC cholinesterase activity than humans (Anonymous, 2000b). This would make these species more sensitive to nerve agents unless they have an alternative system to combat cholinesterase inhibition.

The LCt_{50} (50% lethal concentration via inhalation route) for military personnel for different nerve agents has been estimated from animal studies. Humans appear to be more sensitive to tabun than other animals. The LCt_{50} for tabun is 70 mg min/m^3 for humans, 320 mg min/m^3 for dogs, 450 mg min/m^3 for rats, and 960 mg min/m^3 for rabbits (Anonymous, 2000a; NRC, 2003). Goats are more sensitive than humans to VX. The LCt_{50} for goats is 9.2 mg min/m^3, while the human LCt_{50} is 15 mg min/m^3 (Anonymous, 2000a; NRC, 2003).

Even though the lethal nerve agent exposure level for animals may be higher than that for humans, it is still possible that animals could exhibit other nonlethal effects sooner and more noticeably than humans. Rabbits develop 90% miosis at a lower inhaled concentration of cyclohexyl sarin when compared to humans (2.71 mg min/m^3 versus 13.85 mg min/m^3) (NRC, 2003).

Another way that animals could provide an early warning is via their sense of smell. Most animals have much more sensitive olfactory systems than humans. Animals may be able to be trained to sense low nerve gas concentrations (Dalton, 2003).

It is unknown if animals may have decreased dermal absorption compared to humans due to their protective fur or feathers. Comparing dermal exposures in humans and other species also shows differences for the various nerve agents. Mice appear to be more sensitive to dermal sarin than humans; the LD_{50} values for each are 1.08 and 28 mg/kg, respectively (Sidell et al., 1997; RTECS, 2008). The mouse may also be a good sentinel animal for tabun. The dermal LD_{50} for mice is 1 mg/kg; for rats, it is 18 mg/kg, and for humans, it is 14 mg/kg. Mice may be slightly more resistant to dermal soman, with LD_{50}s of 7.8 mg/kg for mice and 5 mg/kg for humans.

There are a few cases where shared exposures to nerve agents have been reported for both animals and humans. In 1968, there was an accidental release of two different nerve agents (one is thought to have been VX) in Utah. A flock of sheep that was grazing near the base was noted to be acting "crazy in the head," and thousands died less than 24 h later (Boffey, 1968). Nearby humans, cattle, dogs, and horses did not develop symptoms. The sheep had severely depressed cholinesterase levels, as did the cattle and horses. Cholinesterase testing of dogs and humans was normal. The sheep may have been more affected due to higher exposures through ingestion of contaminated pasture, or by spending more time in the vicinity of the chemical release.

3-Quinuclidinyl Benzilate (bz)

Clinical Signs

3-quinuclidinyl benzilate (BZ) is a centrally acting synthetic anticholinergic agent. BZ is used as a hallucinogenic and incapacitating CWA. It is about 25 times more potent than atropine and has a very long duration of action. BZ is disseminated as an aerosol, with the primary route of absorption through the respiratory system.

BZ affects both the peripheral nervous system (PNS) and CNS. Peripheral anticholinergic effects can be summarized by the mnemonic "dry as a bone, red as a beet, hotter than Hades and blind as a bat." BZ inhibits glandular secretions ("dry as a bone"), leading to a dry mouth and foul breath (Holstege, 2006). Cutaneous vasodilation and skin flushing may be noted ("red as a beet") due to decreased capillary tone. Hyperthermia ("hotter than Hades") is due to elevated body temperature secondary to inhibition of sweating and inability to dissipate heat. Vision loss ("blind as a bat") is from a loss of accommodation reflexes and decreased depth of field secondary to ciliary muscle paralysis and mydriasis (Holstege, 2006). Paralytic ileus is commonly seen as a result of anticholinergic toxicity (Ketchum and Sidell, 1997; Holstege, 2006). This can lead to fatal colic in equids. Urinary retention is also a common anticholinergic effect following exposure to BZ (Ketchum and Sidell, 1997; Holstege, 2006).

CNS signs of disorientation, agitation, tremor, ataxia, stupor, coma, and seizures may occur from inhibition of central muscarinic receptors (Ketchum and Sidell, 1997;

Holstege, 2006). It is unknown if animals hallucinate like people, but they do appear distressed. Rhabdomyolysis can be seen secondary to seizures and agitation. If severe, myoglobinuric renal failure could develop (Holstege, 2006).

Other systemic signs may also occur. Sinus tachycardia is common (Ketchum and Sidell, 1997; Holstege, 2006). Moderate hypertension may occur, and tachypnea may be expected following an acute exposure. Nausea and vomiting may also occur.

Kinetics

BZ easily crosses the blood–brain barrier (BBB), leading to mostly CNS effects. Signs are dependent on the dose and time after exposure. Prolonged effects may occur depending on the dose of BZ absorbed. Tachycardia and dry mouth develop within 15 min to 4 h after exposure (Ketchum and Sidell, 1997). Peak effects occur at 8–10 h (Ketchum and Sidell, 1997).

BZ accumulates in the cerebrum following an IV injection in rats (Sawada et al., 1990). It is highly lipophilic and has a high degree of plasma protein and red cell binding. Steady state was reached in the cortex and caudate within 1–5 min. Binding to high-affinity muscarinic acetylcholine receptors (mAChRs) is essentially irreversible for the first 6 h.

BZ is excreted via the kidneys (Holstege, 2006). Only about 3% is excreted unchanged in rat urine (Byrd et al., 1992). The two major metabolites are 3-quinuclidinol and benzilic acid. Without treatment following an incapacitating dose, recovery is gradual, requiring 72–96 h (Ketchum and Sidell, 1997).

Decontamination and Treatment

Move into fresh air. Monitor animals for respiratory distress. If cough or dyspnea develops, administer supplemental oxygen. Assisted ventilation may be required. Nebulized beta-adrenergic agonists should be used if bronchospasm develops.

Due to the method of distribution (aerosol), most exposures are expected to be via inhalation. However, with animals' tendency to groom, dermal exposures can also become oral exposures. Emesis is not recommended due to potential seizures and coma. Activated charcoal or gastric lavage can be helpful after oral exposures. These procedures can be successful even if delayed, as anticholinergics slow gastrointestinal motility.

Flush eyes with copious amounts of tepid water for at least 15 min. Animals should be bathed with soap and water. Bathing will not only remove the BZ, but will also provide external cooling to combat hyperthermia. BZ may be detected in urine, serum, or blood, but there are no rapid tests to diagnose exposure to BZ, so clinical use is minimal.

Benzodiazepines can be used to control agitation. Avoid phenothiazines due to possible hypotension. Animals may need to be heavily sedated so that they do not injure themselves or others. Control seizures with a benzodiazepine, phenobarbital, or propofol. Rhabdomyolysis can occur if agitation or seizures are not controlled.

Treat arrhythmias symptomatically (lidocaine, propranolol, etc.). Physostigmine can be used to treat severe arrhythmias, but long-lasting reversal of anticholinergic toxicity does not occur. Physostigmine is ineffective if given during the first 4–6 h following the onset of BZ effects. If physostigmine is discontinued, recovery from BZ may be slightly prolonged (Ketchum and Sidell, 1997).

Monitor electrolytes and renal function tests in symptomatic patients. Administer IV fluids to maintain urine output and to protect the kidneys from myoglobinuria. The prognosis is good if animals do not develop rhabdomyolysis or secondary infection. No chronic problems are expected from BZ itself (Holstege, 2006).

BZ is stable and environmentally persistent. "Off gassing" may occur from contaminated patients. Goggles and masks should be worn by all personnel until the animals are decontaminated (Holstege, 2006). Remove contaminated collars, leashes, harnesses, halters, and other devices, and discard them, as leather and fabrics absorb BZ.

Species Susceptibility

There are no studies demonstrating that animals are more sensitive than humans.

RCAs (Lacrimators)

Clinical Signs

Chloroacetophenone (i.e., CN, mace, tear gas), chlorobenzylidene malonitrile (i.e., CS, Paralyzer, super tear gas), and oleoresin capsicum (OC; pepper spray) are lacrimators used in riot control. They are solid chemicals administered as a fine dust or aerosol spray rather than being true gases. Exposure to lacrimators causes immediate pain, blepharospasm, lacrimation, rhinorrhea, coughing, and sneezing, but usually no permanent tissue damage (Grant and Schuman, 1993; Blain, 2003). With higher ocular concentrations, chemical burns with keratitis and loss of the corneal epithelium may occur (Hoffman, 1967). A 4% weight/volume CN product has caused permanent corneal injury and ocular necrosis in animals, but no such injury was seen in a 10% w/v CS product (Gaskins et al., 1972; Grant and Schuman, 1993). Oral ingestion will also cause eye irritation, lacrimation, vomiting, and diarrhea (Blain, 2003). Laryngospasm may occur in some cases due to the irritant effects; it

can progress to pulmonary edema, bronchospasm, and bronchopneumonia.

Dermal contact with lacrimators is very painful (Pinkus, 1978). Erythema and blisters are common. The extent of dermal effects depends on the thickness of the stratum corneum, and the extent of exposure (Blain, 2003). High concentrations can cause first- and second-degree burns of the skin (Stein and Kirwan, 1964; Hu et al., 1989). No teratogenicity or carcinogenicity has been demonstrated in humans or animals (Folb and Talmud, 1989; Blain, 2003). For further details on toxicity of RCA, see chapter 11.

Kinetics

The effects of lacrimators occur very quickly. Pain, salivation, coughing, rhinorrhea, sneezing, and erythema begin within seconds of exposure and can last approximately for an hour (Blain, 2003). Delayed effects may also be seen. Conjunctivitis and ocular edema may be noted from 1–2 days following exposure. Laryngospasm and pulmonary edema may occur up to 48 h (usually 12–24 h) post-exposure. Bronchospasm may also be delayed up to 48 h post-exposure and become chronic (Folb and Talmud, 1989).

One of the CS metabolites is cyanide, but cyanide toxicosis does not appear to happen with routine use (Cucinell et al., 1971). Gastrointestinal signs resolve over 24 h (Solomon et al., 2003). Erythema disappears over 48 h and coughing may persist for weeks after exposure (Blain, 2003).

Decontamination and Treatment

Animals may need to be sedated to be able to treat them safely. Move animals into fresh air and monitor for respiratory distress. If cough or dyspnea develops, monitoring is necessary for oxygenation status. Supplemental oxygen may be needed. Laryngospasm may require intubation to permit adequate ventilation. Inhaled beta-2 agonists (i.e., albuterol and salbutamol), corticosteroids, and aminophylline may help reduce bronchospasm (Folb and Talmud, 1989). Thoracic radiographs should be monitored if pulmonary edema is expected (Stein and Kirwan, 1964).

Flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Diphoterine solution can be used for decontamination of both eyes and skin after exposure to lacrimators (Viala et al., 2005). Ocular signs resolve approximately 3–7 min after decontamination with diphoterine.

Animals should be bathed with soap and copious amounts of cold water. Using small amounts of water can actually increase irritation (Lee et al., 1984). Topically applied magnesium hydroxide–aluminum hydroxide–simethicone suspension (Maalox Max) caused resolution of signs within 2 min of application following exposure to OC. If chemical burns develop, clean wounds with a mild, disinfectant soap and water. Pain control and antibiotics may be needed. Tetanus toxoid should be given if burns are present. With oral ingestions, antacids may help decrease gastrointestinal signs.

Secondary contamination is common and personnel should wear aprons, rubber gloves, and masks as needed. Contaminated items can be washed in cold water (hot water will cause residual gas to vaporize) with soap or allow nonwashable items to air out for a few days. Most lacrimators dissipate quickly, but CS may be micronized and mixed with an antiagglomerant agent (CS1), which remains active for up to 5 days. A similar formulation mixed with silicone (CS2) remains in the environment for up to 45 days (Hu et al., 1989).

Species Susceptibility

There are no controlled studies showing that animals are more sensitive to lacrimators than humans.

Ricin and Abrin (Toxalbumins)

Clinical Signs

Ricin and abrin are toxalbumins, which are plant lectins with a specific affinity for animal cell receptors. *Ricinus communis* (including the castor bean plant, castor oil plant, koll, mole bean, moy bean, and palma christi) contains ricin. The castor bean plant is grown throughout the United States as an ornamental. The brown and white seeds, which resemble large ticks, contain the ricin. Most animals are exposed to ricin by eating the seeds, but ricin has been used as a CWA, a reagent for pepsin and trypsin, an experimental antitumor and immunosuppressive agent, and a commercial mole killer (Sax and Lewis, 1989; Budavari, 2000; HSDB, 2008). Abrin is found in the *Abrus precatorius* plant (including the Buddhist rosary bead, crab's eyes, Indian bead, Indian licorice seed, jequirity bean, jungle bead, love bean, lucky bean, mienie-mienie, ojo de pajaro, prayer bead, rosary pea, seminole bead, and weather plant). This tropical ornamental vine produces colored seeds that are popular for use in jewelry and other decorative items. The seeds come in three different colors: red with a black eye, black with a white eye, and white with a black eye (Niyogi, 1969).

Variable toxicity is seen in cases of oral ingestion. Seeds that are chewed are much more likely to cause clinical signs. Seeds swallowed whole may not cause any problems at all (Kinamore et al., 1980). The amount of toxalbumins in the seeds will vary by size, weight, moisture content, region, season, and the period of plant growth at the time of harvesting. The ricin content of castor beans can vary from 1% to 10% (Balint, 1974). The

abrin content in *Abrus precatorius* seeds is estimated at 0.15% (Lin et al., 1971). Toxicity and death have occurred with ingestions of one to two chewed beans of either plant. Ricin levels can be measured in plasma and urine but are not clinically useful (Kopferschmitt et al., 1983).

Vomiting, abdominal pain, and bloody diarrhea are the most common signs seen after ingestion (Kopferschmitt et al., 1983). Toxalbumins cause severe gastrointestinal lesions of the oropharynx, esophagus, and stomach. The lesions are clinically similar to alkaline burns. Fluid losses can lead to dehydration, electrolyte disturbances, hypotension, and tachycardia (Ingle et al., 1966).

Liver damage occurs in toxalbumin toxicosis. Histopathology reveals injury to the smooth endoplasmic reticulum and depletion of liver glycogen (Balint, 1978). The liver values for alanine aminotransferase (ALT), total bilirubin, aspartate aminotransferase (AST), alkaline phosphatase, and gamma-glutamyl transferase (GGT) can all be elevated. Glucose metabolism is affected by ricin. Not only do glycogen stores decrease, but gastrointestinal absorption of glucose decreases and glucose concentrations fall (Lampe, 1976). Hypoglycemia is a common finding.

Mild to moderate CNS depression is commonly observed. Seizures are reported more frequently in animals than in people (Hart, 1963). While hematuria is commonly reported, the hemagglutination that is seen in animal and laboratory work is almost never seen in actual toxicities (Waller et al., 1966).

Ricin is among the most toxic compounds known when given parenterally. Parenteral toxicity is much greater than oral toxicity. The oral lethal dose of ricin is estimated to be 1 mg/kg (Kopferschmitt et al., 1983). When given by injection, the lethal dose of ricin drops to about 1 μg/kg (Budavari, 2000). With an inhalation exposure, signs seen are cough, dyspnea, arthralgias, fever, and death (Griffiths et al., 2007). Other organ system dysfunctions may not occur. For more details on toxicity of ricin and abrin, see chapters 27 and 53.

Kinetics

Gastrointestinal effects usually develop in under 6 h, but vomiting can begin in 1–3 h postingestion of castor beans (Kopferschmitt et al., 1983). In some cases, signs may be delayed for several days. The cytotoxic effects on the liver, CNS, kidney, and adrenal glands may not occur for 2–5 days post-exposure. With an inhalation exposure symptoms begin within 8 h. Death occurs about 48 h after a parenteral or oral exposure (Budavari, 2000).

Glycoproteins are large molecules and are poorly absorbed from the gastrointestinal tract. Many cell surfaces contain receptors specific for ricin and the toxin is taken up by active transport. The primary site of distribution of ricin is the liver, spleen, and adrenal cortex in the mouse. A high concentration was also found in the bone marrow (Godal et al., 1984). In rats, abrin is distributed primarily to the liver (12%) and kidney (3%; Lin et al., 1971).

The metabolism and elimination of toxalbumins is poorly understood. Ricin is eliminated by first-order kinetics when injected via IV into mice and human cancer patients (Godal et al., 1984). The plasma half-life in humans is about 2 days (Kopferschmitt et al., 1983).

Decontamination and Treatment

There are no specific treatments for toxalbumin exposure. Aggressive decontamination is recommended. With ingestions, emesis should be induced in species that can vomit (e.g., dogs, cats, ferrets, and swine), if the animal is not already vomiting. Activated charcoal can bind orally ingested toxalbumins. Monitor asymptomatic animals for 8 h following any exposure to toxalbumins. Monitor for dehydration, electrolyte disturbances, elevated liver enzymes, and hypoglycemia.

Intravenous fluids are very important to maintain normovolemia and urine output. If hematuria is present, consider alkalinization of the urine. A goat anti-ricin polyclonal and a mouse anti-ricin A-chain monoclonal antibody have been tested and shown to neutralize ricin in castor bean extract, but they are not clinically available (Lemley and Wright, 1991; Wannemacher et al., 1991).

If the toxalbumin was distributed by air, move the animal into fresh air and monitor for respiratory distress. If cough or dyspnea develops, administer supplemental oxygen. Wash animals thoroughly with soap and water. Eyes should be flushed with copious amounts of tepid 0.9% saline or water for at least 15 min. Other treatments are the same as for an oral exposure.

Species Susceptibility

There are no controlled studies demonstrating that animals are more susceptible to developing acute effects than humans.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The lack of controlled studies comparing animals and humans needs to be addressed if animals are to be used as sentinels. The existing studies and anecdotal reports do not provide enough convincing evidence (Rabinowitz et al., 2008). Sentinels would need to demonstrate easily recognizable signs before the emergence of human illness. A good sentinel could have either greater susceptibility to a particular toxin relative to humans or a shorter latency time from exposure to onset of signs (Cottrell and Morgan, 2003).

With any suspicious outbreaks, the local health department, poison center, law enforcement agencies, and the US Federal Bureau of Investigation (FBI) should be contacted

immediately. Due to the absence of a nationwide surveillance system for animal diseases, the responsibility for detecting possible outbreaks of unusual symptoms in animals will fall to several different groups of people. Farmers, agriculture officials, veterinarians, animal control officers, wildlife rehabilitators, (animal) poison control centers, and the lay public (such as animal owners) may all be involved in detecting outbreaks.

During a chemical warfare incident, the human casualties should be addressed first, followed by the animal casualties. Situations in which herds of livestock or flocks of poultry are affected are going to be much more complex to manage than exposures to house pets. The simple logistics in getting the personnel and equipment to the site of exposure can be daunting, if not impossible. Handling livestock can be dangerous, especially to untrained personnel. The use of antidotes and other pharmaceuticals must be documented in food-producing animals and withdrawal times for meat and milk must be followed. In many herd situations, humane euthanasia may be the best solution.

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CHAPTER

55

Threats to Wildlife by Chemical and Warfare Agents

Robert W. Coppock and Margitta Dziwenka

INTRODUCTION

Wildlife as a target of terrorist activity is a relatively new concept. Wildlife has been the object of criminal activity, especially removal of unwanted predators and poaching, and wildlife has suffered collateral damage during war activities. Wildlife has traditional value as a source of food, for big game hunting, as materials for garments, and as other necessities and luxuries. There is also an increased ambition to view and photograph wildlife in its natural habitat. Ecotourism is a growing industry, especially if a country has unique or rare wildlife species or spectacular habitats. Wildlife is a commercially exploited source of income in sport and trophy hunting. In some countries, wildlife-based ecotourism provides an important source of revenue. The role of government is to protect wildlife from overkilling and to protect the environmental necessities for wildlife to feed and breed; this role is generally enforced by using a variety of laws and regulations. Additional laws and enforcement measures can be used to protect wildlife from human disturbances. Terrorist activities against wildlife can be undertaken to disrupt or destroy regional income and to remove a food source from aboriginal peoples that depend on wildlife for subsistence and culture preservation. Terrorist activities by their nature are generally performed for a variety of reasons to destabilize society (Gleick, 2006). Environmental terrorism is a term that has been used to negatively label individuals or groups that want to change the manner is which natural resources are exploited, permanently altered, or destroyed (Gleick, 2006).

BACKGROUND

Wildlife can be a direct or indirect target for terrorist attack. Wildlife has a similar sensitivity to the toxic effects of physical and chemical agents as domestic animals. Essentially the same laboratory infrastructure is needed to diagnose the etiology of diseases in wildlife and domestic animals. Terrorist attacks against governmental and commercial operations could occur in more remote areas, and such attacks could have an immense impact on wildlife, including fish and other aquatic organisms. Impoundments of toxic water could be a target for terrorists for the primary purpose of contaminating potable water sources. Not linked to terrorism, there are recent examples of a large volume of toxic water being released into waterways spanning several countries with subsequent public outcry and economic impacts. The release of 50–100 tonnes of cyanide from a tailings pond into the Sasar River and eventually reaching the Danube River had a devastating impact on wildlife in Eastern Europe (Cunningham, 2005). One can only imagine the public and political reaction that would have occurred if this incident was claimed to be an act of terrorism.

Terrorist attacks could have a collateral cascading impact on wildlife. Fish and other aquatic organisms can be impacted by run-off water from fire fighting. This water could contain toxic substances and these substances could have negative impacts on wildlife. Run-off waters from burnt-over lands draining into waterways also are high in toxic substances.

Terrorist attacks can create wildlife problems. Birds and rodents can impact the recovery of biological

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evidence during forensic investigations (Chipman et al., 2002).

ILLICIT AND RESTRICTED SUBSTANCES

Background

There are concerns regarding illicit pesticides being used for clandestine activities (Whitlow et al., 2005). Because of their extreme toxicity, over time the use of certain substances was limited and in some cases they were even deregistered. These compounds are especially dangerous to wildlife because residual levels in carcasses can cause relay toxicity in scavenging and opportunistic feeders.

Tetramethylenedisulfotetramine

Background

Tetramethylenedisulfotetramine (TETS; 2,6-dithia-1,3,5,7-tetraazatricyclo[3.3.1.13,7]-decane,2,2,6,6-tetraoxide, CAS No. 80-12-6, "tetramine") is emerging as an illicit neurotoxin (Barrueto et al., 2003; Whitlow et al., 2005). TETS is an odorless, white, crystalline power that is readily soluble in water, mixes with feedstuffs, and is generally considered to be tasteless. It is estimated that 50% of the rodenticides in China contain TETS (Owens et al., 2009).

Mechanism of Action

TETS selectively and irreversibly binds with the chloride channel on the γ-aminobutyric acid receptor and disrupts regulation of chloride in the neuron. Inhibitory activity in the central nervous system (CNS) is decreased and clinical manifestations are seizures, with onset occurring soon after TETS ingestion. The toxicity of TETS is generally considered to be more potent than sodium monofluoroacetate (SMFA; Compound 1080). The oral LD_{50} of TETS for most species is 0.1–0.3 mg/kg of body weight, and the total lethal dose for a human is considered to be 10 mg. Rabbits dosed with TETS 0.4 mg/kg body weight and killed 1 h later had detectable levels (0.07–0.238 μg/g) in the liver, kidney, heart, and lung (Xiang et al., 2001). TETS is excreted in urine, and urine can be used for forensic investigations (Zeng et al., 2006). Scavenging animals and birds can be poisoned with TETS because of the stability of TETS in tissues and body fluids.

Pathology and Detection

The pathology of TETS in humans has been reported (Zhou et al., 1998). Pathological observations in poisoned humans were edema of the brain, hemorrhages in the brain stem, and myocardial degeneration in the papillary muscles. Cardiac myopathies of varying distribution are a common finding in the majority of species. Human tissues and urine can be assayed for TETS (Xiang et al., 2001; Zeng et al., 2006). A reasonable assumption is that similar pathology and levels in tissues and urine would occur in other species. Analytical methods include liquid chromatography mass spectrometry and gas chromatography–mass spectrometry (Owens et al., 2009).

Sodium Monofluoroacetate and Sodium Fluoroacetamide

Background

Sodium monofluoroacetate (SMFA; Compound 1080) and sodium fluoroacetamide (Compound 1081) are candidate chemicals for terrorists and criminal activities targeting humans, domestic animals, and wildlife (Holstege et al., 2007). The toxicology of sodium fluoroacetamide is similar to SMFA (Osweiler et al., 1976). SMFA and sodium fluoroacetamide were used as pesticides (Shlosberg and Egyed, 1975; Proudfoot et al., 2006). In addition to being a synthetic chemical, SMFA and closely related compounds are found in *Dichapetulum* and other species of plants. The use of SMFA as a poison to eliminate unwanted animals and birds is restricted in many countries, primarily because of large kills in nontarget species (Ataria et al., 2000; Eason, 2002). Countries with limited indigenous mammal populations use SMFA to control imported animal species that disrupt and endanger indigenous wildlife (Eason, 2002). SMFA is water-soluble and can be easily added to water, baits, and feedstuffs. The use of SMFA can be limited to kill problem predators. For example, SMFA in some jurisdictions has been limited to sheep collars containing SMFA. In these applications, the SMFA is contained within bladders that are punctured during a predator attack (Burns and Connolly, 1995). Diagnosis of intoxication with SMFA could be delayed because the majority of attending medical professionals would not immediately suspect intoxication with a restricted substance. SMFA is essentially not detected by the sense organs. It is a white powder that has the appearance of flour or sugar, and is stable in storage over a long period of time. SMFA is heat-stable to 200°C. It is soluble in water and insoluble in ethanol and lipids. In water, it is degradable by microorganisms.

Toxicology of Sodium Monofluoroacetate

Compound 1080 is absorbed from the gastrointestinal tract, respiratory tract, mucous membranes, and wounds (Holstege et al., 2007). Different routes of exposure do not have a remarkable effect on toxicity. The mechanism of action for SMFA is blockage of the tricarboxylic acid cycle. Metabolic activation by the formation of fluorocitrate is required, a process known as lethal synthesis. Fluoroacetate is converted to fluoroacetyl-CoA and then converted by citrate synthase to fluorocitrate. Aconitase catalyzes the

TABLE 55.1 Estimated Lethal Dose of SMFA in Selected Animals

Mammal or Bird	Lethal Dose (mg/kg body wt)
Dog	0.1–0.2
Coyote and fox	0.1–0.3
Cattle	0.2–0.7
Pig	0.3–0.4
Cat	0.3–0.5
Horse	0.5–1.8
Human	0.5–5.0
Goat	0.5–0.7
Magpie	0.6–2
Rabbit	0.8
Chicken	6–18
Ducks	7–9

Source: Robinson, 1979; Ataria et al., 2000; Goh et al., 2005; Collicchio-Zuanaze et al., 2010

conversion of citrate to isocitrate and aconitase is inhibited by fluorocitrate. Inactivation of aconitase blocks the tricarboxylic acid cycle. Serum citrate levels increase and serum calcium generally decreases. Fluorocitrate also blocks the transport of citrate through the mitochondrial membrane. The heart and CNS are simultaneous targets for SMFA. Animals poisoned by SMFA are a hazard to scavengers, opportunists, and carnivores. The sensitivity of species to SMFA is given in Table 55.1.

Clinical Signs of Intoxication

Clinical signs of SMFA toxicity are due to the effects on the CNS and heart (Osweiler et al., 1976; Proudfoot et al., 2006). Herbivores can show more cardiac signs. The sequence of cardiac events is arrhythmias, tachycardia, inefficient pumping (weak pulse), and death when the heart fibrillates. Clinical signs observed are ataxia, collapse, and a short interval of agonal struggling (Robinson, 1979). Field observations are usually the discovery of a dead animal with the appearance of sudden collapse and limited evidence of agonal struggling. Carnivores generally have neurological signs of hyperesthesia, aimless wandering, frenzied running, vocalization, incoordination, emesis, opisthotonus, coma, and death. Thermoregulation may be disrupted. Death occurs from 1 to 24 h after ingestion of SMFA. Birds fall from the sky and die soon after hitting the ground, or show nervous system signs and an inability to fly. Relay intoxication by SMFA and its metabolites can occur. An animal that has ingested SMFA and vomits can be lethally intoxicated, and the animal ingesting its vomit can also be lethally intoxicated. The tissues of an animal poisoned by SMFA can be lethal to animals and birds that consume them.

Pathology

Pathological findings in SMFA poisoning are those of acute heart failure and hypoxia. Hypocalcemia can be observed in SMFA intoxication (Proudfoot et al., 2006). Tissues can be assayed for SMFA by gas chromatography methods (Okuno et al., 1984).

CYANIDE

Background

Cyanide (CN^-) is poisonous to essentially all animal species and is a listed chemical for terrorists and criminal activities. Cyanide can be placed in a variety of baits, including water bait. Sodium and potassium cyanide along with other forms of cyanide can be used. Industrial activities using water impoundments can be conducted at a narrow margin of safety and could be targets for terrorists and criminals. Cyanide is also used in the extraction of gold and silver ores. The cyanide used in the process is recycled, but some cyanide escapes into the tailing pond along with unrecovered heavy metals (Donato et al., 2007). Birds, bats, and other animals using the tailing ponds as part of their ecosystem have been poisoned (Donato et al., 2007; Griffiths et al., 2014). Catastrophic release of a large volume of tailing pond slurry containing high concentrations of cyanide and heavy metals into river systems has occurred (UNEP, 2000; Macklin et al., 2003; Cunningham, 2005). The rupture in January 2000 of a Romanian dam holding 100,000 m^3 of cyanide-contaminated slurry water caused an environmental disaster (Macklin et al., 2003; Cunningham, 2005). An estimated 50–100 tonnes of cyanide and heavy metals were released into the Sasar River and then flowed through the Lapus River to the Somes River, to the Tisza River, and emptied into the Danube River. The breach in the dam was attributed to a number of factors, including a sequence of perception events. This incident was evaluated as causing a cascade of severe wildlife mortalities. It is also feasible that terrorist or criminal activities could target tailings ponds and cause a huge sudden release of contaminated water, creating a down-river environmental disaster that targets drinking water safety, wildlife, tourism, and international relations. Cyanide also has a history of being used to control unwanted wildlife and is toxic in the marine environment (Wiemeyer et al., 1986).

Toxicology

The pathophysiology of acute cyanide poisoning is due to its rapid diffusion into tissues and impairment of

TABLE 55.2 Estimated LD_{50} of Cyanide in Wildlife

Bird or Mammal	LD_{50} (mg/kg body wt)
Black vulture	4.8
American kestrel	4
Coyote	4.1
Eastern screech owl	8.6
Japanese quail	10
European starling	17
Chicken	21

Source: Sterner, 1979; Wiemeyer et al., 1986

oxidative phosphorylation and other biochemical pathways (Beasley and Glass, 1998). The primary enzyme target is cytochrome oxidase by the high affinity for ferric iron in the oxidized enzyme. Oxygen utilization is inhibited and lactate accumulation rapidly leads to metabolic acidosis. Clinical signs of cyanide intoxication in birds have been described (Wiemeyer et al., 1986). The progression of clinical signs of cyanide poisoning in vultures is ataxia, eye blinking, head bowing, wing droop, increased ataxia, seizures with tail fanning, opisthotonos gasping, and death. The progression of clinical signs in kestrels, owls, and quail is more violent. Time from ingestion to death is dose-dependent. The lethal dose 50 (LD_{50}) for some species is given in Table 55.2.

Terrorist and criminal actions that release cyanide into the environment can kill large numbers of wild animal species. Release of cyanide-polluted water can have long-term consequences on a freshwater system (Lakatos et al., 2003). Cyanide poisoning of wildlife can also be collateral damage due to its presence in run-off water from fire fighting and in run-off from burnt-over lands (Barber et al., 2003). Freshwater fish are highly sensitive to free cyanide. Fish mortality can occur at less than 20 µg/L (Barber et al., 2003; Eisler and Wiemeyer, 2004). The lethal level of cyanide in rainbow trout is changed by water temperature and exercise (McGeachy and Leduc, 1988). Young fish can also be sensitive to the effects of cyanide on the thyroid gland (Brown et al., 2004). Cyanide can reduce the number of viable eggs produced by sexually maturing rainbow trout (Lesniak and Ruby, 1982).

Readers are referred to Chapter 23 for more details on cyanide poisoning.

RICIN (CASTOR BEAN)

Background

Castor beans (*Ricinus communis*) and ricin have been identified as potential terror agents of terrorism. Castor beans and castor bean by-products could be used as terrorist agents targeting wildlife. Castor beans do not initiate unique clinical signs of intoxication and in waterfowl can be confused with other acute causes of death (Jensen and Allen, 1981). There are inconclusive reports of Canada geese being killed due to castor beans. In Texas, castor bean intoxication could have been the cause of deaths of 10,000 ducks in 1967, and 2,000 ducks in 1969–1970 (Jensen and Allen, 1981). The death of 1,673 ducks occurred again in Texas in 1971. A castor bean was found in the gastrointestinal tract of one duck during pathological examination. Castor beans had been grown in the area. Although inconclusive, this report does incriminate voluntary ingestion of castor beans as the cause of death in ducks.

Pathology and Toxicology

Ducks taken from the area where 2,000 ducks had died in 1969 through 1970 were examined by diagnostic procedures (Jensen and Allen, 1981). The toxins of *Clostridium botulinum* and pathogenic bacteria were not identified. Catarrhal enteritis was identified with hemorrhage into the intestine. Seed parts suggestive of *Ricinus communis* were found in scrapings of the wall of the proventriculus. Fatty degeneration of hepatocytes was observed.

In a study in which ducks were force-fed whole castor beans (Jensen and Allen, 1981), progressive signs of acute intoxication were passage of blood-streaked mucus, leg paresis, loss of mobility with wings, sitting, prone recumbency, and death. Necropsy findings were whole and fragments of castor beans and congestion of the liver. Histopathology changes included severe fatty degeneration of hepatocytes, granulocytic infiltration into portal areas, pulmonary congestion, and peribronchial hemorrhage; necrosis and hemorrhage were observed in the spleen. These findings support the field observation that some waterfowl may consume castor beans that can result in fatal intoxication.

The observation that wild ducks voluntarily ingest castor beans provides evidence that there is some risk that terrorists could successfully use ricin against wild ducks and other wild waterfowl species.

PESTICIDES

Background

The neurotoxic cholinesterase inhibitors developed for chemical warfare, also used by terrorists, can kill wildlife species. Birds and other wildlife are well-known nontarget species for insecticides through intentional poisonings and contaminated seeds, water, forage, and

prey (Schafer et al., 1983; Stone et al., 1984; Fleischli et al., 2004). Granular insecticides can be voluntarily ingested. Insecticides and neurotoxic chemical warfare agents could be used in acts of terrorism against a targeted wildlife population, and wildlife could be killed as collateral damage.

Vertebrates can be nontarget species when insecticides are applied according to the registered label (Marian et al., 1983; Hunt et al., 1995; Allen et al., 1996; Poppenga, 2012). Vertebrate deaths have been reported from illegal and off-label use of insecticides. Models can estimate the probability of bird deaths resulting from field application of pesticides (Mineau, 2002). Birds, mammals, fish, and other aquatic organisms can be nontarget species from pesticides that enter water systems from surface water.

Incidents of Intoxication

Waterways and flood areas can be contaminated with pesticides, especially insecticides, and result in bird deaths (Hunt et al., 1995; Elliott et al., 1996). Granules of carbofuran in flooded fields have been incriminated as the cause of duck deaths and the cause of death in raptors scavenging dead or debilitated ducks. Illegal use of insecticides resulting in the death of wildlife has also been reported (Allen et al., 1996). Rodenticides have been used to control unwanted rodents and caused collateral deaths (Ruder et al., 2011). The toxicology of pesticides has recently been reviewed (Poppenga, 2012; Gupta, 2012).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Wildlife generally has not been considered in the emergency management of terrorist events using chemical agents. Wildlife can be a direct target because they can be important sources of food and income for some regions and countries. Disruption of ecotourism could also be an objective of terrorists. Wildlife can also be collateral damage from terrorist attacks and acts of war. The need to manage problem wildlife at the remote investigation site for wreckage from the World Trade Center brought attention to this aspect of emergency management planning. Regardless of the cause, wildlife can be intoxicated when chemicals and physical agents are released into the environment. Emergency planning needs to include terrorist attacks on wildlife, especially in areas where wildlife is concentrated during breeding and migration, and where wildlife congregates during the nonbreeding season. Shooting of the mountain gorillas hopefully is not an indication that these events will become more common (Jenkins, 2008). There is a need to develop better models for investigating and understanding the total impact of all causes of incidents involving wildlife (Etterson, 2013).

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SECTION 6

TOXICOKINETICS, TOXICODYNAMICS AND PHYSIOLOGICALLY-BASED PHARMACOKINETICS

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CHAPTER

56

Toxicokinetic Aspects of Nerve Agents and Vesicants

Harald John, Frank Balszuweit, Kai Kehe, Franz Worek and Horst Thiermann

INTRODUCTION

Despite the long history of chemical warfare agents (CWAs), which were first used in large quantities during World War I, vesicants and nerve agents still represent a commonly admitted threat for military and civilian communities. Therefore, effective medical countermeasures and state-of-the-art medical care are mandatory prerequisites to take the threat of poison attacks seriously. Rising interest in effective treatment of sulfur mustard, and especially nerve agent poisoning, leads national and governmental authorities to continue programs of medical defense against toxicants. The development and continuous improvement of therapy rely on intensified knowledge of provoked pathophysiological effects, toxicology, and toxicokinetics. The latter is important when understanding the reactivity of poisons in organisms at the molecular level. Awareness of predominant biotransformation and efficient elimination processes is essential for the design of a therapeutic regimen for poisoning. For example, the development of protective scavengers is a current challenge in nerve agent defense research.

The toxicokinetic profile of a CWA in mammalian organisms depends on numerous factors, including the nature of the poison, route of exposure, and differences among species. Sophisticated study design, modern technical and analytical monitoring tools, and reliable data from literature are indispensable quality criteria that should be met when performing toxicokinetic studies. The present chapter is focused on this topic.

We will first introduce the reader to typical invasion processes of exogenous poisons. The anatomy of exposed areas of the body is discussed, emphasizing interactions with nerve agents and vesicants invading on similar routes following common absorption principles. Afterward, both classes of CWAs are discussed individually. Organophosphorus (OP) nerve agents are distinguished in terms of their physicochemical properties and toxicity, as well as invasion and distribution processes *in vivo*, which are relevant to toxicokinetic characteristics. Subsequently, special focus is given to the basics of elimination and biotransformation, describing the phenomena of detoxification and concentration decrease at the molecular level. Enzymatic hydrolysis and binding to proteins and enzymes are discussed in comprehensive detail. A few comments on excretion will finish this "journey through the poisoned body." The next several sections of the chapter are dedicated to concentration-time profiles exemplarily selected for typical routes of nerve agent exposure, followed by short introductions to predictive mathematical toxicokinetic models and to most modern bioanalytical methods that allow poison quantification and identification of biotransformation products.

Vesicants, including sulfur mustard and lewisite, are the subject of the second main part of this chapter. Coherences of invasion and distribution are presented, and the major processes of biotransformation and elimination caused by binding to proteins [and more prominently, to deoxyribonucleic acid (DNA)] are discussed. Finally, we make some comments about current bioanalytical approaches. This chapter provides readers with a comprehensive overview of the toxicokinetics of OP nerve agents and vesicants.

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OVERVIEW OF INVASION PROCESSES OF CWAS

Toxicokinetics is a subfield of toxicology that studies how, how fast, and to what extent toxicants are absorbed by, distributed in, biotransformed by, and eliminated from the bodies of living organisms. These processes depend on multifaceted conditions, such as the species and gender of intoxicated organisms, the physicochemical properties of the toxicant (e.g., hydrophobicity, charge, and molecular weight), its dose and concentration, and its chemical reactivity and stability. Furthermore, the route of exposure is a crucial parameter affecting the effectiveness of incorporation and time for distribution within an organism.

Elaboration of toxicokinetic data of CWAs is essential for designing effective antidotes, improving first aid, and optimizing therapeutic regimen and medical care. It has to be considered that data obtained from *in vitro* or *in vivo* animal studies need careful extrapolation to humans, which at least requires sophisticated mathematical models to consider basic interspecies differences (Langenberg et al., 1997; Sweeney et al., 2006; Levy et al., 2007; Worek et al., 2007).

For controlled toxicological studies, the poison is most often administered subcutaneously (s.c.), intravenously (i.v.), or intramuscularly (i.m.), whereas more realistic scenarios of CWA uptake are percutaneous (p.c.) through unprotected skin, by inhalation of aerosols and vapor, or by ingestion (p.o.) of contaminated food and drink.

Such exposure events that lead to poison uptake and its distribution in an organism are part of the invasion process, whereas all steps causing a decrease in poison (e.g., elimination by degradation, biotransformation, and excretion) are part of the evasion process. For a better understanding of the pathophysiology and toxicokinetics of CWAs, an overview of the routes of poison incorporation is given in the next section, with a special emphasis on OP nerve agents and vesicants.

Percutaneous Uptake by Contact with Skin

The total skin surface of an adult human is approximately 1.8 m^2, which is very small when compared to the adsorptive surface of the lungs (100 m^2) and the gastrointestinal tract (GIT, 200 m^2; Marquardt et al., 1999). The primary function of the skin is to protect an organism against exogenous compounds present in the external environment. Nevertheless, percutaneous uptake is the predominant route of poisoning by nonvolatile and rather lipophilic agents [e.g., *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX)], which exhibits low vapor pressure, thus making respiratory incorporation very unlikely except for the inhalation of aerosols (Czerwinski et al., 2006). In the case of vesicant agents, such as sulfur mustard and lewisite, the skin is both a target organ, susceptible to severe local effects, and a pathway for absorption of the agent, leading to its distribution and subsequent systemic effects. The protective skin architecture is provided by a sophisticated and effective barrier built of two main components: the outer epidermis and the underlying inner dermis.

Epidermis

The epidermis is composed of various consecutive complex layers, including the stratum corneum (horny layer), stratum ludicum (clear layer), stratum granulosum (granular layer), and stratum germinativum (germinative layer), which itself is subdivided into two parts; stratum spinosum (spinous or prickle layer) and stratum basale (basal layer) (Marquardt et al., 1999). For a general overview of percutaneous poison uptake, we will now restrict the introduction to the horny and germinative layers, which are in this sense the most significant epidermal strata.

The stratum corneum is the upper stratum of the epidermis. It consists of several avascular, stratified cellular layers of dead keratinocytes characterized by a very low water content (5–10%), thus making the surface hydrophobic and reducing its permeability for polar compounds. This layer, which exhibits the character of a multilayer lipid membrane, is the major barrier hindering hydrophilic substances from invading an organism. Penetration through the horny layer is most often a passive diffusion-controlled process following Fick's law in a good approximation. Because of their lipophilic nature, liquid nerve agents and vesicants can readily penetrate the horny layer, and absorption of liquids is much more effective than that of their corresponding vapors (Blank et al., 1957). VX was shown to form a reservoir in the stratum corneum, enabling continuous delayed release of the poison (Rolland et al., 2013). Accordingly, while in contact with the skin barrier nerve agents might also react with skin proteins, thereby forming adducts and detoxifying the agent, as shown for human callus reacting with VX *in vitro* (Verstappen et al., 2012). To estimate the capability of nerve agents for skin penetration the octanol:water partition coefficient, (listed as dimensionless log *P*; see Table 56.1) can be used, which correlates to the lipophilicity of the agent (Czerwinski et al., 1998, 2006). This parameter can also be used to predict the distribution of OP agents in other tissues and blood (Langenberg et al., 1997; Sweeney et al., 2006).

Skin permeation velocity of toxic substances increases with (i) decreasing molecular weight, (ii) increasing lipophilicity, (iii) increasing area of contaminated skin, and (iv) decreasing thickness of contaminated horny layer. The thickness of the stratum corneum varies depending on the area of the body and species. Heavily strained

TABLE 56.1 Physicochemical Properties of VX and G-Type OP Nerve Agents

Agent	CAS No.	NATO Code	MW (g/mol)	Boiling Point (°C)	Vapor Pressure (mbar)	Water Solubility (g/L)	Hydrolysis Rate, $\tau_{1/2}$ (h)	log *P*(–)
Cyclosarin	329-99-7	GF	180.2	239	0.059 (25°C)[a]	3.7 (20°C)	n.a.	1.04[b]
Sarin	107-44-8	GB	140.1	158	2.8 (20°C)	Miscible	39 (pH 7.0)	0.30[b]
Soman	96-64-0	GD	182.2	190	0.53 (25°C)	21 (20°C)	45 (pH 6.6)	1.82[b]
Tabun	77-81-6	GA	162.1	237–240	0.049 (20°C)	98 (25°C)	8.5 (pH 7.0)	0.38[b]
VX	50782-69-9	VX	267.4	298	$9 \times 10 - 4$ (20°C)	30 (20°C)	1000 (pH 7.0)	2.09/0.68[b]

Source: Data are taken from Munro et al. (1999) unless otherwise noted,
log *P*, octanol:water partition coefficient; MW, molecular weight; n.a., not available; $\tau_{1/2}$, period of half-change for hydrolysis.
[a]*Committee on Gulf War and Health (2004).*
[b]*Czerwinski et al. (2006).*

areas (e.g., the sole of the foot, palm of the hand, and insides of the fingers) are protected by a 400–600-µm horny layer in humans, whereas the arms, legs, and body are covered by a barrier of 8–15 µm in thickness. Since the first incidents of chemical warfare in World War I, it was observed that skin areas with a very thin horny layer, in particular the axillae and the scrotum, were most susceptible to the effects of sulfur mustard. Effects in these regions, even though they were rarely exposed to the liquid agent, were severe.

Considering thickness and structural composition of skin (e.g., the diameter and density of hair follicles, as well as the number of cell layers) is essential for choosing appropriate *in vitro* models for skin penetration. Pig skin, exhibiting a stratum corneum thickness in the ear of 8.6–28 µm, is similar to human abdominal skin (5.5–40 µm), so it offers a good model to study the agent's permeation as recently shown for VX (Vallet et al., 2008). The best model for p.c. *in vivo* exposure studies is thought to be the pig after inner ear-skin application of the agent (Chilcott et al., 2003). In contrast to persistent VX, the major fraction (98%) of a small amount of liquid sarin, which was applied p.c. to 6 human subjects, evaporated very rapidly without passing the skin because of its high volatility (Marrs et al., 2007). In the presence of organic carrier solutes, skin penetration may be enhanced, as shown for VX dissolved in isopropanol, thus doubling the permeation velocity for animal and human skin *in vitro* (Dalton et al., 2006a,b). In contrast, cooling of exposed skin regions was shown to delay entry of the agent into circulation, thus representing an optional support for the treatment of cutaneous nerve agent poisoning (Mikler et al., 2011). At the least, experimental conditions should also consider that moisture, heat, and abrasions force the transfer and uptake of permeable CWAs (Blank et al., 1957).

The germinative layer, located under the stratum corneum, consists of living keratinocytes that are responsible for the regeneration and proliferation of skin. It exhibits the highest biotransformation activity of all strata toward endogenous and exogenous substances initiating binding to carboxylesterases (CaE or CarbE). Nevertheless, sorption to cutaneous tissue may form a depot, as demonstrated for sarin (Fredriksson, 1958; Satoh and Hosokawa, 2006), which explains the delayed local fasciculation of muscles observed after s.c. administration of nerve agents (Czerwinski et al., 2006).

Dermis

The dermis (corium) consists of connective vascularized tissue composed of collagen, elastic and reticular fibers anchoring sweat and sebaceous glands, and hair follicles. Capillaries pervading the dermis and hypodermis (subcutis, located beneath the dermis) allow systemic distribution of toxic compounds once they have passed the epidermis. Therefore, percutaneously incorporated poison may be directly transported by circulation to any compartment of an organism, or it may be temporarily retained within the skin layers. In addition, it might penetrate with first-order kinetics into subcutaneous tissues and muscles, thus creating a poison depot for delayed release (Wolthuis et al., 1981; Chilcott et al., 2005). If nerve agents are kept in fat tissue, degradation by biotransformation appears rather unlikely, thus maintaining an active release system (Sweeney et al., 2006). Correspondingly, Van der Schans et al. (2003) reported that it took more than 3 h to reach a maximum concentration in blood of lipophilic VX after percutaneous administration of $1 \times LD_{50}$ to hairless guinea pigs (see the section "Percutaneous uptake," later in this chapter).

Respiratory Uptake by Inhalation

Airways and Absorption

Gaseous poison or aerosols of toxic substances are incorporated following inhalation and contact with the respiratory tract. This appears to be the predominant route of poison intake for volatile G-type nerve agents (e.g., tabun, sarin, and soman) exhibiting relatively high

vapor pressure (Table 56.1). The respiratory tract is composed of three main compartments, distinguishable by the areas and organs involved in the breathing process: (i) the extrathoracic or nasopharyngeal compartment, comprising the mouth, nose, and throat (pharynx); (ii) the tracheobronchial compartment, containing the voice box (larynx), windpipe (trachea), and right and left bronchi; and (iii) the alveolar or pulmonary compartment of the lung, which includes the bronchioles connecting the bronchi with the lobes of the lung and alveoli. The sacs and cavities of the alveoli allow diffusion-controlled oxygen and carbon dioxide exchange in the blood of pulmonary capillaries (blood–air barrier). This diffusion process follows Fick's law, whereupon the diffusion layer exhibits a thickness of 0.4–2.5 µm composed of surfactant and alveolocapillary membranes. The lung of a healthy adult person possesses 300–400 million alveoli spread over a surface of approximately 100 m^2 (Marquardt et al., 1999).

Depending on the physicochemical nature of the toxic gas or aerosol, absorption will take place at different areas of the respiratory tract. Apart from the chemical properties, the size of particles and aerosols will also affect the targeted area. Materials with diameters less than 2 µm will reach the alveoli, whereas larger ones (about 20 µm in diameter) are retained in the upper respiratory areas of the throat and bronchi, preventing gas exchange in the lung.

Absorption in the Upper Respiratory Tract

In contrast to highly lipophilic compounds, hydrophilic toxicants, characterized by higher water solubility (e.g., hydrogen chloride and fluorine), are primarily adsorbed by the wet mucosa membranes of the throat and trachea in the upper respiratory tract. This is also the primary area for G agent first-order absorption according to higher water solubility (Sweeney et al., 2006; Marrs et al., 2007). Early studies have shown that 80–90% of inhaled sarin was readily absorbed by humans when exposed to concentrations ranging from 7–43 mg min/m^3 (Oberst et al., 1959, 1968). Slightly lower rates (approximately 70%) were obtained for sarin applied to guinea pigs, dogs, and monkeys (Benschop and de Jong, 2001). More aggressive and reactive agents (e.g., vesicants and pulmonary agents) may cause harm by chemical burns, provoking time-delayed inflammation and scarring in these areas. Langenberg et al. (1998b) found significant effects of sulfur mustard in the upper respiratory tract of guinea pigs, caused by a large fraction of inhaled agent.

The nasal system of guinea pigs is more complex than that of other mammalian species, including humans. In those species, the fraction of agent absorbed or deposited in the upper respiratory system may be smaller. Consequently, the middle and lower respiratory systems are likely to be exposed to a larger amount of the agent.

Absorption in the Middle Respiratory Tract

Toxic agents with medium water solubility (e.g., chlorine and sulfur dioxide) reach farther into the middle respiratory tract, being absorbed in the bronchi and its junctions. As a result, elevated excretion of mucus, coughing, and bronchoconstriction will lead to heavy dyspnea. The respiratory tube system from the nose to the bronchioles is lined with ciliated epithelium, allowing transportation of the mucous layer produced by cells of the bronchial system from the lung into the oral cavity. This mechanism, which may be supported by coughing, allows the binding and removal of dust particles, thus protecting the alveoli from deterioration. However, once the mucus has reached the oral cavity, spontaneous swallowing might transport poison into the digestive tract of the stomach and gut (i.e., the GIT).

It is the middle respiratory tract that apparently is the most significant target in the case of respiratory exposure to sulfur mustard. Local effects, such as the formation of pseudomembranes, may both be life-threatening in the acute phase of illness after poisoning and cause long-term disability. The systemic absorption from the middle respiratory tract has never been investigated separately. However, findings from Langenberg's guinea pig model (Langenberg et al., 1998b) suggest that the largest part of inhaled sulfur mustard is deposited in that particular area.

Absorption in the Alveoli

Several toxic compounds, particularly gases of low water solubility or increased lipophilicity (e.g., carbon monoxide, hydrocyanic acid, nitrogen oxides, phosgene, and further inorganic and organic irritants) reach the alveoli and are absorbed by blood. Aggressive and reactive compounds that are inhaled may damage the epithelial cells of the alveoli, causing the liberation of edema fluid, which fatally prolongs the diffusion layer and minimizes the permeability for oxygen and carbon dioxide, finally leading to unconsciousness or death by asphyxia (toxic lung edema). Only very few hints exist for OP-induced lung edema following a yet-unraveled mechanism of action (Lainee et al., 1991; Delaunois et al., 1992; Niven and Roop, 2004). Similarly, in the case of sulfur mustard exposure, the alveoli are only of minor importance in terms of target organ for both local effects and systemic absorption (Langenberg et al., 1998b).

Nose-only Exposure Model for Controlled Respiratory Uptake in Animal Studies in Vivo

To elucidate the velocity of nerve agent absorption via the respiratory tract, which may take seconds to hours, Langenberg et al. (1998a) designed an apparatus that challenged guinea pigs by nose-only exposure with a constant stream of nerve agent vapor in air held for several minutes. As guinea pigs were not ventilated, artificially sublethal doses of soman and sarin (0.4–0.8 LC*t*50)

were applied, thus enabling the affected respiratory frequency and minute volume to be monitored. A typical concentration–time profile detected for C(–)P(+)-soman after administration of C(±)P(±)-soman is characterized by a discontinuous curvature reflecting concentration increase during absorption and concentration decrease by elimination (see the section "Respiratory uptake (nose-only model)," later in this chapter). For more detailed technical data on the nose-only apparatus, see Langenberg et al. (1998a,b) and Benschop and de Jong (2001). The same researchers also used this apparatus in subsequent experiments to investigate the respiratory uptake of vesicant agents, results of which will be discussed in the section "Respiratory uptake," later in this chapter.

Gastrointestinal Uptake by Ingestion

When an animal drinks poisoned liquids or ingests contaminated food, toxicants will be incorporated and directly transferred from the mouth, through the gullet (esophagus) and into the stomach and bowels (intestine) representing the GIT, where transfer into the circulation occurs. The physiological function of the digestive tract includes intake, breakdown, transport, and digestion of food and creation of waste (excrement). Food ingredients, as well as toxicants and their digested (biotransformed) forms, are either absorbed through the walls of the intestine and enter circulation or are eliminated by feces. The surface of the small intestine of an adult human covers more than 200 m^2 and is made up of 4–5 million tiny, fingerlike projections (villi and microvilli) covering the surface of the mucous membrane. In contrast, the resorbing areas of the large intestine (0.5–1 m^2), stomach (0.1–0.2 m^2), rectum (0.04–0.07 m^2), and oral cavity (0.02 m^2) are explicitly smaller due to lack of villi; therefore, they are of minor importance for poison uptake. Uptake by diffusion through lipid layers (hydrophobic molecules) and pores (small hydrophilic molecules) of the intestinal mucosa (following Fick's law) are the most common processes. Nevertheless, facilitated diffusion and active transport, based on affinity binding of the toxicant to carrier molecules, might also occur (Marquardt et al., 1999). The processes of pinocytosis and phagocytosis are unlikely with small CWAs. Following intestinal uptake into circulation, toxicants are directly transported to the liver, where further biotransformation by cytochrome P450 enzymes may happen. This is of essential relevance for toxification of OP pesticides, including parathion, chlorpyrifos, diazinon, and dimethoate, which are transferred into their more toxic oxon derivatives (Butler and Murray, 1997; Furlong, 2007). The liver and intestine are supposed to play very important roles in elimination of free soman, as deduced from rabbit studies at high soman dosing (Li et al., 2002).

In contrast, for sarin, it has been shown that the kidney is more important for detoxification than the liver (Little et al., 1986). However, only very limited research efforts have been undertaken to characterize this route of poison uptake for nerve agents (Sim et al., 1971; Sidell and Groff, 1974). Very high lethal oral doses were reported for tabun (rabbit: 16,300 µg/kg; rat: 3700 µg/kg; dog: 200 µg/kg) and sarin (rat: 550 µg/kg), indicating extensive hydrolysis in the GIT (Marrs et al., 2007). Very few investigations of the toxicokinetics of vesicants after ingestion have been conducted. Nevertheless, this route of exposure is supposed to result in a significant systemic absorption of an agent. The rationale behind this apparent "blind spot" of toxicokinetic investigation might be the rarity of gastrointestinal exposure during conditions of military operations. Moreover, should a GIT exposure occur, the local, rather than the systemic, effects would be life-threatening. See the section "Invasion," later in this chapter, for an in-depth discussion.

In contrast, ample work has been done with OP pesticides to address the troubling statistic of more than 500,000 deaths per year worldwide caused by accidental and suicidal ingestion (Eddleston et al., 2005, 2008a,b).

Uptake by Intravenous Injection

Intravenous uptake of CWAs is highly unlikely for realistic exposure scenarios, except perhaps for the contamination of open and bleeding wounds. Nevertheless, numerous scientific studies investigating toxicity and therapeutic treatment of CWAs made use of this route of administration. The rationale behind this design is to constitute a defined amount of poison in blood, which is immediately systemically distributed by circulation to the target compartments under conditions of 100% bioavailability. Therefore, i.v. studies are undoubtedly relevant to elaborate systemic toxicity and to characterize the impact on the whole organism. It was shown that sarin was distributed in the central nervous system (CNS) within 20–30 s after i.v. administration to mice (Waser and Streichenberg, 1988). Therefore, poison acts without delayed uptake caused by diffusion or permeation through skin, tissue, and organs, which would increase the extent of degradation and hydrolysis by biotransformation. The latter processes reduce the amount of toxic agents and hamper making a direct correlation to poison concentrations in blood. As demonstrated by Van der Schans et al. (2003) only 2.5% bioavailabilty was observed in guinea pigs, resulting from a 7-h permeation period after percutaneous administration of VX.

As outlined in previous sections, the amount of incorporated poison and its fraction interacting with target molecules to induce more- or less-specific toxicological effects may vary drastically, even under controlled

conditions of administration. However, monitoring of quantitative concentration-time profiles of the original poison and its biotransformation products is needed to elucidate the kinetic behavior and to unravel pathophysiological mechanisms. Due to the broad range of such essential variations causing a number of uncertainties, many *in vivo* and *in vitro* studies have been performed during the last 30 years of sophisticated research on protection against OP compounds (OPCs) and blister agents. The following sections will summarize the most important consolidated findings underlining the current status of toxicokinetics of the most prominent CWAs: nerve agents and vesicants.

NERVE AGENTS

OPCs as Nerve Agents

For important background information addressing the history, synthesis, basic chemical data, decontamination, toxicity, military use, and political relevance of nerve agents, we refer readers to a number of textbooks, reports, and monographs that go beyond the scope of this toxicokinetic contribution (e.g., Koelle, 1963; Franke, 1977; Munro et al., 1999; Augerson, 2000; Committee on Gulf War and Health, 2004; Langford, 2004; Marrs et al., 2007; Richardt and Blum, 2008). Nevertheless, in this section, selected chemical and physicochemical properties are considered that are of importance for understanding the toxicokinetic behavior of OPCs.

Well-known representatives of nerve agents are cyclosarin (*o*-cyclohexyl methylphosphonofluoridate; GF), sarin (isopropyl methylphosphonofluoridate; GB), soman (1,2,2-trimethylpropyl methylphosphonofluoridate; GD), tabun (ethyl dimethylphosphoramidocyanidate; GA), VX (*S*-2-diisopropylaminoethyl *o*-ethyl methylphosphonothioate), and Russian VX [*o*-isobutyl *S*-(*N*,*N*-diethylaminoethyl)methyl phosphonothioate; RVX, VR] (see Table 56.1 and Figure 56.1). Apart from these substances, a large number of additional OP toxicants with high structural similarity have been seen on experimental and laboratory levels, especially when pesticides for civilian use are included. However, the abovementioned compounds are the most prominent nerve agents in literature. This fact is obviously due to political instructions for national security and defense programs that consider historical development, large-scale production, and intended military use and focus on agents that most likely would be relevant chemical threats. Soman, for example, was produced in large amounts during the Cold War by the Soviet Union in particular, thus menacing the rest of the Western world. Therefore, toxicokinetic studies on nerve agents are mainly restricted to sarin, soman, and VX. In addition, following United Nations (UN) inspections in Iraq in the 1990s, which revealed the weaponizing of cyclosarin, some studies on the toxicokinetic properties of cyclosarin were also conducted.

Physicochemical Properties

If they are present in a highly pure state, all nerve agents are colorless and odorless liquids characterized by different vapor pressures, thus causing either very rapid evaporation with increased inhalational risk (e.g., G-type agents) or relatively high resistance abating p.c. absorption (e.g., V-type agents and cyclosarin; see Table 56.1). Therefore, visual or sensory recognition of these substances by smell or taste is nearly impossible.

Water Solubility

Water solubility is a crucial parameter affecting the toxicological potency of a compound. Solubility of nonhydrolyzed nerve agents is fundamentally determined by the hydrophobicity and extent of organic substituents. On one hand, hydrophilic compounds exhibiting great water solubility are less effectively absorbed by skin in the absence of organic carrier solutes, but they are easily distributed once they have reached aqueous biological fluids. Therefore, skin penetration of less lipophilic sarin (miscible in water) is not favored, whereas nerve agents of significant lipophilicity with low water solubility (VX: 30 g/L; GF: 3.7 g/L; see Table 56.1) penetrate skin and other hydrophobic biological membranes and mucosa unhampered (Winkenwerder, 2002). Nerve agent transfer into blood and its systemic distribution are limited, potentially provoking accumulation in fatty tissue. Nevertheless, noncovalent binding to carrier proteins of circulation (e.g., γ-globulin and albumin) may support systemic transport of not very water-soluble compounds in blood, as would be expected for VX (Vallet et al., 2008), and as commonly known for endogenous fatty acids or exogenous drugs (John and Schlegel, 1999; Li et al., 2007; Weiss et al., 2008). Table 56.1 denotes corresponding measures of solubility in water.

Octanol:Water Partition Coefficient

The lipophilicity of nerve agents and their expected partition behavior are characterized by the octanol:water partition coefficient ($\log P$). This parameter is often used as an estimate for the tendency of the toxicant to bioaccumulate in an organism. Furthermore, it is helpful to predict the penetration of skin and distribution between tissue and blood (Poulin and Krishnan, 1995; Czerwinski et al., 1998, 2006). The $\log P$ for the more polar sarin was determined to be 0.30, whereas this measure for lipophilic VX is 2.09, which documents a 50-fold higher lipid solubility. However, this ratio might be slightly different when considering the inconsistency of data

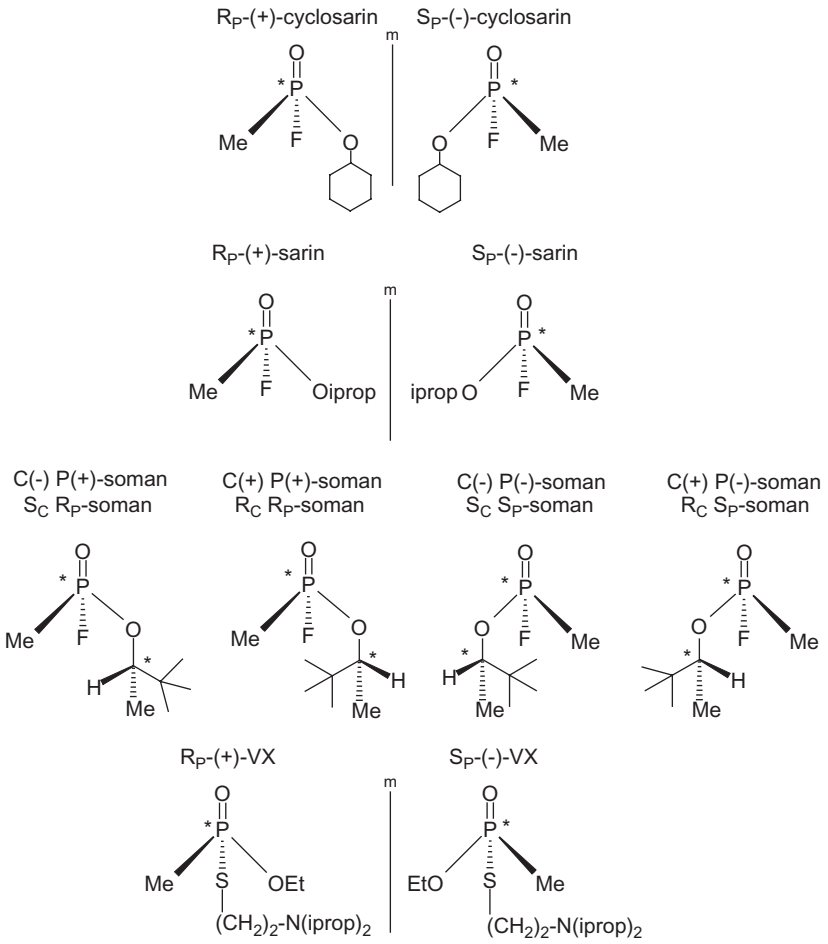

FIGURE 56.1 Structures of stereoisomeric OP nerve agents. Chirality was assigned according to the rules of Cahn, Ingold, and Prelog (Cahn et al., 1966; Prelog and Helmchen, 1982) considering oxygen of the P═O bond with minor priority compared to alkoxy substituents (Quin, 2000). Empirically found opticity is, according to Benschop and de Jong (1988), correlated to compounds obtained from stereoselective synthesis (Li et al., 2001). Chirality emerged as a crucial parameter for toxicokinetic properties. SP-isomers (P(−)-forms) of illustrated G agents (cyclosarin, GF; sarin, GB; soman, GD) are characterized by higher toxicity combined with higher stability against hydrolyzing mammalian wild-type enzymes as their corresponding RP-isomers, P(+)-forms (Table 56.3).

from literature. Table 56.1 summarizes the log P values of selected nerve agents.

Hydrolysis

In aqueous media, nerve agents undergo nonenzymatic hydrolysis, which is accelerated by very acidic and basic pH values (Franke, 1977). Hydrolysis primarily substitutes the reactive leaving group of the OPC for a hydroxy group, thus making the molecule more soluble (Figure 56.2). After cleavage of diisopropyl ethyl mercaptoamine (DESH) from the phosphorus atom of VX (Figure 56.1), the remaining ethyl methylphosphonic acid exhibits a water solubility of 180 g/L, which is six times higher than VX itself (Munro et al., 1999). Depending on pH, the alkoxy-group of VX might also be cleaved from the phosphorus atom. Hydrolysis of the reactive electronegative leaving group, which is essential for primary toxicity (inhibition of acetylcholinesterase (AChE)), deactivates the molecule, reducing its toxicity dramatically. The major hydrolysis product of sarin is isopropyl methylphosphonic acid (IMPA), which is about 10,000 times less toxic than its precursor when administered orally to rats (Munro et al., 1999). IMPA is also the most prominent biotransformation product of sarin produced *in vivo* (Little et al., 1986). The dramatically increased rate of hydrolysis under acidic conditions is presumably the most important reason for extraordinarily high lethal dose (LD_{50}) values found in laboratory animals after oral administration of nerve agents (Marrs et al., 2007). Table 56.1 summarizes hydrolysis rates expressed as periods of half-change in aqueous solutions of nerve agents near neutral pH.

Chirality

Typical production batches of nerve agents formerly intended for military use are mixtures of enantiomers

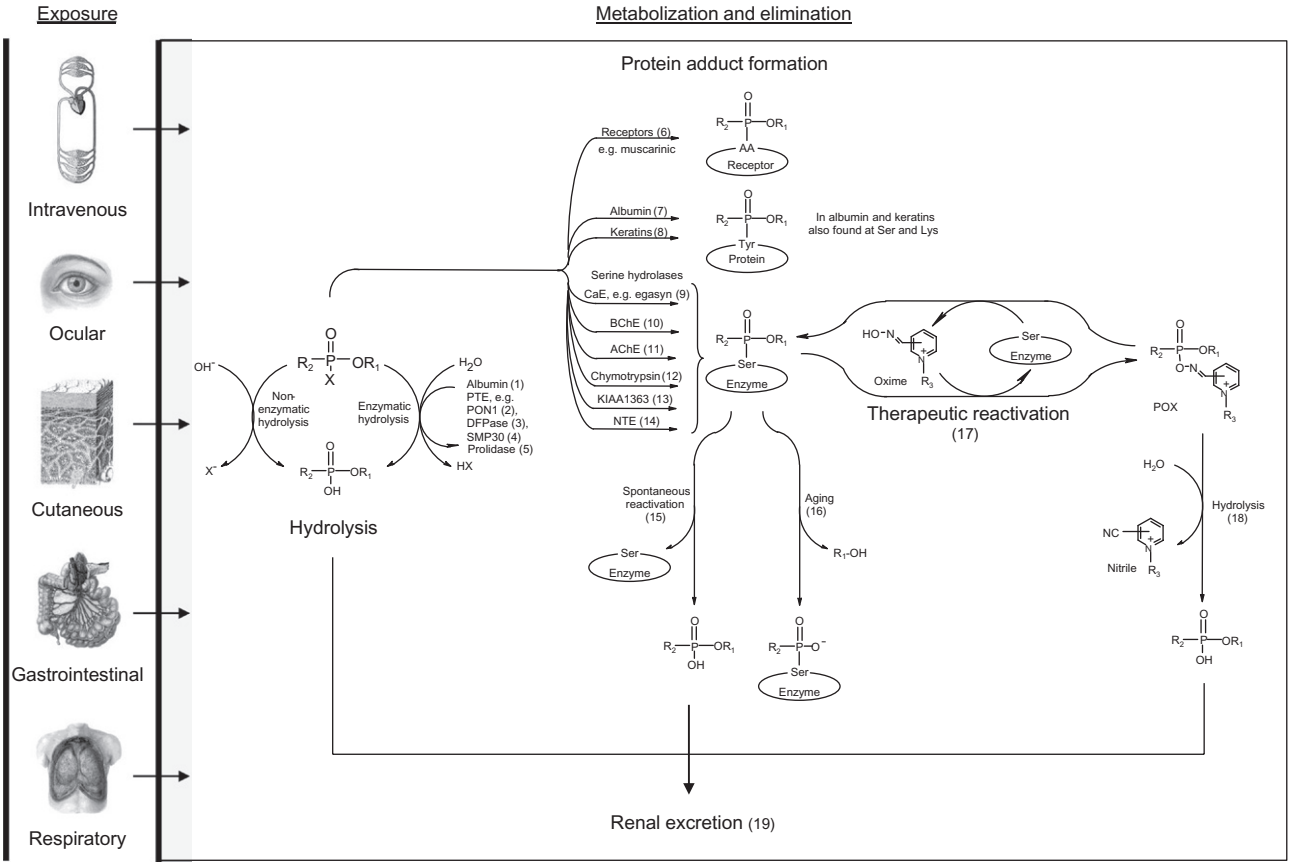

FIGURE 56.2 Elemental steps of toxicokinetics of OP nerve agents in mammalian organisms. OP nerve agents are incorporated following different routes of exposure. Whereas G agents are predominantly uptaken by the respiratory tract via inhalation, V agents mainly follow percutaneous invasion. Once the poison has reached circulation, it is distributed systemically, causing poisoning of the CNS and PNS by inhibition of AChE. Several processes of biotransformation (e.g., enzymatic and nonenzymatic hydrolysis) and elimination (e.g., formation of adducts by binding to proteins and multiple serine esterases, followed by marginal spontaneous reactivation and more prominent aging) reduce the amount of circulating poison. Therapeutic causal intervention by oximes reactivates cholinesterases under liberation of a toxic phosphoryloxime intermediate (POX), which itself undergoes immediate hydrolysis. Hydrolyzed nerve agents emanating from these chemical conversions are excreted by the kidney more prominently than by liver. Legend: AA, amino acid; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CarbE, carboxylesterase; DFPase, diisopropyl fluorophosphatase; KIAA1363, acetyl monoalkylglycerol ether hydrolase; Lys, lysine; NTE, neuropathy target esterase; PON1, paraoxonase 1; POX, phophoryloxime; PTE, phosphotriesterases; $R_{1/2}$, organic substituents, e.g., methyl, cyclohexyl, isopropyl; Ser, serine; SMP30, senescence marker protein 30; Tyr, tyrosine; X, nucleophilic leaving group; e.g., F, CN, (CH2)2-N(iprop)2. (1) Vilanova and Sogorb (1999); Manoharan and Boopathy (2006); Li et al. (2007); (2) Billecke et al. (2000); Amitai et al. (2006); Furlong (2007); (3) Blum et al. (2006); Nordgren et al. (1984); (4) Kondo et al. (2004); (5) diTargiani et al. (2010); (6) Silveira et al. (1990); (7) Black et al. (1999); Li et al. (2007, 2008); Grigoryan et al. (2009); John et al. (2010); (8) Grigoryan et al. (2009); Schopfer et al. (2010); Verstappen et al. (2012); (9) Maxwell and Brecht (2001); Satoh et al. (2002); Fujikawa et al. (2005); (10) Bartling et al. (2007); Kolarich et al. (2008); (11) Benschop and de Jong (2001); Aurbek et al. (2006); (12) Casida and Quistad (2005); (13) Nomura et al. (2008); (14) Gordon et al. (1983); Costa (2006); (15) Worek et al. (2005); Aurbek et al. (2006); (16) Worek et al. (1998, 2005); Bartling et al. (2007); (17) Sidell and Groff (1974); Kiderlen et al. (2005); Aurbek et al. (2006); Bartling et al. (2007); (18) Kiderlen et al. (2005); (19) Waser and Streichenberg (1988); Shih et al. (1994); Minami et al. (1997).

obtained from nonchiral synthesis (Figure 56.1). Sarin, cyclosarin, tabun, and VX consist of mixtures of two enantiomers; each of which differs in the chirality at the central phosphorus atom. This enables the rotation of linearly polarized light clockwise [P(+)-enantiomers] or anticlockwise [P(−)-enantiomers; see Figure 56.1]. In contrast, chirality of soman appears more complex based on two chiral centers, which reside at the phosphorus atom, P(+) and P(−), and additionally in the pinacolyl moiety, C(+) and C(−). Hence, soman occurs in four stereoisomeric conformations as two pairs of diastereomers: P(+)C(+), P(+)C(−), P(−)C(+), and P(−)C(−); (Figure 56.1). To denominate stereoisomers of nerve agents, the experimentally found rotational direction of light is typically provided, whereas assignments according to the R and S nomenclature are rare. Therefore, Figure 56.1 summarizes both absolute configurations and related optical activities of the most common nerve agents. Chirality was assigned according to the established rules of Cahn, Ingold, and Prelog (Cahn et al.,

1966; Prelog and Helmchen, 1982), considering oxygen of the P═O bond with minor priority to alkoxy substituents (Quin, 2000). Empirically found opticity is, according to Benschop and de Jong (1988), correlated to compounds obtained from stereoselective synthesis (Li et al., 2001).

It is well known that chirality of OP nerve agents causes significantly differing toxicological properties determining poison elimination by hydrolysis and kinetics of enzyme inhibition (Benschop and de Jong, 1988). Therefore, special attention should be paid to the corresponding stereoisomers when elaborating toxicokinetics. A number of studies took care of these conformational differences by administration of pure stereoisomers (Benschop and de Jong, 2001) or by enantioselective detection and quantification of poison molecules (Spruit et al., 2001; Li et al., 2003a,b; Van der Schans et al., 2003; Reiter et al., 2007, 2008, 2011; Yeung et al., 2007; Tenberken et al., 2010). Different techniques were established to produce pure (or at least enriched) enantiomers of nerve agents; e.g., (i) synthesis by use of chiral adducts subjected to reactions of defined strereochemical outcome; (ii) fractional crystallization, chromatographic separation, and isolation of mixed enantiomers; (iii) stereoselective binding to serine esterases (e.g., α-chymotrypsin); and (iv) stereoselective enzymatic hydrolysis by phosphorylphosphatases (chemoenzymatic preparation; Benschop and de Jong, 2001; Li et al., 2001).

Stereoselective enzymatic degradation of nerve agents is also a current issue in developing both novel noncorrosive decontamination systems and new therapeutics using recombinant mutated enzymes optimized for fast and exhaustive hydrolysis of most toxic isomers (Tsugawa et al., 2000; Li et al., 2001; Ghanem and Raushel, 2005; Furlong, 2007; Blum and Richardt, 2008).

Toxicity

OPCs, especially nerve agents, represent a class of highly reactive compounds undergoing nucleophilic substitution of their leaving group [e.g., F, CN, S-$(CH_2)_2$-N(iprop)$_2$; Figure 56.1] by nearly irreversible coupling to nucleophiles [e.g., strongly polarized hydroxyl groups in amino acid side chains or OH functions in aqueous media (Figure 56.2)]. Toxic effects are mainly due to derivatized enzymes which were subjected to this reaction.

Although most toxicological studies were performed as animal studies, some data about humans exist, which were obtained from military volunteers exposed to nonlethal doses of sarin (NRC, 1982, 1985), accidentally intoxicated industrial workers (Duffy et al., 1979), and poisoned civilians affected by terrorist attacks in Tokyo in 1995, Matsumoto in 1994, and Osaka (Morita et al., 1995; Okumura et al., 1996; Tsuchihashi et al., 1998). For detailed data on these events, see the reports of the Committee on Health Effects Associated with Exposures During the Gulf War (2000).

Inhibition of AChE

Phosphylation of the OH moiety of serine residue, being part of the catalytic triad in the esteratic center of AChE, represents pathophysiologically the most important reaction, resulting in enzyme deactivation. Inhibition of AChE was proved to be the predominant major reaction *in vivo*, which causes death within minutes in mammals, insects, and other species depending on acetylcholine (ACh)-mediated signal transduction. Maxwell et al. (2006) found compelling arguments that inhibition of AChE by nerve agents is the primary mechanism of OP toxicity. They correlated the median LD_{50} of highly toxic nerve agents determined from rats after s.c. administration with the corresponding bimolecular rate constants of AChE inhibition determined *in vitro*, using a probit model for interpreting the mathematical relationship.

AChE is present in the nervous system, where it is most important for toxic effects, and on the surface of red blood cells (RBCs), where its biological function is still unknown. Inactivation of AChE hinders the degradation of the neurotransmitter ACh in the synaptic cleft, which is of major importance for regulation of presynaptic and especially postsynaptic effects. Rising ACh concentrations cause permanent overstimulation of muscarinic (subtypes m1–m5) and nicotinic receptors of effector cells, leading to cholinergic crisis and, ultimately, death. Clinical symptoms of poison-induced AChE inhibition include (i) muscarinic effects (e.g., miosis, bradycardia, increased secretion of urine, saliva, tears and sweat, bronchoconstriction, and increased gastrointestinal motility); as well as (ii) nicotinic effects (e.g., muscular weakness, twitching and tremors, elevated blood pressure, and tachycardia); and (iii) central effects (e.g., headache, impaired memory and alertness, anxiety, insomnia, and most important, respiratory depression and paralysis). Death is caused by respiratory failure, as elicited by flaccid paralysis of respiratory muscles, and bronchoconstriction, together with increased bronchial secretion and central respiratory depression (Costa, 2006). Liquids or vapors from these agents can cause death within minutes after exposure. Table 56.2 summarizes the LD_{50} values for different species and routes of administration for the most common nerve agents. The relative lethality of these substances, determined in animal studies, is as follows, listed in descending order: VX, soman, cyclosarin, and tabun (Sidell and Borak, 1992).

Local irritations do not occur except by fasciculation of underlying muscles after percutaneous uptake or miosis caused by excessive stimulation of muscarinic receptors on the papillary sphincter muscles, resulting from ocular exposure (Sidell and Borak, 1992; Dabisch et al., 2008). However, clinical symptoms related to massive restraints in motoric and respiratory abilities require the reduction of AChE activity by more than

TABLE 56.2 Acute Lethality of OP Nerve Agents

	LD_{50} (μg/kg)			
	Sarin	**Soman**	**Tabun**	**VX**
INTRAVENOUS				
Human	14[a]		14 (LD_{Lo})[b]	1.5 (TD_{Lo})[c]
Rat	45–63[b]	44.5[d]	70[b]	7–10[b]
Mouse	83[e]	35[d]	150[c]	20[e]
Guinea pig		27.5[e]		
Rabbit	15[c]		63[c]	
PERCUTANEOUS				
Human	24–28 × 103[b]	18,000 (LD_{Lo})[c]	14–21 × 103[b]	86 (LD_{Lo})[c]
Rat	2,500[b]		18,000[d]	
Mouse	1,080[d]	7,800[c]	1,000[d]	
Guinea pig	8,750[f]	9,930[f]	25,840[f]	34[f]
Rabbit	925[d]		2,500[c]	
SUBCUTANEOUS				
Human				30 (LD_{Lo})[c]
Rat	103–108[a]	70–165[b]	162[b]	12[c]
Mouse	170[a]	156[e]	250[c]	22[d]
Guinea pig	30[c]	24[d]	120[c]	8.4[d]
Rabbit	30[c]	20[d]	375[c]	14–66[d]
RESPIRATORY[g]				
Human	50–100[a]	70 (LD_{Lo})[c]	150 (LD_{Lo})[c]	5–15[h]
Rat	80–300[a]		30.4[d]	
Mouse	240–380[a]	33.3[c]	0.5[c]	
Guinea pig	100–200[a]		197[c]	
Rabbit	75–144[a]		84[c]	

LD_{Lo}, lethal dose, low: the minimum amount of a chemical which has shown to be lethal to a specified species; TDLo, lowest toxic dose. Data do not consider chiral distinctions.
[a]*Winkenwerder (2002).*
[b]*Subcommittee on Chronic Reference Doses for Selected Chemical Warfare Agents, National Research Council (1999).*
[c]*Maynard and Beswick (1992).*
[d]*ToxNet, Toxicology Data Network.*
[e]*Benschop and de Jong (2001).*
[f]*Czerwinski et al. (2006).*
[g]*Given as LCt_{50} [mg min/m^3].*
[h]*Augerson (2000).*

70%, as deduced by Thiermann et al. (2005) from murine diaphragm experiments. Accordingly, acute cholinergic syndromes in humans were not observed until the RBC AChE activity was inhibited by 75–80% (Sidell and Borak, 1992). Clinical symptoms may depend on the gender of the animal, as recently documented for the extent of miosis after vapor exposure to soman, cyclosarin, and VX (Dabisch et al., 2008). Male rats were approximately three times less sensitive than female rats, whereas miniature pigs show the reverse effect. This phenomenon refers to different activities of ocular AChE and butyrylcholinesterase (BChE; Dabisch et al., 2008).

Additional Targets with Potential Clinical Relevance

As binding to proteins other than AChE reduces the amount of free OP poison, these alternative targets are

to be considered when discussing toxicokinetic behavior. Albumin (Black et al., 1997a,b; Li et al., 2007, 2008; Williams et al., 2007; John et al., 2010), receptor/channel complexes (Pope, 1999), muscarinic and nicotinic ACh receptors (Bakry et al., 1988; Silveira et al., 1990), and other secondary serine hydrolase targets were shown to be chemically modified by OPCs; changing their functionality into potential pathophysiological situations by affecting noncholinergic mechanisms (Duysen et al., 2001; Casida and Quistad, 2005). In general, chemical modification of any protein and enzyme requires very high OPC concentrations that are far beyond the lethal dose of nerve agents, thus being of only minor relevance for acute poisoning scenarios (Pope, 1999). In contrast, elaboration of toxicity of the much-less-toxic OP pesticides is more and more focused on these additional targets; investigating, for example, genetic susceptibility, developmental toxicity and neurotoxicity, delayed neurotoxicity, and organophosphate-induced delayed polyneuropathy (OPIDP), a distal sensorimotor axonopathy (Costa, 2006; Balali-Mood and Balali-Mood, 2008). OPIDP is associated with OP-inhibited enzyme neuropathy target esterase (NTE), which undergoes an essential aging process of phosphylated NTE (elimination of an organic substituent from the central phosphorus atom, as shown in Figure 56.2; Costa, 2006). However, Gordon et al. (1983) demonstrated that, despite NTE inhibitory potency of the nerve agents tabun, soman, and VX, no OPIDP was induced. Nerve agent concentrations inhibiting half of the NTE activity were about three orders of magnitude higher (micromolar range) than for AChE (nanomolar range).

Using microarray technology, Gao et al. (2013) performed a toxicogenomic study on neural cells that had been exposed to VX. A huge number of affected gene expressions relevant in numerous physiological and pathophysiological processes were identified, indicating a broad variety of targets for potential harm. Specific long-term changes in the brain were also observed after exposure to nerve agents (Zhu et al., 2010; Spradling et al., 2011; Oswal et al., 2013). In addition, epigenetic changes were found in cell culture models after paraquat exposure (Song et al., 2011). The aerotoxic syndrome in humans is discussed as possibly being associated with the incorporation of OP tri-O-cresyl-phosphate (TOCP) as an additive in jet hydraulic and engine fluids (Carletti et al., 2013). However, the mechanism causing signs and symptoms is still unknown.

Elemental Steps of Nerve Agent Toxicokinetics

Although toxicological characterizations in terms of mean acute lethal doses for different species are available for all nerve agents, detailed and extensive toxicokinetic data are rare in literature for most of them, with the exception of soman and sarin (Benschop and de Jong, 2001). For VX, and in particular GF, only a very limited extent of data is available (Van der Schans et al., 2003; Reiter et al., 2007).

Invasion

Supplementary to the more common discussion on invasion processes given in the introductory overview (see the section "Overview of invasion processes of CWAs," earlier in this chapter), we now present some recent findings on skin penetration models. Nerve agents are readily absorbed through the skin, eyes, lung, and GIT. Depending on individual vapor pressures of nerve agents, different routes of poison uptake are preferred. G agents (such as sarin, soman, and tabun) are very volatile, limiting percutaneous uptake due to significant evaporation from the skin (approximately 98% for sarin). In contrast, VX exhibits high persistency due to its vapor pressure being 3,000 times lower than that of sarin (Marrs et al., 2007; see Table 56.1). Therefore, percutaneous uptake is most prominent for V agents, characterized by an absorption rate of at least 600 µg/cm^2/h, as shown for VX after inner ear-skin droplet application of 2 LD_{50} (Figure 56.2; Chilcott et al., 2005). Nevertheless, VX penetration through skin is characterized *in vitro* by a significant lag time of at least 1 h and a moderate penetration rate of about 1–2%/h (Vallet et al., 2008). These data are in accordance with early studies on human subjects demonstrating that, 3 h after percutaneous exposure of VX, only 0.4–0.6% was incorporated (Vallet et al., 2008). *In vitro* studies using guinea pig and human skin, as well as dermatomed, abdominal skin from domestic pigs, revealed significant differences in VX permeability. The highest permeability was observed for the skin of the guinea pig, whereas no significant differences in penetration kinetics were found for human skin and skin taken from a pig's flank (Dalton et al., 2006a,b). Therefore, pig skin may serve as an appropriate *in vitro* model for human skin (Vallet et al., 2008), as already established in pharmaceutical research using Franz-type diffusion cells (Franz, 1975; Simonsen and Fullerton, 2006). To predict *in vivo* human VX absorption via skin, full-thickness human abdominal skin has also been demonstrated to be appropriate (Vallet et al., 2008).

Distribution

Once nerve agents have penetrated the blood, systemic distribution, including crossing of the blood–brain barrier (BBB), causes toxicity within the CNS and peripheral nervous system (PNS). In mouse and rat studies, it has been shown that within 1 min after sublethal single-dose i.v. administration of sarin, the nerve agent was present in many other compartments (e.g., the diaphragm, heart, lung, and brain) and in much higher concentrations in the plasma, liver, and kidney (Little et al., 1986; Waser

and Streichenberg, 1988). Similar results were observed for VX (Chilcott et al., 2005) and soman, which was also found in cerebrospinal fluid with 100% bioavailability after i.v. bolus injection into pigs (Göransson-Nyberg et al., 1998; Augerson, 2000). For a mathematical description, Langenberg et al. (1997) calculated related tissue/blood partition coefficients for the distribution of soman in guinea pigs, revealing a measure of approximately 2 for liver and 1.1 for kidney; whereas the value for the lung and brain were calculated as 0.5. Sarin present in the brain did not severely inhibit AChE activity in the cortex (60%), striatum (40%), and hippocampus (56%) within 24 h (Whalley and Shih, 1989). In contrast, soman has caused more severe inhibition in these three areas (83–99%), lowering the synaptosomal sodium-dependent, high-affinity choline uptake (SDHACU) within the first 4 h in the hippocampus; whereas from 2–24 h after exposure, SDHACU increased in the striatum (Whalley and Shih, 1989). The differences are thought to be due to different aging rates and different AChE-inhibiting potencies. However, the brainstem and midbrain were influenced by neither sarin nor soman. It is assumed that active sites of the brain are affected primarily due to increased metabolic action, vasodilation, and increased blood flow in these regions (Scremin and Jenden, 1996). In contrast, 50% inhibition of AChE activity in brain was associated with death or serious signs of toxicity in mice after subcutaneous exposure (Duysen et al., 2001).

The presence of sarin, soman, and VX in the brain demonstrates the necessity for antidotes, especially AChE reactivators, to be capable of passing the BBB, representing a current scientific challenge (Lorke et al., 2008; Okuno et al., 2008).

Concentrations of sarin found in different tissues were decreased by 85% within 15 min after exposure (Committee on Gulf War and Health, 2004). Apart from active sarin, its inactivated biotransformation product, IMPA, was found in these tissues in a predominant ratio, indicating rapid *in vivo* biotransformation. This finding is discussed in the next section. Soman was mainly accumulated in the lung after s.c. challenge of rats (Shih et al., 1994) and disappeared from blood and liver 2 min after i.p. administration of 0.75 × LD_{50} to mice (Nordgren et al., 1984). In contrast, VX is more persistent *in vivo* than sarin or soman, causing delayed systemic distribution after s.c or p.c. administration. This provoked maximum concentrations of VX in blood several hours after p.c. exposure in guinea pigs (Van der Schans et al., 2003).

As outlined in the section "Toxicity," earlier in this chapter, the toxicological mechanism of action of nerve agents is based on the chemical reactivity of the nucleophilic leaving group. Therefore, biotransformation in terms of degradation by hydrolysis and binding to proteins determines bioavailability and elimination processes, regulating toxicity.

Biotransformation and Elimination

Enzymes from plasma and tissue are mainly responsible for hydrolysis of OPCs producing derivatives of phosphoric and phosphonic acids characterized by high water solubility and nearly no toxicity. *In vivo* studies in mice have shown that, within 1 min after injection, 50% of sarin was rapidly biotransformed, generating both free hydrolyzed IMPA and bound IMPA attached to esterases by phosphylation as predominant in plasma (Little et al., 1986). In contrast, V-type agents are more stable against enzymatic hydrolysis, but may undergo additional biotransformation pathways, including oxidation of nitrogen, sulfur, or both (Van der Schans et al., 2003). Due to the slow reaction velocity of nonenzymatic hydrolysis, this process is of minor importance for elimination kinetics of nerve agents under physiological conditions near neutral pH. As listed in Table 56.1, periods of half-life for nerve agent hydrolysis range from approximately 9 h for tabun to 6 weeks for VX. In contrast, the velocity of enzymatic hydrolysis in blood is much faster, defining the rate-determining step for poison elimination.

Enzymatic Hydrolysis

Enzymes that hydrolyze OPCs cleave the reactive leaving group of OPCs (e.g., F or CN) from the central phosphorus atom, initiating nucleophilic substitution by a hydroxyl group. Hydrolyzing enzyme activity is present in plasma, and to a much higher extent in kidney and liver, enabling the removal of toxicants from circulation (Sweeney et al., 2006). Early studies using an isolated hydrolyzing rat liver enzyme demonstrated degradation efficacy for nerve agents in the following order: sarin, soman, and then tabun (Little et al., 1989). Biotransformation products deactivated by hydrolysis are nearly nontoxic and easily eliminated from the organism via renal excretion (Munro et al., 1999). Despite this capability to hydrolyze OPCs, the original physiological functions and substrates of different enzymes vary significantly, not allowing assignment to a specific class of enzymes (Figure 56.2). The following section presents the most important enzyme systems relevant for nerve agent biotransformation.

Phosphotriesterases Based on historical development, different (and sometimes inconsistent) nomenclatures were used to assign enzymes that lead to the degradation of OPs. Meanwhile, the International Union of Biochemistry has introduced systematic rules and numbering to classify the relevant enzymes in the following way: hydrolases (group 3), which cleave ester links (group 3.1), that may represent carboxylester

hydrolases (group 3.1.1) or phosphoric triester hydrolases, phosphotriesterase (PTE) (group 3.1.8). The latter group contains aryldialkylphosphatase (EC 3.1.8.1) and diisopropyl fluorophosphatase (EC 3.1.8.2; Vilanova and Sogorb, 1999). As these enzymes catalyze substrate cleavage without self-inhibition, they are representatives of A-esterases.

- Paraoxonase 01 (PON1)
  Paraoxonase (PON1, EC 3.1.8.1; formerly EC 3.1.1.2) is a calcium-dependent, liver-expressed P450 PTE belonging to the class of A-esterases with broad substrate specificity toward various lactones and esters, which is present in liver and plasma associated with high-density lipoprotein particles (Vilanova and Sogorb, 1999). P450 isozymes and variants are well known for their dual role in OP biotransformation. On one hand, they bioactivate less toxic phosphorothioates to their highly toxic oxon derivatives via monooxygenase activity. On the other hand, they hydrolyze and detoxify OP insecticides, such as paraoxon, chlorpyrifos, and diazinon by dearylation, as well as nerve agents (e.g., sarin and soman) by defluorination (Davies et al., 1996; Kiderlen et al., 2005; Furlong, 2007; Figure 56.2).

  The original physiological function of PON1 is involved in inactivation of toxic products produced by lipid oxidation (Draganov and La Du, 2004). In addition, PON1 hydrolyzes OPCs, such as sarin, cyclosarin, tabun, and soman, thus accomplishing enzymatic protection against nerve agents in circulation (Billecke et al., 2000; Amitai et al., 2006; Figure 56.2). Levels and genetic variability of PON1 influence sensitivity to these specific substrates caused by Glu/Arg point mutation at position 192 of the human wild-type enzyme (Billecke et al., 2000; Dragonov and La Du, 2004). The Glu^{192} mutant is about three times more active than the Arg^{192} variant (Draganov and La Du, 2004). Despite these differentiations, rather low catalytic activity of recombinant human PON1 expressed in HEK cells occurred as a moderate stereoselective process characterized by preferred cleavage of the less toxic P(+)C(+)-enantiomer of soman (k_{cat} 1,030 min^{-1}), which happens twice as fast as for the other three stereoisomers (Yeung et al., 2007, 2008; see Table 56.3). Nordgren et al. (1984) used an enzyme isolated from swine kidney, which they called "phosphoryl phosphatase," to incubate purified enantiomers of soman *in vitro*. Whereas the less toxic P(+)-isomers (R_PS_C- and R_PR_C-soman) were hydrolyzed very rapidly ($\tau_{1/2}$ = 2 min under experimental conditions), both highly toxic P(−)-forms (S_PS_C- and S_PR_C-soman) showed much higher stability ($\tau_{1/2}$ 60–120 min). It appears likely that the predominant enzyme isolated was PON1. No stereoselective effects in the catalytic PON1 mediated hydrolysis of cyclosarin and soman using recombinant mammalian material from *E. coli* were detected by Amitai et al. (2006). No (+)-cyclosarin was detected in hemolyzed blood samples taken from swine after i.v. administration of racemic (±)-cyclosarin, whereas the (−)-cyclosarin enantiomer was present for at least 20 min after exposure (Reiter et al., 2007). These authors suppose that this phenomenon was due to rapid enzymatic and nonenzymatic hydrolysis in blood *in vivo*, but they do not discuss the potential role of PON1 explicitly. Yeung et al. (2007) conclude that variations in catalytic efficiency of hPON1 toward soman enantiomers are due to differing Michaelis–Menten constants (K_M), characterizing the stability of the enzyme–substrate complex. Consequently, site-directed mutagenesis of recombinant enzymes could cause reduction of K_M for the more toxic enantiomers, improving its hydrolyzing capacity. A tenfold to hundredfold increase in catalytic activity of wild-type hPON1 is expected to allow effective protection against incorporated nerve agents (Amitai et al., 2006; Rochu et al., 2007; Masson and Rochu, 2009). Masson and Rochu provided Chapter 70 of the previous edition. Following a directed evolution process, Goldsmith et al. (2012) succeeded in producing a recombinant variant with more than 340-fold increased catalytic activity. In addition, stereoselectivity was reversed to preferred hydrolysis of the more toxic S_P(-)-isomer of cyclosarin. Recently, the aforementioned PON1 mutant was successfully tested against 2*LD_{50} cyclosarin in a guinea pig model. Prophylactic administration of the enzyme prevented death, as well as signs and symptoms of GF poisoning (Worek et al., 2014a). In addition, this enzyme showed a catalytic activity against a broad spectrum of alkyl methylfluorophosphonates. Engineering efficient recombinant human PON1 is a current challenge in medical defense research, intending to yield an effective and biocompatible therapeutic applicable for a wide range of nerve agents exhibiting sufficient activity toward all relevant isomers (Amitai et al., 2006; Valiyaveettil et al., 2011; Kirby et al., 2013). Figure 56.2 displays its role in biotransformation and elimination.

- Senescence Marker Protein-30 (SMP30)
  The human senescence marker protein-30 (SMP30 or regucalcin, primary Swiss-Prot accession No.

Q15493) is expressed by hepatocytes and plays a role in regulation of plasma membrane Ca^{2+}-pumping activity, with the potential to rescue cells from high calcium level-induced apoptosis (Kondo et al., 2004). The expression of this liver enzyme decreases with aging. SMP30 was originally identified in rat liver and exhibits a 65% amino acid similarity to PON1 in rat species, but does not show catalytic PON1 activity toward the hydrolysis of paraoxon (Billecke et al., 1999). In contrast, mouse and rat SMP30 hydrolyze diisopropylfluorophosphate (DFP), an OPC related to nerve agents (Kondo et al., 2004). It may be speculated that this enzyme is also involved in nerve agent biotransformation in liver. This assumption is supported by the findings of Little et al. (1989), who observed hydrolysis of sarin, soman, and tabun by an enzyme derived from rat liver homogenate. Recombinant human material might also represent a valuable countermeasure for nerve agents and was expressed in *E. coli* as a properly folded and active protein (Choi et al., 2010).

- Prolidase
  Prolidase is a Mn^{2+}-dependent enzyme of 54 kDa that was purified from fibroblast cells, erythrocytes, kidney, and liver (EC 3.4.13.9) and exhibits a primary catalytic activity toward peptide-bond cleavage of dipeptides containing a C-terminal proline or hydroxyproline residue. In addition, it was found to hydrolyze G-type nerve agents tabun, sarin, soman, and cyclosarin by liberating the respective leaving groups *in vitro* with K_M values in the millimolar range. Unfortunately, recombinant human material expressed in *E. coli* exhibited a stereoselective preference for less toxic S_P-isomers; thus, it may not degrade the poison efficiently (diTargiani et al., 2010). This enzyme will affect the toxicokinetics of nerve agents and may represent an additional bioscavenger candidate when activity can be enhanced and stereoselectivity reversed.
- Diisopropyl Fluorophosphatase (DFPase)
  Some enzymes were isolated, but not unambiguously identified, from swine kidney, which exhibit DFP-cleaving activity and were denominated diisopropyl fluorophosphatase (DFPase) in older swine (Nordgren et al., 1984). This DFPase belongs to the class of A-esterases acting on DFP, tabun, and organofluorophosphates (e.g., cyclosarin, sarin, and soman). Nowadays, most recent studies define DFPase (EC 3.1.8.2, formerly assigned as EC 3.8.2.1) as a calcium-dependent PTE identified in squid *Loligo vulgaris* exhibiting unique structural properties (Blum et al., 2006). This squid-type DFPase has not yet been identified in mammalian organisms.

Nonmammalian Enzymes Several other enzymes from bacteria [e.g., PTEs or OPH, from *Pseudomonas diminuta* or *Flavobacterium* sp., OP acid anhydrolase (OPAA), from *Alteromonas* sp. and prolidases from *Pyroscoccus furiosus*, or squid (DFPase)] are also known to detoxify nerve agents, and it is of interesting relevance for novel noncorrosive decontamination approaches (Blum and Richardt, 2008; Theriot et al., 2011). So far this does not play a role in the toxicokinetics of OPCs in humans (Amitai et al., 2006; Rochu et al., 2007; Masson and Rochu, 2009). In contrast, promising studies were performed in rodents demonstrating a protective effect of OPH (Wales and Reeves, 2012). However, potential application as detoxifying antidotes remains a future challenge.

Nonproteinaceous Scavengers and Hydrolyzing Compounds Scavenging and hydrolysis of OP nerve agents *in vivo* is a major challenge in therapeutic treatment of poisoning. Compounds that enable degradation of poison to nontoxic products in the body help to minimize the toxic impact of nerve agents. In contrast to bioscavengers like PON1 or BChE, small molecules are much less immunogenic and might be of higher stability and longer half-life, representing more favorable properties for antidotal treatment. Following this idea, structures of modified β-cyclodextrins (β-CD) are currently optimized for improved hydrolysis of G- and V-type nerve agents (Estour et al., 2013). *In vitro* studies indicate reversible binding to β-CD as noninclusion (interaction with the outer surface) and as inclusion-complexes (interaction inside the hydrophobic cavity) initiating hydrolysis that might occur with enantioselective preference (Müller et al., 2013). Recently, a β-cyclodextrin derivative bearing a pyridinium oximate in 6-position of one glucose unit was synthesized and shown to possess a promising detoxification potential against a variety of alkyl methylfluorophosphonates *in vitro* (Bierwisch et al., 2014). Prophylactic i.v. injection of the β-cyclodextrin derivative prevented systemic toxicity in cyclosarin (~$2LD_{50}$) poisoned guinea pigs and preserved brain AChE activity (Worek et al., 2014b). Such compounds will seriously affect toxicokinetic behavior of poisons and support the elimination process.

Formation of Protein Adducts

Besides direct enzymatic hydrolysis of nerve agent substrates, numerous additional proteins are present in organisms that allow covalent binding to OPCs, contributing to detoxification of the poison load (Figure 56.2). Serine esterases are especially predominant targets of

nerve agents mostly undergoing irreversible adduct formation. Nevertheless, more than 75% of serine hydrolases (B-esterases) present in plasma and tissues are essentially unknown with respect to their interaction with OPCs (Casida and Quistad, 2005).

Carboxylesterase Ubiquitous glycosylated carboxylesterases (CarbE, EC 3.1.1.1), formerly named *ali-esterases*, are B-esterases belonging to the multigene enzyme superfamily of α/β hydrolases (Hosokawa and Satoh, 2006; Satoh and Hosokawa, 2006). In principle, this class of isozymes plays a major role in pharmacokinetics by hydrolytic biotransformation of exogenous ester-drugs and ester-prodrugs. However, their physiological function still remains unclear (Satoh and Hosokawa, 2006).

Carboxylesterases are very important serine esterases in plasma of nonhuman species that bind nerve agents with broad specificity, representing the major determinant for *in vivo* detoxification, especially in mice and rats (Maxwell and Brecht, 1991; Figure 56.2). Apart from catalytic CarbE activity in plasma, it is also found in several tissues and organs, including the brain, lung, kidney, and liver, thus realizing poison decrease was not only in the circulation (Satoh et al., 2002). Microsomal liver carboxylesterase 1, which is also referred to as *egasyn*, is loosely associated with a β-glucuronidase (BG) complex (Fujikawa et al., 2005). Organophosphate-inhibited egasyn causes cleavage of this complex, releasing elevated quantities of BG into circulation. Thus, BG plasma activity may serve as a sensitive biomarker for OP poisoning (Inayat-Hussain et al., 2007).

Interaction of CarbE with nerve agents follows a kinetic of first order characterized by inhibition of CarbE at the active site serine residue described by a bimolecular rate constant, k_i (Maxwell and Brecht, 2001). For noncharged nerve agents (e.g., sarin and soman), the k_i of rat serum CarbE was found to be greater than $10^6\,M^{-1}\,min^{-1}$, whereas cationic substrates (e.g., VX) are converted with poor reactivity ($k_i < 10^4\,M^{-1}\,min^{-1}$). This specificity is explained by the electrostatic characteristics of the large active site containing only a few cation-Π bonding and anionic residues (Maxwell and Brecht, 2001; Satoh and Hosokawa, 2006).

Covalent binding of OPCs to CarbE is considered an irreversible reaction of 1:1 stoichiometry, resulting in adducts that do not age (Maxwell and Brecht, 2001). In contrast, spontaneous pH-dependent reactivation liberates the enzyme, making it accessible for additional detoxification (though only to a very limited extent). At physiological pH *in vitro*, spontaneous reactivation (specified by the rate constant kr) is a poison-dependent process showing significantly different velocities depending on the size of the inhibitor (steric demand). Whereas reactivation of VX- and sarin-inhibited rat serum CarbE was faster (k_r $4.2 \times 10^{-3}\,min^{-1}$ and $3.8 \times 10^{-3}\,min^{-1}$), reactivation for soman and VR was about 10 times slower (k_r $0.44 \times 10^{-3}\,min^{-1}$ and $0.52 \times 10^{-3}\,min^{-1}$; Maxwell and Brecht, 2001). Meanwhile, Hemmert et al. (2011) produced a recombinant variant of human CarbE1 that spontaneously dephosphonylates 33,000-fold faster than the wild type after inhibition with sarin, soman, and cyclosarin. This effect was achieved by implementing a pair of histidine and glutamic acid residues proximal to the catalytic triad without changing the high-affinity binding. In addition to these differences, CarbE exhibits stereoselective properties. Nordgren et al. (1984) have demonstrated that CaE from swine liver binds to the less toxic P(+)C(+)-enantiomer of soman (R_CR_P) most efficiently, whereas conversion of highly toxic P(−)C(+)-soman (R_CS_P) happens much more slowly (Table 56.3). This fact indicates that degradation of incorporated nerve agents is primarily targeted to less toxic enantiomers, minimizing the protective effect of CaE. In contrast to G agents, no prominent enantiomeric selectivity was evident for sequestration of VX after p.c. administration (Van der Schans et al., 2003).

Consequently, striking species-dependent differences in LD_{50} values of OPCs are mainly due to variable concentrations of endogenous CarbE acting as a bioscavenger in blood (Table 56.2). Whereas mice and rats exhibit high CarbE activities, activity in rabbits and guinea pigs appears moderate. In contrast, dogs and primates, as well as humans, possess only little or no CarbE (Benschop and de Jong, 2001). These relations are obvious from s.c. soman LD_{50} values obtained from mice and guinea pigs after inhibition of CarbE (10.2 and 12.2 μg/kg), which were very similar to those obtained for dogs and primates without inhibition (9.1 and 13.0 μg/kg; Maxwell and Brecht, 1991). In contrast, the corresponding soman s.c. LD_{50} values for untreated CarbE are much higher for mice (113 μg/kg) and guinea pigs (28.2 μg/kg; Maxwell and Brecht, 1991). For these reasons, guinea pigs are most often used for *in vivo* toxicity and toxicokinetic studies intending to examine a small laboratory animal model predictive for humans. Missing CarbE activity in humans hinders efficient detoxification *in vivo*, thus keeping the toxic effect of nerve agents (Due et al., 1993). Consequently, the value of CarbE as a bioscavenger for effective clearance of OP poison from circulation has led to therapeutic concepts that were applied successfully to rodents and nonhuman primates (Maxwell and Brecht, 2001). Feasibility for human organisms has yet to be demonstrated.

Acetyl Monoalkylglycerol Ether Hydrolase KIAA1363 (primary Swiss-Prot accession No. Q6PIU2) is a human serine hydrolase derived from acetyl monoalkylglycerol ether hydrolase (AcMAGE, EC 3.1.1.) present

TABLE 56.3 Catalytic Constants for Hydrolysis of Nerve Agents and Rate Constants of Esterase Inhibition

	Inhibition rate constant, k_i ($M^{-1}min^{-1}$)								k_{cat} (min^{-1})
	Bovine	**Electric Eel**	**Human**	**Minipig**	**Pig**	**Equine**	**Human**	**Rat Plasma**	**hr wt**
	AChE	**AchE**	**AChE**	**AChE**	**AChE**	**BChE**	**BChE**	**CarbE**	**PON1**
SOMAN									
C(±)P(±)-soman	5×10^{7}[a]	1.5×10^{8}[a]	9.2×10^{7}[bc]			1.29×10^{7}[d]	2.8×10^{8}[b]	0.51×10^{7}[d]	7,500[ef]
C(+)P(+)-soman	$<1 \times 10^{4}$[a]	$<5 \times 10^{3}$[a]	2×10^{3}[gh]			1.7×10^{6}[g]	6×10^{6}[g]		1,030 ± 94[i]
C(−)P(+)-soman	$<1 \times 10^{4}$[a]	$<5 \times 10^{3}$[a]	2×10^{3}[gh]			1.2×10^{5}[g]			593 ± 54[i]
C(+)P(−)-soman	1.75×10^{8}[a]	2.8×10^{8}[a]	8×10^{7}[gh]			1×10^{7}[g]	5×10^{6}[g]	3×10^{7}[j]	553 ± 163[i]
C(−)P(−)-soman	2.7×10^{7}[a]	1.8×10^{8}[a]	1.5×10^{8}[gh]			6×10^{7}[g]	4×10^{7}[g]	1×10^{6}[j]	501 ± 45[i]
SARIN									
(±)-sarin	1.51×10^{7}[d]		3.2×10^{7}[bc]			0.56×10^{7}[d]	3.2×10^{7}[b]	0.30×10^{7}[d]	
(+)-sarin	$<3 \times 10^{3}$[k]								
(−)-sarin	1.4×10^{7}[k]								
CYCLOSARIN									
(±)-cyclosarin			4.21×10^{8}[lc]	4.84×10^{8}[l]	4.84×10^{8}[l]		7.2×10^{8}[b]		25,400[ef]
VX									
(±)-VX	3.23×10^{7}[d]		9.91×10^{7}[lc]	5.61×10^{7}[l]	4.43×10^{7}[l]	6.3×10^{7}[d]		1.51×10^{3}[d]	
(+)-VX	2.0×10^{6}[k]								
(−)-VX	4.0×10^{8}[k]								
VR									
(±)-VR			4.60×10^{8}[lc]	1.95×10^{8}[l]	1.88×10^{8}[l]				
CVX									
(±)-CVX			3.06×10^{8}[mc]		1.46×10^{8}[m]				

Rate constants for inhibition of most prominent serine esterases by nerve agents differ significantly depending on the nature of enzyme, its originating species and stereoisomers of the agent. It appears obvious that for the depicted G agents and VX, the P(−)-isomers are much more effective inhibitors than their corresponding P(+)-forms. In contrast, hydrolysis of the latter isomers happens faster than that of the toxic P(−)-variants. Hydrolytic stability is determined by PON1 activity characterized by its catalytic constant k_{cat} (last column). Both properties (inhibition and hydrolysis) cause higher toxicity for P(−)-agents *in vivo* selectively (Table 50.4). AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CarbE, carboxylesterase; CVX, Chinese VX; hr wt, human recombinant wild type; k_{cat}, catalytic constant for hydrolysis; PON1, paraoxonase 1; VR, Russian VX.

[a] *Benschop et al. (1984).*
[b] *Bartling et al. (2007).*
[c] *Human RBC AChE.*
[d] *Maxwell and Brecht (2001).*
[e] *Amitai et al. (2006).*
[f] *Recombinant rabbit PON1 from E. coli.*
[g] *Ordentlich et al. (1999).*
[h] *Recombinant human AChE.*
[i] *Yeung et al. (2007).*
[j] *Sweeney et al. (2006).*
[k] *Benschop and de Jong (2001).*
[l] *Worek et al. (2008).*
[m] *Aurbek et al. (2006).*

in the brain, lung, heart, and kidney, which is involved in tumor cell invasiveness and lipid metabolism, but can also detoxify OPCs by hydrolysis of the reactive leaving group following transient binding to the active site serine residue (Nomura et al., 2008; Figure 56.2). This capability has been demonstrated for the pesticide chlorpyrifos and its more toxic oxon derivative. Activity toward nerve agent hydrolysis is expected, but it has not been shown so far.

Acetylcholinesterase AChE (EC 3.1.1.7) from vertebrates is deduced from a single gene (Massoulie, 2002) expressing identical enzyme primary structures on the surface of RBCs, in synapses and different organs.

Inhibition of this serine-esterase causes the most prominent pathophysiological effects, determining the severity of poisoning. Covalent binding to AChE can also be considered a step in poison elimination (Figure 56.2), which reduces the amount of incorporated poison, though only to a small extent.

AChE activity in whole blood differs significantly among mammalian species, which makes it relevant for toxicokinetic consideration. Humans exhibit a very high AChE activity in blood (651 mU/μmol hemoglobin, Hb) in contrast to smaller values for miniature pigs (297 mU/μmol Hb) and pigs (190 mU/μmol Hb; Worek et al., 2008). The bimolecular rate constants (k_i) for nerve agent-induced inhibition of AChE vary among different species. Table 56.3 gives an overview of corresponding inhibition kinetics. The k_i for inhibition of human AChE by VX, for example, is about twice as high as for pig AChE (Aurbek et al., 2006). These differences must be considered when assessing interspecies toxicokinetic data.

Nerve agents show enantioselective inhibition kinetics when reacting with AChE. Chirality at the central phosphorus atom plays the predominant role affecting higher rates of inhibition for the P(−)-isomers (S_P) than for the P(+)-forms (RP; Nordgren et al., 1984). Benschop and de Jong (2001) made great efforts to look at this stereoselectivity topic, and they determined the corresponding inhibition rate constants (k_i) for electric eel AChE *in vitro* (Table 56.3). Both P(−)-diastereomers of soman (SpS_C- and S_PR_C-soman) exhibit very high k_i values ranging from 1.8–2.8 × $10^8\,M^{-1}\,min^{-1}$, which documents their high toxicity. In contrast, the less toxic P(+)-diastereomers (RpR_C- and R_PS_C-soman) are characterized by k_i values that are 100,000 times smaller (<5 × $10^3\,M^{-1}\,min^{-1}$; Benschop and de Jong, 2001). Similar results were obtained for sarin enantiomers, which differ by a factor of 5000, revealing a k_i of 1.4 × $10^7\,M^{-1}\,min^{-1}$ for the SP-(−)-form (Table 56.3). VX enantiomers exhibit the smallest differences, because the k_i of the more toxic SP-(−)-VX (4 × $10^8\,M^{-1}\,min^{-1}$) is 200 times higher than for the RP-(+)-enantiomer (Table 56.3; Benschop and de Jong, 2001). These k_i data correspond to the order of experimentally determined LD_{50} values in mice, not considering the impact of hydrolyzing enzymes *in vivo* which also exhibit stereoselective kinetics for hydrolysis (Table 56.4; Benschop and de Jong, 2001).

AChE on the surface of RBCs shows a turnover rate of about 1% per day, enabling complete exchange within approximately 100 days after exposure (Sidell and Borak, 1992). In contrast, regeneration of synaptic AChE is assumed to be faster, reaching 7–10% turnover rates, an estimation determined by animal experiments (Grubić et al., 1981; Brank et al., 1998; Eddleston, 2008b). Both are of importance for therapeutic monitoring of poisoning, as well as for verification of exposure following bioanalytical methods.

TABLE 56.4 Acute Lethality in Mice Caused by Stereoisomers of OP Nerve Agents

Nerve Agent	LD_{50} (μg/kg)
Stereoisomers	**Mouse**
SOMAN	
C(±)P(±)-soman	156 (s.c.)[a]
C(+)P(+)-soman	<5,000 (s.c.)[b]
C(−)P(+)-soman	<5,000 (s.c.)[b]
C(+)P(−)-soman	99 (s.c.)[a]
C(−)P(−)-soman	38 (s.c.)[a]
SARIN	
(±)-sarin	83 (i.v.)[b]
(+)-sarin	
(−)-sarin	41 (i.v.)[b]
VX	
(±)-VX	20.1 (i.v.)[b]
(+)-VX	165 (i.v.)[b]
(−)-VX	12.6 (i.v.)[b]

Higher rate constants of P(−)-isomers for inhibition of AChE in combination with their minor susceptibility to enzymatic hydrolysis by mammalian PTEs cause eminently different lethal doses *in vivo*.
[a]*Benschop et al. (1984).*
[b]*Benschop and de Jong (2001).*

- Spontaneous Reactivation of AChE
  Although inhibition of AChE is mainly discussed as an irreversible reaction, detailed kinetic investigations consider the process of spontaneous reactivation re-releasing an active enzyme and a hydrolyzed detoxified agent (Figure 56.2). Rate constants for spontaneous reactivation (k_s) of human AChE adducts are very small, as measured for some sarin analogs (0.01–0.052 h^{-1}), whereas the k_s for sarin, cyclosarin, and VX was too small to provoke an experimentally detectable effect (Bartling et al., 2007). Sidell and Groff (1974) demonstrated that spontaneous reactivation of RBC AChE inhibited by VX happens much faster than for sarin, which is in accordance with recent human k_s data on V agents VX 0.021 h^{-1}, VR 0.039 h^{-1}, and Chinese VX (CVX) 0.171 h^{-1} (Aurbek et al., 2006). Following point mutations in recombinant human AChE spontaneous reactivation rates for VX, VR, and CVX could be increased up to 110-fold (Trovaslet-Leroy et al., 2011). However, the toxicokinetic relevance of spontaneous reactivation is obvious from the liberation of free esterase accessible for reinhibition with 1:1 stoichiometry.

- Aging of AChE
  The cleavage of an organic substituent from the phosphorus atom, which is bound to the side chain of an amino acid residue from a protein, is described by the aging process (Figure 56.2). Promoted by specific ionic interactions of neighboring amino acid residues in AChE (and BChE), the remaining P-OH function will undergo deprotonation, leaving a negatively charged phosphorus moiety (Figure 56.2). Chandar and Ganguly (2013) investigated the aging process of soman inhibited AChE *in silico* by density functional theory (DFT) and found that dealkylation of the pinacolyl-group and migration of the methyl-group take place simultaneously, catalyzed by histidine 440 and tryptophan 84 residues located in the catalytic triad and catalytic anionic subsite, respectively. Whereas esterase adducts of sarin and soman age by cleaving alkoxy moieties from the central phosphorus atom, tabun loses its dimethylamine moiety (P–N scission) instead of its ethoxy group (Elhanany et al., 2001).

  These aged enzyme adducts are not accessible for reactivation by common antidotal oximes, limiting the success of therapeutic treatment of poisoning with OPCs. The soman–AChE adduct is known for very rapid aging ($\tau_{1/2} \approx 2$ min), causing severe problems in clinical treatment (Worek et al., 2005). Aging half-lives of phosphylated AChE and phosphylated BChE depend on the nature of the inhibiting OPC. Whereas GF-inhibited AChE exhibits a half-life of 8.7 h, the corresponding BChE derivative ages much more rapidly (2.2 h; Worek et al., 1998). The order of $\tau_{1/2}$ for aging of human AChE–nerve agent adducts was found to be, in ascending order, soman (2 min), sarin (3 h), cyclosarin (7 h), tabun (19 h), and VX (36.5 h; Worek et al., 2005). V agent-inhibited RBC-AChE ages very slowly in humans, allowing therapeutic intervention with oximes for a longer period lasting several days (Sidell and Groff, 1974; Thiermann et al., 2007).

Butyrylcholinesterase BChE (EC 3.1.1.8), formerly named *pseudocholinesterase,* is synthesized in the liver and present in blood (5 μg/mL), the synapse of neuromuscular junctions, and glia cells and axons of white matter in the brain in numerous allelic variants (Massoulie, 2002). Although BChE has long been considered a nonfunctional vestigial analog of AChE, recent findings point out a possibly more prominent role; especially in mice, where the total amount of BChE in the body is 10 times as high as AChE (Duysen et al., 2001). Correspondingly, it was observed that the activity of BChE in human whole blood was significantly higher than of AChE (Worek et al., 2008). This led to speculation that BChE may play a backup role for insufficient AChE activity in neurotransmission as deduced from the physiology of AChE knockout mice (Duysen et al., 2001) and may serve as a safeguard against diffusion of ACh into the bloodstream (Massoulie, 2002). However, mandatory experimental evidence is still missing.

Highly glycosylated BChE is a prominent target of OPCs, acting as a protective biological stoichiometric scavenger averting damage to neuronal AChE (Kolarich et al., 2008). However, common nerve agents may exhibit significantly differing inhibition rate constants (k_i); for AChE and BChE, they are approximately in the range of 10^7–$10^9\,M^{-1}\,min^{-1}$ (Bartling et al., 2007). Table 56.3 shows comparison of inhibitory potency of nerve agents against AChE and BChE. *In vivo* studies in humans suggest that VX preferentially inhibits RBC AChE much more effectively than BChE, resulting in 70% and 20% inhibition, respectively (Sidell and Groff, 1974). In addition to agent-dependent k_i values, there is a striking stereoselective dependency in BChE inhibition. The more toxic soman P(−)-enantiomers (SP) inhibit with preference when compared to their corresponding P(+)-forms (RP; Table 56.3; Nordgren et al., 1984). However, differences are not as pronounced as for AChE, but point out effective detoxification properties of endogenous BChE.

Interestingly, no clinical features result from inhibited BChE *in vivo* (Eddleston et al., 2008b). The status of plasma BChE activity is, despite all concerns, a commonly recommended measure to monitor the progress of chemical injury (Eddleston et al., 2008a).

In contrast to all studies on VX toxicokinetics and toxicodynamics published so far, Dorandeu et al. (2008) reported an unexpected and not-yet-clarified phenomenon that emerged after i.v. administration of VX to isoflurane-anesthetized and ventilated swine without oxime therapy. Time-resolved measurement of esterase activities during the experimental period of poison application revealed very fast rebound of BChE activity (from 70% inhibition to 30% within 1 h), while retaining nearly complete inhibition of whole blood cholinesterase. Design and control experiments allowed the following explanations to be excluded: (i) spontaneous reactivation, which should happen much more slowly; (ii) induced hypoalbuminemia liberating albumin as scavenger competing with BChE; and (iii) stimulated release of hydrolyzing enzymes like PON1. More probably, this observation is explained by an increased biosynthesis of BChE in the liver, an elevated release of BChE from other organs (e.g., heart, lung, or pancreas), or both. Future studies will possibly shed more light on this curious and interesting phenomenon.

As total replacement of BChE by synthesis in the liver happens within a couple of weeks, this rather long period allows experimental verification of OP poisoning, even when blood samples from poisoned humans are

collected with significant delay to the time of exposure (Sidell and Borak, 1992). Therefore, detection of BChE adducts by means of modern mass spectrometric methods is the state-of-the-art technique for proving exposure to OP nerve agents (Carol-Visser et al., 2008; Noort et al., 2006; John et al., 2008).

The relevant enzyme adducts may undergo the previously described consecutive reactions: spontaneous reactivation and aging. Jiang et al. (2013) demonstrated that human BChE can be inhibited by both enantiomers of tabun, resulting in stereospecific aging processes showing deamination for S_P-tabun and O-dealkylation for R_P-tabun. In general, aging of BChE nerve agent adducts occurs with highly different agent-dependent periods of half-change ($\tau_{1/2}$) as determined *in vitro* by Worek et al. (2005). Whereas the soman adduct typically exhibits by far the shortest $\tau_{1/2}$ (less than 1 min), cyclosarin appears much more stable ($\tau_{1/2}$ 2.2 h), followed by tabun ($\tau_{1/2}$ 7 h) and sarin ($\tau_{1/2}$ 12 h). The adduct of VX was the most stable BChE derivative characterized by a $\tau_{1/2}$ of 77 h. Spontaneous reactivation by simple hydrolysis of the serine-phosphorus bond was observed for cyclosarin, with a half-life of 20 h and much longer times for VX (63 h) and sarin (63 h; Worek et al., 2005). These data demonstrate that inhibition of wild-type BChE is nearly irreversible, lowering the amount of incorporated toxic OPCs significantly in a stoichiometric manner. Therefore, protection against nerve agent doses of up to $5.5 \times LD_{50}$ (soman and VX) was achieved with exogenous BChE from different species applied prophylactically i.m. to the guinea pig, rhesus monkey, and cynomolgus (Lenz et al., 2007). It is supposed that a dose of 200 mg BChE/70 kg will be sufficiently protective in humans against $2 \times LD_{50}$ of soman (Saxena et al., 2008). Based on this valuable protective capacity, current efforts are under investigation to use BChE from recombinant (e.g., transgenic plants or milk of transgenic animals) or natural sources for therapeutic/prophylactic treatment of nerve agent poisoning (Chilukuri et al., 2005; Lockridge et al., 2005; Huang et al., 2007; Lenz et al., 2007, 2010; Geyer et al., 2010; Saxena et al., 2011; Mumford et al., 2013). The plasma half-life of recombinant preparations is significantly prolonged by fusion to albumin (Huang et al., 2008) or pegylation (attachment of polyethyleneglycol; Chilukuri et al., 2005; Sun et al., 2013), or by polysialylation (Ilyushin et al., 2013). In addition, enhanced rapid spontaneous reactivation will deblock the enzyme, thus being accessible for subsequent binding to another OP molecule. This strategy is followed by site-directed mutagenesis of BChE, leading to a 10^5-fold increase of dephosphorylating activity (Casida and Quistad, 2005). Accordingly, substitution of histidine 117 by glycine in engineered human BChE generated a significant amount of OP hydrolase activity (Nachon et al., 2011). Feasibility for the human body still has to be shown.

Albumin Although albumin is present in blood in high concentrations (0.6 mM; 41 g/L; 50–60% of total plasma protein), it does not represent an effective scavenger for nerve agent detoxification *in vivo*, hampered by its slow reaction velocity (Li et al., 2008). Nevertheless, sarin and soman bind to albumin at active site tyrosine residue 411, as shown in neat buffered solution and crude human plasma. This inhibits the enzymatic acylamidase and esterase activity of albumin (Black et al., 1999; Li et al., 2007, 2008; Figure 56.2). Besides tyrosine 411, additional amino acid residues capable of phosphylation by nerve agents and pesticides were described by John et al. (2010) after matrix-assisted laser desorption/ionization (MALDI)–mass spectrometry (MS) analysis. Albumin also exhibits slight hydrolyzing catalytic activity against OPCs (Vilanova and Sogorb, 1999). Concerning enantioselectivity in phosphonylation of albumin by soman, contradictory results have been presented in recent studies. Li et al. (2008) did not find any enantiomeric preference, and Yeung et al. (2008) demonstrated that human serum albumin preferentially binds to the less toxic C(±) P(+)-enantiomer. However, the albumin adduct is very stable in terms of spontaneous reactivation, exhibiting extended periods of half-life (6.5 days at 25°C, pH 8.0 and 20 days at 22°C, pH 7.4); in addition, it does not show any aging phenomena (Li et al., 2007, 2008; John et al., 2010). Derivatized albumin serves as a biomarker for nerve agent exposure detectable by modern mass spectrometric techniques (Black et al., 1999; Peeples et al., 2005; Li et al., 2007, 2008; Williams et al., 2007).

Keratins Keratins are the most abundant proteins in the stratum corneum, exhibiting molecular weights of about 50–100 kDa. Formation of intermediate filaments assembled from bundles of keratin monomers cause durability and insolubility in water, representing the protective barrier of the body. Verstappen et al. (2012) found that VX and OP pesticides covalently bind to tyrosine residues in human keratins when exposed *in vitro*. MS/MS analysis revealed phosphylation in keratin 1, 6, and 10, results that are very similar to a report from Schopfer et al. (2010) documenting phosphorylation of tyrosine induced by *in vitro* incubation of chlorpyrifos-oxon with human keratin 1, 2, 9, and 10. No distinct binding motifs or consensus sequences have been identified so far that explain preferred phosphylation at specific tyrosine residues (Schopfer et al., 2010). In addition, adducts were also found at lysine residues in keratin 1 and 10. Corresponding phosphoamidate bonds showed unexpected stability (Grigoryan et al., 2009). Such binding processes might contribute to general skin absorption phenomena as observed after cutaneous exposure to VX (Chilcott et al., 2005). No pathophysiological consequences are obvious at present. These phosphylation reactions minimize the total amount of toxic nerve

agents penetrating into circulation, representing a kind of natural scavenging barrier.

Ubiquitin Ubiquitin is a small endogenous and ubiquitous protein (approximately 8,600 Da) with a nearly globular confirmation (Welchman et al., 2005). *In vivo*, it appears either as free (extracelluar space, e.g., plasma, urine, cerebrospinal fluid) or anchored molecules covalently bound to any target protein (intracellular space). Such conjugates often contain multiple ubiquitin molecules connected to long chains (poly-ubiquitinylation). The kind and extent of ubiquitin chains are crucial parameters that determine the fate of the protein with respect to proteasomal degradation. Chain prolongation requires 1 out of 7 free lysine residues to react with the C-terminal glycine of another ubiqiuitin molecule. Therefore, the presence of free, chemically unaltered ε-amino side chains is essential for correct biological function.

Recently, adducts of ubiquitin produced *in vitro* with VX, VR, and CVX were identified by different mass spectrometric methods, documenting that phosphonylation untypically occurred at at least 6 out of the 7 lysine residues selectively (Schmidt et al., 2014). An additional adduct at a tyrosine residue was also found, but to a much lesser extent. Intramolecular cyclization between the derivatized lysine and adjacent glutamic acid residues was detected. Such a reaction has never been described before for any other phosphylated protein. Cyclization will dramatically change the tertiary structure of ubiquitin. All these chemical and structural changes after reaction with V agents will surely affect biological function. It is not clear whether phosphylated ubiquitin is produced in *in vivo* poisoning scenarios or whether it is relevant to any pathophysiological situation.

Additional Proteins By means of modern, highly selective, and high-resolving mass spectrometry coupled to effective chromatographic separation, a number of mammalian proteins were shown to form covalent adducts *in vitro*. These targets include, e.g., α_2-glycoprotein 1, human transferrin, kinesin 3C motor domain 5, bovine tubulin α and β, actin α skeletal muscle, chymotrypsinogen, mouse transferrin, ATP synthase, adenine nucleotide translocase I, and porcine pepsin (Grigoryan et al., 2009; Schopfer et al., 2010). Nerve agents were found to phosphylate tyrosine, serine, threonine, and lysine residues predominantly. However, no correlation to any specific disease or evidence for any pathophysiological consequence has been reported. Nevertheless, numerous signs and symptoms in OP poisoning suggest that AChE inhibition is not the only reason behind the entire clinical picture.

Besides diverse nucleophilic side chains of amino acids from a number of endogenous proteins, OP nerve agents also form adducts with other small, nucleophilic molecules relevant for toxicokinetic analysis. Gäb et al. (2010, 2011) demonstrated that sarin, soman, and cyclosarin react with phosphate anions. Such ions are present *in vivo* or in typical phosphate buffers used for dilution of biological fluids or as solvents for kinetic measurements. G-type nerve agents (Gäb et al., 2011) and VX (Creasy et al., 2012) produce very stable pyrophosphate-like adducts fast enough to compete with sample hydrolysis in aqueous media. Similar reactivity was found for amino alcohol buffers like tris(hydroxymethyl)aminomethane (TRIS), and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), generating O-bound esters of methyl-O-alkyl phosphonates derived from sarin, soman, and cyclosarin (Gäb et al., 2010). These reactions might be relevant when planning and performing any kind of quantitative and kinetic analysis of G-type nerve agents.

Muscarinic Receptors It has been shown that tabun, sarin, soman, and VX bind to the muscarinic receptor subtype m_2, leading to the assumption that the ACh binding site is deactivated, causing potential additional vulnerability (Figure 56.2; Silveira et al., 1990). Despite the high affinity of nerve agents, pathophysiological effects seem to be of only minor relevance compared to AChE inhibition. However, binding to receptors will lower the concentration of toxic and reactive OPCs.

Excretion

Sarin and its corresponding nontoxic hydrolysis products (IMPA and additional methylphosphonic acids) are predominantly eliminated via the kidneys, so those organs play a more important role in detoxification than the liver (Little et al., 1986; Waser and Streichenberg, 1988). Urinary excretion happens very rapidly, as demonstrated for single-dose s.c. application of sarin, cyclosarin, and soman to rats (Shih et al., 1994). The terminal elimination half-life was found to be 3.7 ± 0.1 h for sarin and 9.9 ± 0.8 h for cyclosarin. Soman showed a biphasic elimination, with terminal half-lives of about 18.5 and 3.6 h (Shih et al., 1994). Maximum peak levels of sarin biotransformation products in urine were detected 10–18 h after exposure (Minami et al., 1997) and, after 2 days, hydrolyzed sarin products had been excreted nearly quantitatively (Shih et al., 1994). Even at 5 days, postexposure soman product recovery was only 62% (Shih et al., 1994). Excretion of soman from blood, liver, and kidneys following chemical and enzymatic hydrolysis is considered a first-order elimination process (Sweeney et al., 2006).

Concentration–Time Profiles of Nerve Agents in Blood After Various Routes of Administration

Extensive toxicokinetic studies in animal models using rats, marmosets, and guinea pigs were performed

for sarin and most often for soman, thereby considering individual concentration–time profiles of different enantiomers (Benschop and de Jong, 2001). The resulting concentration curvatures reflect the combination of toxicokinetic factors of distribution and elimination by hydrolysis and protein binding, as described previously in detail. The next section will briefly summarize the most prominent findings about stereoselectivity and related blood concentration–time profiles. In contrast to many OP pesticides, detailed toxicokinetic data on oral uptake of nerve agents is not available. Therefore, we restrict this section to i.v., s.c., p.c., and respiratory exposure. For more detail, a concise and extensive overview is given by Benschop and de Jong (2001).

Intravenous Uptake

Direct poison injection into circulation allows immediate distribution in an organism, but also enables undelayed elimination provoked by plasma components. To illustrate these impacts, we introduce some fundamental results obtained for some G agents, as depicted in Figure 56.3A (Benschop and de Jong, 2001).

As soon as 0.3 min after i.v. administration of C(±)P(±)-soman to rats, guinea pigs, and marmosets (3–6 LD_{50}), the less toxic C(+)P(+)-enantiomer degrades to nondetectable concentration, whereas its diastereomer C(−)P(+)-soman is detectable for a few minutes longer. This rapid decrease of P(+)-enantiomers is caused by fast enantioselective catalytic hydrolysis (Table 56.3). In contrast, the highly toxic P(−)-diastereomers are detected for up to 1 h or longer, showing a steep initial concentration decrease caused by systemic distribution, protein binding, and hydrolysis, followed by a more moderate concentration decline during the later elimination phase (Figure 56.3A). CarbE is expected to be the most important scavenger in laboratory animals, being essential for nerve agent elimination and causing high species-dependent variations in LD_{50} values (listed in descending order, rat, guinea pig, and marmoset), which correlate to individual CarbE concentrations (Table 56.2). Resulting concentration curvatures were fitted best by a three-exponential equation (Benschop and de Jong, 2001). Table 56.3A gives a representative example of the concentration–time profile of C(−)P(−)-soman in guinea pigs. Corresponding experiments, performed with (±)-sarin applied to guinea pigs, demonstrated that initial distribution of (−)-sarin happened an order of magnitude faster than P(−)-soman, whereas elimination was one order of magnitude slower (Benschop and de Jong, 2001). The reasons for these differences are not clarified yet, but they are expected to be due to different kinetics of hydrolysis and protein binding, causing higher persistence of (−)-sarin.

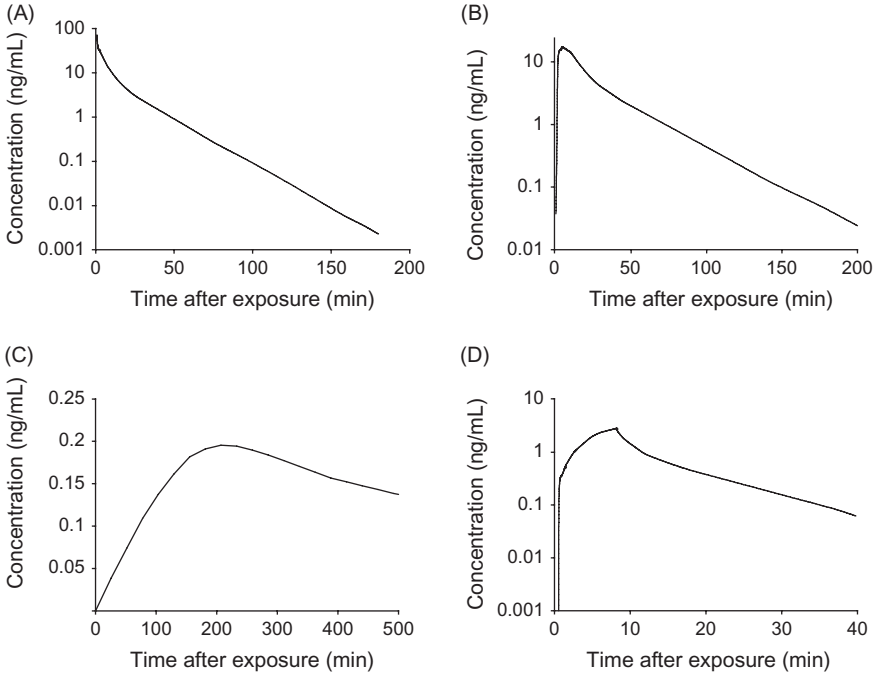

FIGURE 56.3 Concentration–time profiles of highly toxic C(−)P(−) soman and (±) VX in guinea pig blood after administration of nerve agents via different routes of exposure. Guinea pigs were challenged with C(±)P(±)-soman (A, B, D) or (±) − VX (C). A: intravenous, 6 × LD_{50} (165 µg/kg); B: subcutaneous bolus, 6 × LD_{50} (148 µg/kg); C: percutaneous bolus, 1 × LD_{50} (125 µg/kg); D: nose-only for soman vapor in air at 0.8 × LCt50 (48 mg/m^3 for 8 min). Nerve agents were applied to anesthetized, atropinized, and mechanically ventilated animals. Data fits are according to basic studies presented by Benschop and de Jong (2001; A, B, D) and by Van der Schans et al. (2003; C).

Similar results of stereospecific kinetics were also observed for tabun after i.v. challenge in swine, showing a shorter terminal half-life elimination for the less toxic (+)-tabun (Tenberken et al., 2010). In accordance, the (-)-cyclosarin enantiomer could be detected for at least 20 min after i.v. administration of racemic cyclosarin to swine, whereas the (+)-isomer could not be detected in any sample even though the first blood draw was done about 2 min after poisoning (Reiter et al., 2007). Monitoring the concentration-time profile of VX after i.v. challenge in swine, Reiter et al. (2011) also measured the concentration of its toxic hydrolysis product EA-2192 (S-[2(diisopropylamino)ethyl]methylphosphonothioic acid) by means of liquid chromatography-tandem MS (LC-MS/MS). This biotransformation was also observed in heparinized rat plasma *in vitro,* showing a notable stability, and should be considered for therapy of OP poisoning by VX.

Subcutaneous Uptake

Subcutaneous exposure is often used as a substitute for respiratory exposure due to experimental difficulties in performing controlled inhalational poisoning (Benschop and de Jong, 2001). For example, we will note a typical study illustrating the s.c. behavior of soman (Figure 56.3B). A C(±)P(±)-soman bolus injection in the scruff of a guinea pig neck resulted in a discontinuous C(−)P(−)-soman concentration–time profile following a monoexponential equation for the absorption phase and a biexponential fit for the distribution phase (Figure 56.3B; Benschop and de Jong, 2001). The steep initial concentration increase in blood indicated rapid penetration through capillary walls. Maximum concentration was not reached until 7 min after soman injection, yielding an absorption half-life of about 3.5 min. Despite some certain diastereomer-specific differences [decreased bioavailability of C(+)P(−)-soman], both P(−)-forms exhibited comparable kinetic behavior. Bioavailability was found to be 70–80% when comparing the corresponding area under the curve (AUC) to that of i.v. injection. Very similar to the i.v. characteristics mentioned previously, the toxic, more stable P(−)-isomers were detectable in blood for more than 3 h after exposure, whereas the less toxic P(+)-forms of soman could not be determined at any time. This is attributed to the high-hydrolyzing catalytic activity of PTEs in blood, skin, and other affected tissue (Table 56.3).

Percutaneous Uptake

(±)-VX dissolved in isopropanol (1 LD_{50}) was applied p.c. to hairless guinea pigs (Figure 56.3C; Van der Schans et al., 2003). Typically for p.c. exposure, concentration in blood increased very slowly, reaching its maximum between 3 and 4 h after challenge, followed by a slight decrease within the next 4 h. Despite that longer period of monitoring, the illustrated concentration–time profile only reflects the distribution and early elimination phases. This slow release is due to the formation of a depot under and in the skin, as well as to reduced hydrolytic degradation of VX *in vivo*. No prominent effects of stereoselective toxicokinetics were observed; therefore, the depicted curvature reflects the racemic mixture of analytes. Bioavailability of VX at 7 h was found to be very small compared to i.v. data (not exceeding 3%). The long-lasting elimination phase demonstrated high VX persistence in the organism, resulting in threatening concentrations with acute toxicological relevance for a longer period. Such information is important for therapeutic treatment, pointing out the necessity of long time oxime infusion.

Respiratory Uptake (Nose-only Model)

As pointed out in the section "Respiratory uptake by inhalation" earlier in this chapter, respiratory uptake is the most likely route of exposure for G agents, causing 70–80% absorption in the upper respiratory tract. The sophisticated exposure model of the nose-only design applied to guinea pigs challenged the laboratory animals free of potential distortions derived from simultaneous ocular or p.c. uptake, as occurs in vapor chambers (Langenberg et al., 1998a).

The toxicokinetics caused by administration of C(±)P(±)-soman to guinea pigs documented a discontinuous process of monoexponential function for the absorption phase followed by a biexponential fit for distribution and elimination (Figure 56.3D). C(−)P(−)-soman was detected in blood as early as 30 s after challenge, and a short-term maximum was reached immediately after terminating the 8-min exposure period (Figure 56.3D; Benschop and de Jong, 2001). Nevertheless, some depot formation might also occur when applying higher doses, causing maximum levels after completion of exposure. The concentration curvature of C(+)P(−)-soman showed a similar profile, but with consistently smaller concentrations, which is due to a higher degree of enzymatic hydrolysis (Table 56.3). In contrast, less toxic C(−)P(+)-soman was detected in very small concentrations during the exposure phase exclusively, whereas C(+)P(+)-soman was not detected at all. Curvatures for SP-(−)-sarin appeared to be very similar in terms of time for first appearance in blood and for reaching maximum concentration.

These selected representative examples indicate that concentration–time profiles are variable despite common underlying basic chemical reactions of hydrolysis and adduct formation. Despite improving medical treatment of nerve agent poisoning, the concurrence of numerous physiological and pathophysiological parameters should be understood. Therefore, establishment of a descriptive and predictive model is important for medical defense of OPCs.

Mathematical Simulation for Prediction of Nerve Agent Toxicokinetics

As is obvious from the huge number of parameters affecting toxicokinetic behavior (e.g., route of administration, nature of OPCs, hydrolyzing and bioscavenging enzymes, proteins, and distinct compartments for distribution and species specificities), mathematical modeling of this complex situation is a big challenge. Based on numerous experimental data on soman toxicokinetics, Sweeney et al. (2006) introduced a model described by mathematical algorithms that allows the prediction of concentration–time profiles evoked by i.v., s.c., or inhalational soman uptake (≥1 LD_{50}) in common laboratory animals (i.e., rat, guinea pig, and marmoset). This physiologically based pharmacokinetic model (PB/PK) benefits from the combination of relevant pharmacokinetic basics and soman-specific experiences, realizing both an excellent degree of confidence for theoretical and laboratory data, as well as extrapolation to other species. This concept is a further development of an initial model from the same working group introduced by Langenberg et al. (1997). A more detailed description of these models would go beyond the scope of this chapter. Readers are referred to the sources in the reference list at the end of the chapter.

Bioanalytical Techniques Relevant to Toxicokinetics

Elaboration of nerve agent toxicokinetics requires sophisticated analytical tools to detect and, if possible, to quantify the free toxicants, as well as adducts with proteins and enzymes. Analysis of OP nerve agents has been performed by capillary electrophoresis (CE), biosensors, MALDI-MS, desorption electrospray ionization MS (DESI MS), ion mobility time-of-flight MS (IM-TOF MS), nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-ultraviolet (LC-UV), gas chromatography (GC), and many more techniques (Hooijschuur et al., 2002; John et al., 2008).

Determination of Nerve Agents

In contrast to those rather unusual methods, GC coupled to diverse detection systems [e.g., flame ionization detector (FID), nitrogen–phosphorus detector (NPD), flame photometric detector or mass spectrometer, as well as LC methods] represents the most common techniques for OP determination, especially for biological samples. These methods offer high resolution, sufficient limits of detection, good reproducibility, and robust hardware devices. For more detailed information, readers are referred to other review articles (e.g., Hooijschuur et al., 2002; John et al., 2008).

Analysis becomes much more complex when stereoisomers are quantified separately (Figure 56.1). Enantiomers cause identical detector responses in NPD, FID, or MS. Therefore, chiral separation systems are required to overcome these detector limitations. Despite enormous progress in separation media and detector systems within the last two decades, the number of reports on chiral analysis of nerve agents valuable for toxicokinetic studies is still very small. Chiral separations make use of special chromatographic columns modified with chiral ligands.

Isomers of soman were separated by GC on a Chirasil-L-Val column, but lacked baseline separation (Benschop et al., 1981, 1985; Li et al., 2003a,b). In contrast, (+)-sarin and (−)-sarin could be completely separated by the same column (Benschop and de Jong, 2001). Another study presented a modified method using a Chiraldex gamma-cyclodextrin trifluoroacetyl GC-column coupled to an electron impact (EI)-MS, which enabled sufficient baseline separation of all four stereoisomers of soman (Smith and Schlager, 1996; Yeung et al., 2008).

Apart from the Chirasil-L-Val method, sarin enantiomers were also separated by a two-dimensional GC technique on chiral Cyclodex B material prior to NPD monitoring (Spruit et al., 2000, 2001). Additional GC-based approaches allowed baseline separation of cyclosarin enantiomers on a GAMMA DEX column monitored by EI-MS (Reiter et al., 2007), separation of VX stereoisomers on hydrodex-β-TBDAc (β-cyclodextrin; Reiter et al., 2008), and separation of tabun enantiomers on BetaDex 225 coupled to APCI-MS (Tenberken et al., 2010). VX enantiomers were also chromatographed on a Chiracel OD column by LC coupled to an electrochemical detector, yielding a lower limit of quantification of about 10 ng/mL blood (Van der Schans et al., 2003). Another LC method coupled with MS/MS detection for VX made use of CHIRALCEL OD-H and CHIRAL AGP columns (Reiter et al., 2008).

Detection of Enzyme and Protein Adducts of Nerve Agents

In contrast to the measurement of free agents, the qualitative detection of protein adducts is a very novel approach that came about by overwhelming technical progress in bioanalytical mass spectrometry. Electrospray ionization (ESI) and MALDI as soft ionization methods for mass spectrometric detection are highly valuable for the detection of biomacromolecules like DNA, peptides, and proteins (Schulz-Knappe et al., 2001; John et al., 2004, 2005). Therefore, these technologies are also favorable for the analysis of proteins interacting with nerve agents. Consequently, a number of approaches have been published that identify and characterize these adducts. Typically, protein and enzyme adducts are first isolated from complex biological matrices (e.g., blood) using affinity chromatographic methods; subsequently, they are

TABLE 56.5 Physicochemical Properties of Most Common Vesicant Agents

Agent	CAS No.	NATO Code	MW (g/mol)	Melting Point (°C)	Boiling Point (°C)	Vapor Pressure (mbar)	Water Solubility (mg/L)	Hydrolysis Rate, $\tau_{1/2}$ (h)	log P(–)
Sulfur mustard	505-60-2	HD	159.1	14	217	0.147 (25°C)	0.684[a]	14.7 (20°C)	1.37
Lewisite	541-25-3	L	207.3	−18	190 (decomposing)	0.773 (25°C)	500	Rapid, n.a.	n.a.

log P, octanol:water partition coefficient; MW, molecular weight; n.a., not available; $\tau_{1/2}$, period of half-life for hydrolysis. Data are taken from Munro et al. (1999) unless otherwise noted
[a]*Seidell (1941)*

subjected to enzymatic cleavage by adding selected proteases. The resulting internal peptide cleavage products containing the derivatized (phosphylated) residues are chromatographically separated and analyzed by modern mass spectrometry. Using sophisticated MALDI-MS techniques, this general procedure allowed the identification of albumin adducts (Li et al., 2007, 2008; John et al., 2010), as well as the adducts of BChE and their aged products (Jiang et al., 2013). LC-ESI MS was applied to analyze adducts of albumin (Peeples et al., 2005; Williams et al., 2007), BChE (Noort et al., 2006; Tsuge and Seto, 2006), CarbE (Peeples et al., 2005), keratins, tubulin, actin, and transferrin (Grigoryan et al., 2009). For a more detailed description, see John et al. (2008), Schopfer and Lockridge (2012), and Black and Read (2013).

For diagnostic detection of BChE adducts of common G- and V-type nerve agents, enhanced sample throughput was achieved by automated processes in 96-well plate format, extracting plasma by immunomagnetic separation (Knaack et al., 2012). An alternative method, based on the principle of a sandwich enzyme-linked immunosorbent assay (ELISA), was presented by Wang et al. (2011) to determine OP adducts in complete BChE.

This summary underlines the great importance of modern analytical techniques to unraveling pathophysiological situations at the molecular level and to supporting toxicokinetic studies by discovering the most relevant protein-binding elimination processes.

VESICANTS

Sulfur Mustard

Overview of Sulfur Mustard

Sulfur mustard is a blistering or vesicating agent that primarily incurs damage on organs that come into immediate contact with either its liquid or vaporous form. However, severe dermal and respiratory exposure to the agent may also result in absorption of sulfur mustard, which subsequently causes additional systemic damage (Kehe and Szinicz, 2005).

Unfortunately, sulfur mustard has been used in acts of chemical warfare throughout the twentieth century, from World War I to the attacks of Saddam Hussein's former Iraqi regime against Iran and even Kurdish civilians. The simplicity of the agent and its synthesis combined with its devastating medical, psychological, and socioeconomic effects, along with the fact that no causative therapy has yet been established, may convince future aggressors (both state and nonstate parties) to use this agent in their attacks. This, in turn, necessitates medical research efforts, including toxicodynamic and toxicokinetic studies, to develop countermeasures against the effects of sulfur mustard. Table 56.5 displays the basic physicochemical properties for sulfur mustard, which constitute the fundamental reason behind many of its toxicokinetic properties. Data for lewisite, another vesicant agent described in the section "Lewisite," later in this chapter, are also shown in this table.

Toxicity of Sulfur Mustard

Figure 56.4 depicts the basic chemical mechanism by which sulfur mustard incurs its primary damage to biological molecules, which results in subsequent damage to cells, tissues, and organs.

Sulfur mustard forms an intermediate sulfonium ion that further transforms into a carbenium ion, a strong electrophile capable of reacting with nucleophile targets, primarily in DNA and RNA. Examples include the N7 in guanine (61%; Ludlum et al., 1994), the N3 in adenine (16%), and the O6 in guanine (0.1%). Even though the latter is considerably less frequent, it is of significant concern, as the repair enzyme (O6-alkylguanine-DNA-alkyltransferase) is not capable of reversing this reaction. Therefore, DNA mutation due to mispairing may occur during DNA replication, which is proposed to be the origin of subsequent cancer formation (Ludlum et al., 1986). Because of its bifunctional character, sulfur mustard also forms interstrand DNA crosslinks, in particular from one guanine to another (17%). It is estimated that a 100-μM concentration of sulfur mustard accounts for 0.28 crosslinks per 10,000 DNA bases (Shahin et al., 2001).

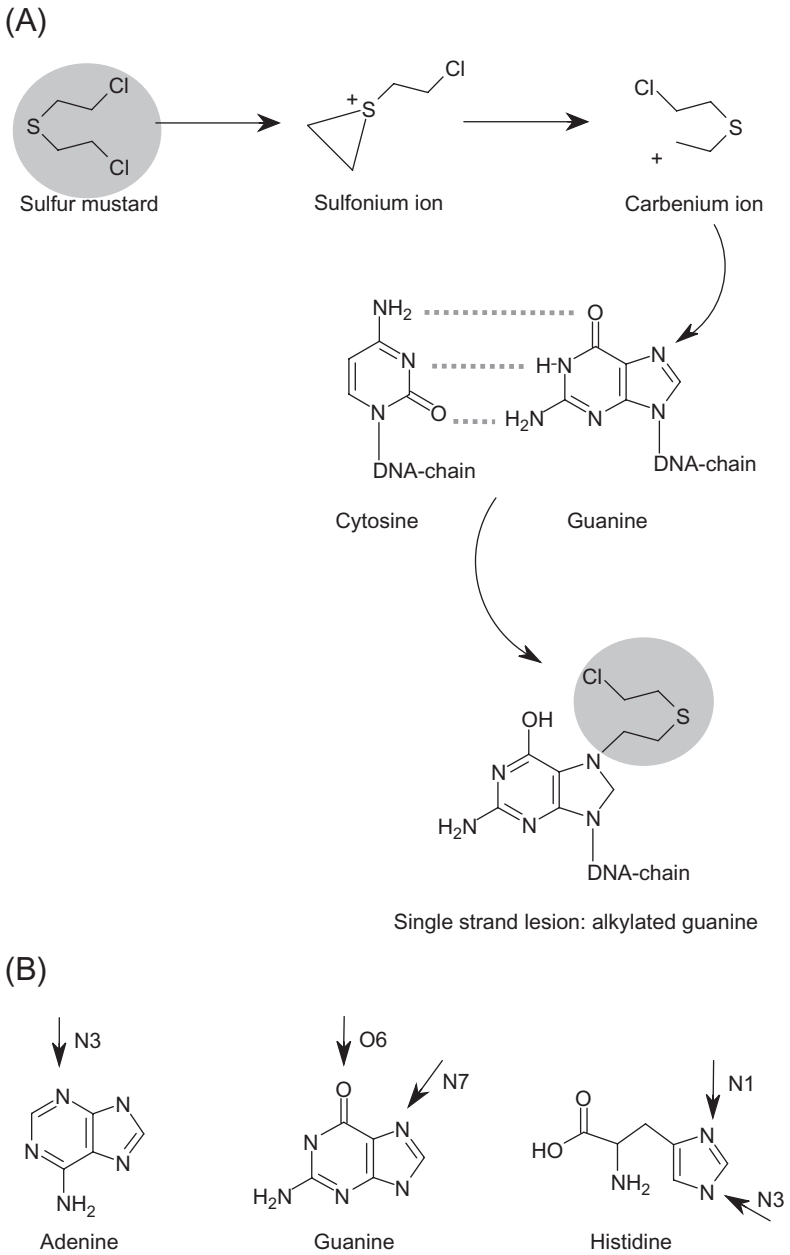

FIGURE 56.4 Sulfur mustard, its structure, mechanism of action, and targets of adduct formation. A: Mechanism of reaction of sulfur mustard and nucleotide guanine. B: Sites of alkylation by sulfur mustard. Arrows mark identified targets in nucleotides and amino acid histidine.

Membrane-bound proteins and enzymes may undergo alkylation in the presence of sulfur mustard (Kehe and Szinicz, 2005). Figure 56.4 depicts targets of alkylation in both DNA bases and amino acids. As sulfur mustard-induced DNA damage has the most significant impact on the cell's short- and long-term survivability, it is DNA alkylation that produces the most significant toxic effects, whereas effects from protein alkylation are usually visible only in the presence of high concentrations of sulfur mustard (Peters, 1947; Lodhi et al., 2001). As systemic concentrations will always be several orders of magnitude below local concentrations at the site of immediate contact, the systemic toxic effects of sulfur mustard are also most likely to result from DNA alkylation. DNA alkylation produces the most evident effects in proliferating cells; tissues with rapidly proliferating cells, such as bone marrow, suffer the most obvious damage. Toxic effects are similar to side effects from alkylating antineoplastic drugs (Dacre and Goldman, 1996). (Actually, the first antineoplastic drug was developed from a structural analog of nitrogen mustard—see Goodman et al., 1984.) Those effects include nausea,

vomiting, fever, fatigue, apathy, and loss of appetite. Bone marrow toxicity results in an initial leukocytosis, which is soon followed by leukopenia, thrombopenia, eosinopenia, and subsequent anemia (Dacre and Goldman, 1996).

Specific toxic effects of sulfur mustard have also been reported in the CNS and may range from agitation to seizures (Balali-Mood and Navaeian, 1986).

Invasion

Significant amounts of sulfur mustard may be absorbed from skin that has been in direct contact with sulfur mustard. Vapors of sulfur mustard may also be absorbed through the respiratory system.

In theory, there are two additional pathways for systemic uptake of sulfur mustard: the surface of the eyes and the gastrointestinal system. They have, however, never been investigated for the following reasons: gastrointestinal exposure to sulfur mustard is a rare phenomenon and may only occur in cases of sabotage (deliberate food poisoning) or a considerable lack of basic chemical defense measures (i.e., food consumption in a contaminated area). The local effects of exposing the gastrointestinal system to sulfur mustard are severe and life-threatening; they include strong abdominal pain, bloody diarrhea and vomiting, and rupture of stomach or duodenum. Subsequent peritonitis is often fatal (Dacre and Goldman, 1996). In comparison to life-threatening local effects of gastrointestinal exposure, any related systemic effect would be of secondary importance.

As opposed to the rarity of gastrointestinal exposure, ocular exposure is frequent among the victims of sulfur mustard attack, and local symptoms of varying severity are likely to occur whenever the individual was not protected by a respirator (mask) at the time of attack (Solberg et al., 1997). As the eye is a very sensitive organ, ocular symptoms are often among the first signs of sulfur mustard exposure. Due to the close proximity of a large number of capillary vessels and the fact that the eye constitutes a relatively weak barrier, xenobiotic substances are often rapidly absorbed (Lama, 2005). As the surface of the eye is small in comparison to the skin and the respiratory tract, the total amount of sulfur mustard that may be absorbed from the eye's surface is low. Self-protective effects of the eye, such as blepharospasm and pronounced lacrimation, may further reduce the amount of an agent that may be absorbed through this pathway. The local effects of exposure appear very severe and are a primary reason for concern in victims of sulfur mustard exposure. Whereas blepharospasm and lid edema cause transitory loss of vision—already resulting in immense distress for victims—heavy exposure may result in permanently opaque cornea and blindness. In general, early effects in the eyes are treatable with therapy and have a relatively good outcome after several weeks. In comparison, any systemic effects from transocular absorption alone would be of only minor concern. It has to be emphasized that isolated ocular exposure on the battlefield or due to terrorist attacks utilizing sulfur mustard is exceedingly unlikely. Ocular exposure would inevitably be accompanied by cutaneous and respiratory exposure. The specific rate of absorption (i.e., the amount absorbed through a given surface over a specified time) may be lower, particularly in the case of initially intact skin. However, the larger surface of the skin (and possibly the respiratory tract) would result in an amount that constitutes the major fraction of sulfur mustard absorbed, which would subsequently determine the occurrence and severity of systemic effects. Research regarding the absorption of sulfur mustard has focused on two major pathways, which are described in further detail in the subsequent sections.

Percutaneous Absorption

Penetration rates of liquid sulfur mustard were determined *in vitro* (71–294 $\mu g/cm^2/h$) on human skin with a Franz-type glass diffusion cell and correspond very well to *in vivo* data derived from human skin (60–240 $\mu g/cm^2/h$; Chilcott et al., 2000). Significant effort has been dedicated to confirm the validity of animal models by comparing data derived from these models with findings from *in vitro* human skin experiments. Chilcott et al. (2001) investigated absorption in *in vitro* models of pig ear and heat-separated human skin. Absorption was determined at $411 \pm 175\,\mu g/cm^2/h$ for pig ear skin and $157 \pm 66\,\mu g/cm^2/h$ for human skin, respectively. These data were considered to agree with earlier *in vivo* findings, even though the authors cautioned that data on effectiveness of decontaminants were completely disparate between human and pig ear skin. Logan et al. (1999) determined that sulfur mustard exposure through skin of the hairless guinea pig was $120\,\mu g/cm^2/h$.

Occlusion of the skin (i.e., covering the skin with a material that is impenetrable to air and moisture) can result in a dramatic increase in p.c. absorption. For example, in a study by Chilcott et al. (2002) using *in vitro* models of human skin, absorption rates in unoccluded controls ($4.41 \pm 1.90\,\mu g/cm^2/h$) increased to $538 \pm 193\,\mu g/cm^2/h$ under occluded conditions.

In contrast, Karvaly et al. (2008) showed that a commercially available barrier cream and a perfluoropolyether oil applied prior to exposure reduced p.c. uptake of sulfur mustard. In particular, perfluoropolyether oil was highly effective, preventing sulfur mustard exposure for a period of 20 min. A petroleum jelly ointment, however, had no protective effect, even when applied prior to exposure.

Benson et al. (2011) percutaneously exposed guinea pigs to ^{14}C-labeled sulfur mustard vapors ($525\,mg/m^3$) for 12 min, applied to three skin areas totaling $19.8\,cm^2$.

A total of 29.8 ± 5.31 µg sulfur mustard per animal was absorbed, 90% of which remained in the skin. This result agrees with an *in vitro* study by Hattersley et al. (2008) using human skin samples, which confirmed that a depot of sulfur mustard existed for at least 24 h following dermal exposure, and estimated concentrations in skin is at least 20 times above the acutely toxic concentration.

Regarding airtight vapor cups frequently used for percutaneous sulfur mustard exposure studies, Dalton et al. (2006b) validated this approach and showed that equilibrium concentrations developed within 2 min after sealing the cups and were not significantly different from calculated saturated vapor concentration (1,363 mg/m^3) when cups were placed on inert control surfaces. However, when cups were placed on samples of pig skin, percutaneous uptake of sulfur mustard lowered actual vapor concentrations to values significantly below saturation (i.e., to 592 ± 246 mg/m^3 on a 10.15 cm^2 skin surface and 740 ± 224 mg/m^3 on a 2.54 cm^2 skin surface). The authors validated the assumption that saturated vapor concentration could be used to calculate concentration—time products for percutaneous absorption studies, but they cautioned that, depending on the size of the reservoir and the skin surface, decreasing vapor concentrations had to be taken into account.

Respiratory Absorption

Langenberg et al. (1997) conducted inhalation exposure experiments in hairless guinea pigs. LCt_{50} was determined at 800 mg min/m^3. Following application of 1 LCt_{50} over 5 min, no unchanged sulfur mustard in blood was found (with a limit of detection of 5 pg/mL). Low concentrations (0.7 adducts per 10^7 nucleotides) of N7-guanine adducts of sulfur mustard were found in the lung. Concentration of N7-guanine adducts was also determined from various tissues along the respiratory tract. Actually, concentrations found were much larger, peaking at approximately 90 adducts per 10^7 nucleotides in the larynx and trachea. Concentration in the carina was lower (approximately 50 per 10^7 nucleotides), but it was still substantially higher than the abovementioned value in the lungs. The authors concluded that most of the sulfur mustard inhaled reacts in the upper airways rather than being absorbed. In animals with complex nasal systems (such as guinea pigs), only very minor fractions would reach the lung. However, they also pointed out that, in humans (along with other species with a less complex nasal system), the proportion of sulfur mustard reaching the lung may be larger.

Following inhalation of 3 × LCt_{50} (300 mg/m^3 over a period of 8 min), sulfur mustard was found in blood. Its peak concentration during the inhalation period was found to be approximately 5 ng/mL. This concentration soon declined. A mean value of 2 ng/mL was found even after 4 h. A mathematical model explaining the data during the distribution and elimination phase could not be developed. These findings were further complicated by the fact that in 2 of 12 animals, no sulfur mustard was found in any of the samples. No sulfur mustard was detected in any of the animals at 15 and 20 min (7 and 12 min postexposure, respectively) before reappearing in most of the animals at the abovementioned concentration of 2 ng/mL. The latter finding may possibly be explained by the hypothesis that the early peak in sulfur mustard concentration is due to direct inhalation (afterward, concentration declines to levels below the limit of detection), whereas the second, long-term increase in sulfur mustard concentration may be attributed to sulfur mustard absorbed from depots in the upper airways. Figure 56.5 depicts the concentration over time, following the respiratory exposure to 3 × LCt_{50} of sulfur mustard. For comparison, concentration over time after i.v. application is also depicted. The authors concluded that toxicity from sulfur mustard inhalation was due to its local, rather than its systemic, effects.

Benson et al. (2011) presented a respiratory exposure study using ^{14}C-labeled sulfur mustard. Anesthetized rats with transorally placed tracheal catheters were exposed to 250 mg ^{14}C sulfur mustard vapor/m^3 for 10 min. A total of 18.1 ± 3 µg sulfur mustard per animal was absorbed. Within 2 h postexposure, inhaled sulfur mustard was distributed and more than 70% were deposited in the carcass and pelt.

Distribution

Sulfur mustard is a strongly lipophilic substance that may accumulate in fatty tissues and has actually been detected at autopsy in a patient who died 7 days postexposure. Detailed data, as published by Drasch et al. (1987), are displayed in Table 56.6.

Obviously, these findings confirm the theoretical assumption that the lipophilic properties of sulfur mustard result in a distribution, primarily in lipophilic tissues. High concentrations found in the brain may also explain why the CNS is one of the organs exhibiting systemic effects of sulfur mustard poisoning, even though it is not a site of rapidly proliferating cells.

It should be noted that some authors have questioned the findings by Drasch et al., considering the absolute concentrations excessively high and therefore unlikely. However, as high-dose sulfur mustard poisoning is a rare event, it is nearly impossible to verify or falsify the data. One might argue that the amounts of sulfur mustard described may not even permit short-term survival. However, if most of an agent entered the organism via p.c. absorption, there is a possibility that large amounts of the agent actually had been absorbed and formed a depot without resulting in instant death, but ultimately leading to death 7 days later. Whether or not the absolute values are accurate, they at least give an impression of

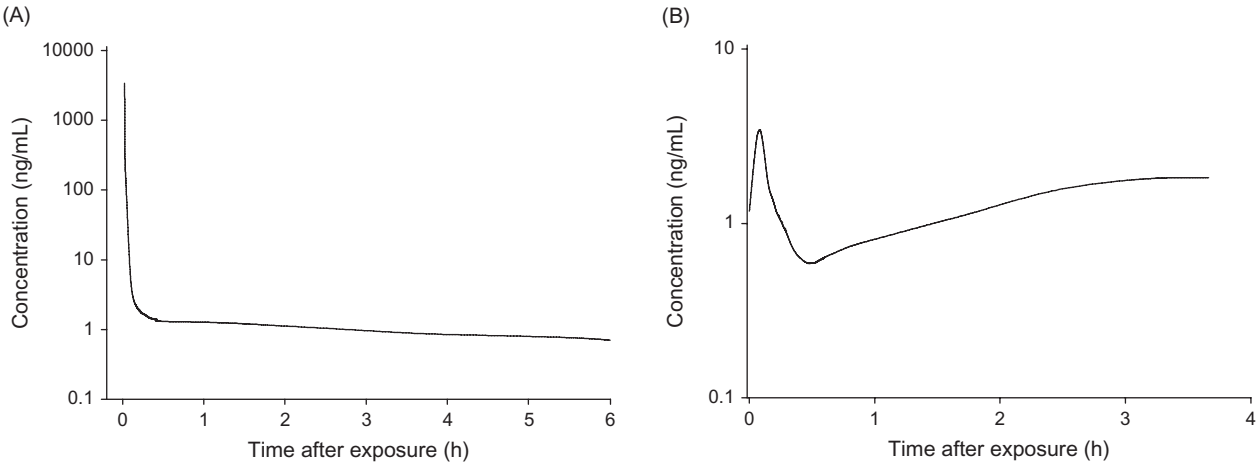

FIGURE 56.5 Concentration over time, following i.v. and respiratory exposure to sulfur mustard in the guinea pig. A: Decline of sulfur mustard exposure after i.v. injection. B: Concentration over time after respiratory exposure: initial increase in the inhalation phase, followed by a decline and a secondary increase; concentration of approximately 2 ng/mL is sustained for 4 h.

TABLE 56.6 Content of Sulfur Mustard in the Tissues of a Deceased Victim

Tissue/Organ	Concentration (mg/kg)
Fat	15.1
Skin with subcutaneous fatty tissue	11.8
Brain	10.7
Skin	8.4
Kidney	5.6
Liver	2.4
Cerebrospinal fluid	1.9
Muscle	3.9
Spleen	1.5
Blood	1.1
Lung	0.8
Urine	Not detected
Blister fluid	Not detected

Data are according to Drasch et al. (1987)

the distribution of sulfur mustard within an organism that correlates with the agent's lipophilic properties.

More recent *in vitro* experiments using human skin have confirmed the presence of unhydrolyzed sulfur mustard in the lipophilic stratum corneum and the upper epidermis. At 24 h post-exposure, the distribution ratio between epidermis and dermis has been determined to be 62–38%. Chilcott et al. (2000) also suggested that efforts to remove or neutralize the agent from these deposits might have a clinical benefit for the patient.

These findings suggest that, despite the presumably brief half-life calculated (see the section "Elimination," later in this chapter), sulfur mustard may be present in an organism for a significantly longer period of time, necessitating measures for medical personnel providing therapy and an awareness for the possibility of secondary blister formation, even 30 days post-exposure (Balali-Mood and Hefazi, 2005), as well as efforts to remove these deposits. In particular, the use of laser debridement (Graham et al., 2008) and mechanical dermabrasion (Rice, 2008) has been recommended to facilitate the healing process after dermal exposure to sulfur mustard. The effects may be partly attributed to the removal of epidermal depots of sulfur mustard.

As mentioned previously, Benson et al. (2011) confirmed that 90% of percutaneously absorbed sulfur mustard was deposited in skin, whereas more than 70% absorbed sulfur mustard was distributed to the carcass and pelt after respiratory exposure. The distribution of sulfur mustard is highly dependent on the original route of exposure. It was confirmed that a considerable amount of sulfur mustard was still present in deep lipophilic compartments even after the end of exposure.

Biotransformation

While the previously discussed findings demonstrate the stability of sulfur mustard in lipophilic tissues, the agent is rapidly hydrolyzed whenever situated in an aqueous compartment (Vycudilik, 1987). Thiodiglycol (TDG) is the primary hydrolysis product, in which the chlorine atoms have been replaced by hydroxyl groups. Karvaly et al. (2008) used subcutaneous microdialysis to monitor TDG in rats exposed to sulfur mustard. Peak concentrations of 7.2–21.7 nmol/L TDG were found, following percutaneous exposure to 2 µM sulfur mustard.

TDG may undergo oxidation to TDG sulfoxide, which is conjugated with glutathione to form 1,1′-sulfonylbis [2-*S*-(*N*-acetylcysteinyl)ethane]. Following the β-lyase pathway, the 1,1-sulphonylbis[2-(methylsulfinyl)ethane] and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl] ethane can be formed (Black and Read, 1995). Figure 56.6 summarizes the pathways of sulfur mustard biotransformation. No sulfo- or glucuronyl-conjugates were detected in urine after i.v. administration of sulfur mustard (Maisonneuve et al., 1993).

Halme et al. (2011) presented a method for the efficient stable isotope dilution LC-HESI-MS/MS method for verification of β-lyase biotransformation products in human urine after sulfur mustard exposure. The β-lyase products 1,1′-sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE) and 1-methylsulfinyl-2-[2-(methylthio) ethylsulfonyl]ethane (MSMTESE) were successfully detected at a concentration of 4 ng/mL, whereas the limit of quantification was established at 10 and 11 ng/mL for SBMSE and MSMTESE, respectively.

Within cells, sulfur mustard forms adducts with DNA, primarily those described above. Adducts can also be formed with nucleophilic sites of amino acids and proteins. Byrne et al. (1996) demonstrated that sulfur mustard, with two highly reactive groups was able to form protein crosslinks between cysteine residues and assumed that protein crosslink formation may actually contribute to sulfur mustard toxicity. Contrary to DNA adducts, there is no specific mechanism to reverse protein adduct formation. For this reason, there is a strong forensic interest in the detection of protein adducts of sulfur mustard, as these may provide evidence of sulfur mustard exposure for prolonged periods after an incident.

Capacio et al. (2008) developed a method to detect sulfur mustard adducts in plasma proteins by hydrolyzation (which produced TDG) and subsequent derivatization of TDG, followed by GS/MS analysis. The method was successfully applied to determine sulfur mustard adducts in rat plasma, following respiratory exposure to sulfur mustard.

A number of adducts to amino acid residues have been identified by Noort et al. (1996) and Black et al. (1997a,b). Six different histidine residues, three glutamic acid residues, and both N-terminal valines were found. Alkylated cysteine, aspartic acid, lysine, and tryptophan were also detected. While N1 and N3 histidine adducts were found to be most abundant, it was the alkylated N-terminal valine adducts that were most useful for subsequent quantification. See the section "Detection of DNA and protein adducts of vesicants," later in this chapter, for analytical details.

Noort et al. (2008) investigated the persistence of sulfur mustard adducts to albumin and hemoglobin in rats. The albumin adduct (*S*-2-hydroxyethylthioethyl)-Cys-Pro-Tyr was detectable up to 7 days after exposure, while the adduct to the N-terminal valine in hemoglobin was still detected after 28 days. The decrease of adduct concentration corresponded with albumin half-life and the lifetime of the rat erythrocyte, respectively.

Following two simultaneous cases of accidental human exposure to sulfur mustard, Smith et al. (2008) investigated the concentration of cysteine-34 adduct to albumin and adducts to glutamic and aspartic acids of plasma proteins. In the case of a more severely exposed patient who required hospitalization, both adducts were detected over a 42-day period, though they decreased by approximately 75% toward the end of that time. In a second patient who had developed a single, small blister, the albumin adduct was found during a 6-day period post-exposure.

Recently, local adduct formation after cutaneous exposure to sulfur mustard has been a focus of investigation in an effort not only to establish new forensic methods, but also to better understand vesication, the most evident symptom of sulfur mustard exposure via the skin. Adducts of sulfur mustard to cytokeratin types I and II, actin stratifin, and galectin-7 were successfully identified by Mol et al. (2008) in sulfur mustard-exposed human epidermal keratinocytes. Sulfur mustard adducts to actin, annexin A2, and keratin 9 were also found in HaCaT cells (immortalized kerationcytes) by Sayer et al. (2009).

Elimination

Following i.v. application of ^{14}C-labeled sulfur mustard in rats, 80% of the radioactivity administered was excreted via the renal pathway. Fecal excretion amounted to less than 3% (Maisonneuve et al., 1993). A study by Benson et al. (2011) in rats and guinea pigs, following respiratory and percutaneous exposure to ^{14}C-labeled sulfur mustard, confirmed predominantly renal excretion. Biotransformation products excreted in urine after accidental human exposure included TDG, TDG sulfoxide, and the bis-mercapturate of mustard sulfone (Barr et al., 2008). When comparing the concentrations of TDG and its sulfoxide, the latter was found in concentrations twice as high. When p.c. exposing weanling pigs to sulfur mustard, Graham et al. (2000) found peak levels of TDG either in the samples drawn after 6–8 h or in other cases after 24–48 h. Findings were considered to be in agreement with earlier data from rodent species and cases of accidental human exposure.

It is worth noting that background levels of TDG and TDG sulfoxide have been found in the urine of healthy individuals never exposed to sulfur mustard. For this reason, their validity as unambiguous markers for sulfur mustard exposure has been questioned. Black and Read (1995) suggested the determination of β-lyase pathway biotransformation products, which in the urine of exposed patients, were found at concentrations similar to those of TDG sulfoxide, but were not found in unexposed individuals.

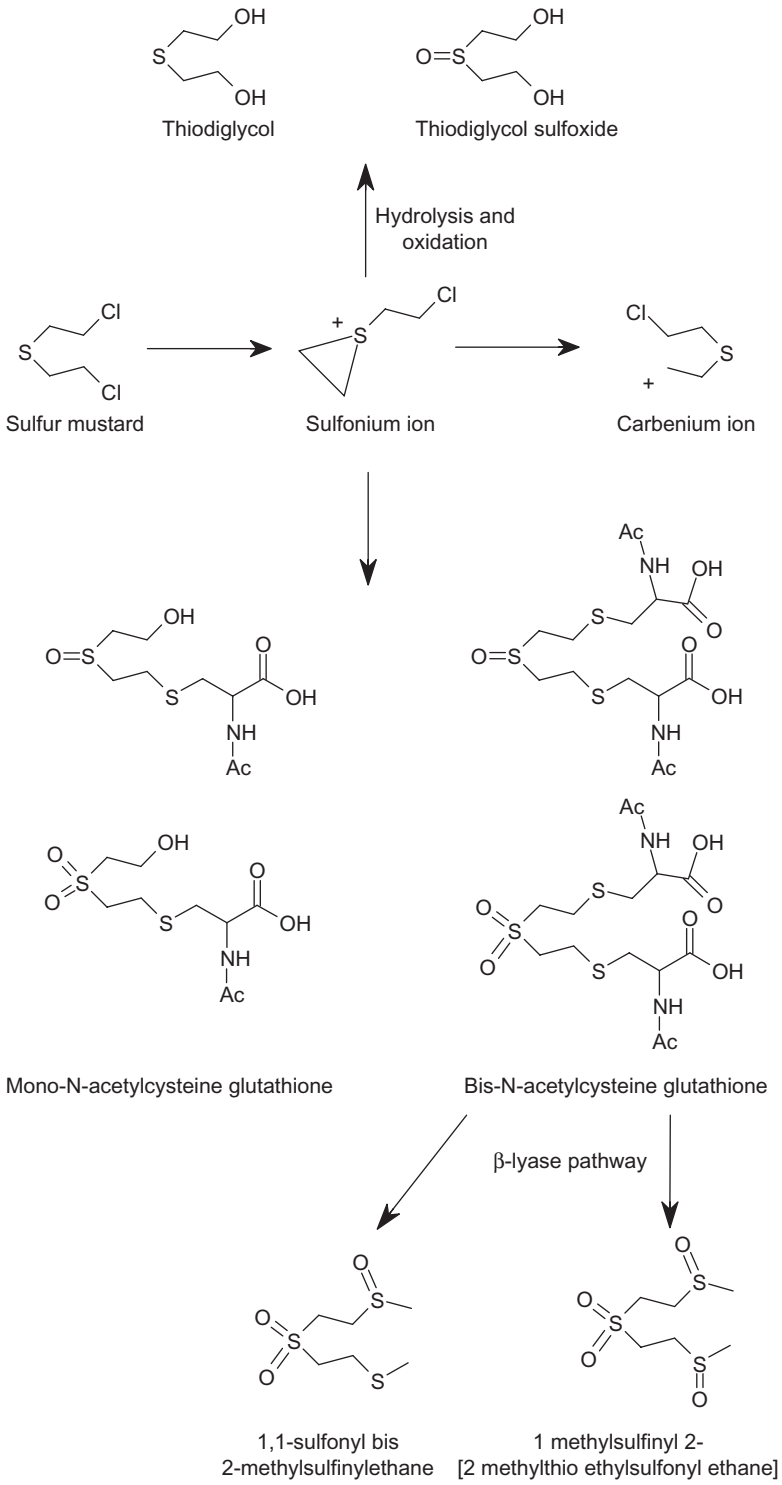

FIGURE 56.6 Biotransformation of sulfur mustard.

Lewisite

Overview of Lewisite

Lewisite (2-chlorvinyldichlorarsin) is another vesicant. It was first synthesized and described by the Belgian priest and chemist Julius Arthur Nieuwland (Nieuwland, 1904). Unlike sulfur mustard, there has never been a documented use of this substance in armed conflict. During World War I, the American military chemist Winford Lee Lewis suggested and initiated its

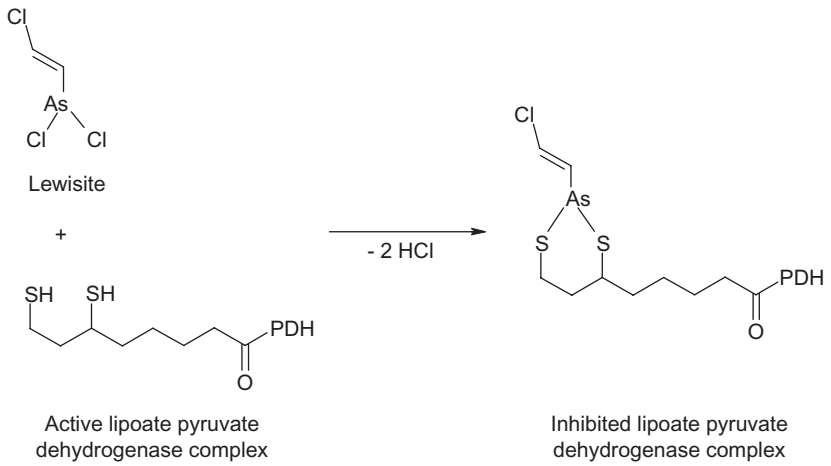

FIGURE 56.7 The structure and mechanism of action of lewisite. Lewisite forms covalent bonds with lipoic acid, inactivating the enzyme PDH.

development into a chemical weapon, but due to the 1918 armistice in Europe, it was not used on the battlefield (Vilensky and Redman, 2003).

Lewisite remains a concern because its physical properties (in particular a melting point of −18°C; see Table 56.5) might facilitate its use in cold climates. A belligerent party willing to use chemical weapons might decide that lewisite would provide a "unique" capability to wage chemical warfare even in winter or in mountainous regions. Large stockpiles of the agent were abandoned by the Imperial Japanese Army during its retreat from China in the latter stages of World War II, creating a chemical hazard that persists decades after the war (Hanaoka et al., 2006). Table 56.5 summarizes the physicochemical properties of lewisite.

Toxicity of Lewisite

The dominant element in lewisite structure is arsenic, which is able to react with sulfhydryl groups of various enzymes, disabling the enzyme in the process (Goldman and Dacre, 1989). Figure 56.7 depicts the structure of lewisite and its toxic mechanism.

Lipoic acid is particularly susceptible to this reaction and one of the most evident consequences is the inhibition of the enzyme pyruvate dehydrogenase (PDH), rapidly disabling the cell's metabolism of glucose and fatty acids. The resulting energy deficiency may lead to swift, necrotic cell death. In comparison to sulfur mustard, the latency period (from exposure to first signs and symptoms) is significantly shorter, and lewisite injuries have been described as extremely painful from an early stage. Moreover, it is a much more lethal agent and has a large systemic toxicity; 0.5 mL of the agent may produce systemic effects, whereas 2 mL (approximately 3.6 g) can be fatal (Marrs et al., 1996).

Invasion

Percutaneous Absorption

Precise data [i.e., a diffusion coefficient expressing the p.c. absorption of lewisite (amount absorbed per area and time)] is not available. However, Inns and Rice (1993) conducted p.c. toxicity studies in rabbits and determined the LD_{50} to be 5.3 mg/kg (3.5–8.5 mg/kg, 95% confidence interval). The exposed area was 2 cm^2, and exposure lasted for 6 h.

Inns et al. (1990) had also determined the LD_{50} of i.v. lewisite administration to be 1.8 mg/kg (1.6–2.1 mg/kg 95% confidence interval). It can be concluded that by exposing 2 cm^2 of rabbit skin to a dose of 5.3 mg/kg for 6 h, a dose producing the equivalent effect of 1.8 mg/kg is absorbed. No further calculations that might exaggerate the reliability of available data shall be conducted here.

King et al. (1992) conducted lewisite absorption studies on isolated perfused porcine skin flaps, using lewisite concentrations from 0.07–5.0 mg/mL. Blister formation was observed, and at higher lewisite concentrations, there was a pronounced increase of lactate dehydrogenase in blister fluid, a sign of necrotic cell death. Cellular glucose utilization was decreased at the same time, underscoring the detrimental effect of lewisite on cellular energy metabolism. At the highest concentration of 5 mg/mL of lewisite, arsenic was detected in venous blood from the perfused skin preparation. At this concentration, systemic uptake of lewisite or its degradation products and toxic effects resulting thereof are to be expected.

Respiratory Absorption

The LCt_{50} of lewisite in humans has been estimated at 1,500 mg min/m3 (ATSDR, 2007), although no

experimental data has been cited. Considering its pronounced local effects lethality can at least partly be attributed to local effects of lewisite on the respiratory tract.

Distribution

High distribution volumes per kilogram indicate extensive distribution in tissues, due to the lipophilicity of the substance. In a rabbit model, more than sevenfold more of the substance was found in some tissues (e.g., the liver, lung, and kidneys) compared to blood concentrations. That ratio was maintained over the sampling period; i.e., for at least 96 h (Snider et al., 1990).

Biotransformation

Once incorporated, unbound lewisite is quickly hydrolyzed. Its predominant biotransformation product is 2-chlorovinylarsonous acid (CVAA; Figure 56.8). Analytical methods to confirm lewisite exposure have focused on detection and quantification of CVAA, at least in the past. However, Noort et al. (2002) also pointed out that, due to the high affinity of arsenic toward sulfhydryl groups, adducts of lewisite/CVAA and cysteine residues of proteins are formed. In an *in vitro* study, incubating 14C-labeled lewisite with human blood samples, 90% of lewisite was found in erythrocytes, whereas 25–50% of arsenic was bound to globin. From these protein adducts, CVAA can be released to form an adduct with the antidote British anti-lewisite (BAL; Fidder et al., 2000). The authors were also able to identify a specific protein adduct of lewisite formed with cysteine residues 93 and 112 of β-globin. See the section "Detection of DNA and protein adducts of vesicants," later in this chapter, for analytical details. Figure 56.8 summarizes the biotransformation and reversal of adduct formation by BAL.

Elimination

Snider et al. (1990) determined the elimination of lewisite from rabbits after p.c. injection. The half-life was determined, ranging from 55–75 h. A clearance of 120 mL/h/kg was found. These findings only describe the overall elimination of arsenic from the organism following a lewisite exposure.

In vivo, unbound CVAA is quickly excreted via the renal pathway and cannot be detected in urine samples taken later than 12 h postexposure. The biological half-life of protein adducts is much longer: in blood samples taken 10 days postexposure and treated with BAL, Fidder et al. (2000) were still able to release 10% of the CVAA-BAL concentration found on day 1. Protein adducts of CVAA play an important role in the verification of potential lewisite exposure.

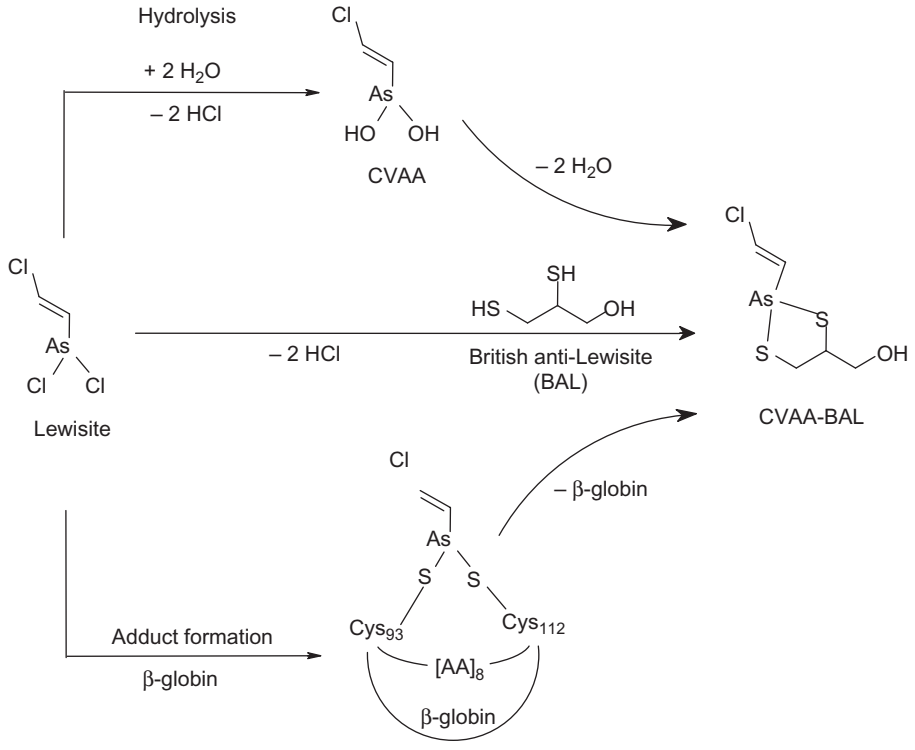

FIGURE 56.8 Lewisite biotransformation, adduct formation, and its reversal.

Bioanalytical Techniques for Quantification of Vesicants

Determination of Vesicants and Direct Biotransformation Products

When Drasch et al. (1987) determined the concentration of sulfur mustard in tissues of a deceased victim, they had to employ a combination of dichloromethane extraction and a thin-layer chromatography cleanup on silica plates, followed by derivatization with gold chloride and quantification by electrothermal atom absorption spectroscopy.

Vycudilik (1985) had already used gas chromatography-mass spectrometry (GC-MS) to confirm the presence of sulfur mustard in urine samples. Sodium chloride was added to the sample to facilitate extraction and slow down hydrolysis in the aqueous sample, and then the analyte was extracted with diethylether. The solvent was evaporated and the residue dissolved in methylene chloride. After purification—by shaking the sample solution for 1 h with silicagel—the solvent was again evaporated. The residue was again dissolved in methylene chloride and used for chromatography.

To detect TDG, TDG sulfoxide, and their acid-labile esters, Black and Read (1991) used $TiCl_3$ reduction, converting these products into single analyte TDG. TDG was then converted to its bis(pentafluorobenzoyl) derivative and quantified by GC-MS using negative ion chemical ionization. TDG sulfoxide could also be extracted directly using solid-phase extraction, followed by a Florisil cleanup. Derivatization and quantification was conducted as described previously. $TiCl_3$ was also used by Daly and O'Hehir (2007) to reduce the β-lyase pathway biotransformation product 1,1-sulphonylbis[2-(methylsulfinyl)ethane] to 1,1′-sulfonylbis[2-(methylthio) ethane] (SBMTE). This was followed by automated solid-phase extraction and LC-positive ion-ESI–tandem mass spectrometry.

Li et al. (2013) presented an ultrahigh-performance LC-tandem mass spectrometry (UPLC-MS/MS) method for simultaneous quantification of seven plasma biotransformation products of sulfur mustard. Limits of quantification ranged from 0.01 to 5.0 µg/L. The main products found in rat plasma were bis-β-chloroethyl sulfoxide (SMO), TDG, TDG sulfoxide (TDGO), 1,1′-sulfonylbis-[2-S-(N-acetylcysteinyl) ethane] (SBSNAE), 1,1′-sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE) and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE).

Methods to detect lewisite exposure have been focused on its main biotransformation product, CVAA. Initial methods were developed for environmental samples (Bossle et al., 1989). Methods for CVAA quantification in serum were described by Fowler et al. (1991), as well as Jakubowski et al. (1993). CVAA was derivatized with 1,2-ethanedithiol and quantified using GC-MS. Logan et al. (1996) employed a similar method to detect CVAA in urine of guinea pigs exposed to lewisite.

Detection of DNA and Protein Adducts of Vesicants

Adducts of sulfur mustard can be hydrolyzed to release TDG. Lawrence et al. (2008) used this procedure, followed by extraction, derivatization, and GC-negative ion chemical ionization–mass spectrometry. A number of methods have been developed to directly detect and quantify the adducts of sulfur mustard. The most prevalent DNA adduct, N7-(2-hydroxyethylthioethyl)-guanine, was directly detected and quantified by Fidder et al. (1994, 1996a), using electrospray LC-tandem MS with multiple reaction monitoring. Benschop et al. (1997) used an immunoslotblot assay to detect this adduct in blood samples from Iranian patients, in order to verify their exposure that had occurred during the Iran–Iraq war in the 1980s.

Initial efforts by Noort et al. (1996, 1997) to detect the protein adducts of sulfur mustard focused on the 4-(2-hydroxyethylthioethyl)-L-aspartate, 5-(2-hydroxyethylthioethyl)-L-glutamate, cysteine and N-terminal valine adduct, and two histidine adducts, N1- and N3-(2-hydroxyethylthioethyl)-L-histidine. Acidic hydrolysis and pronase digestion were used to release these adducts from globin. Pronase is a mixture of proteinases isolated from the extracellular fluid of *Streptomyces griseus*. Adducts were derivatized with 9-fluorenylmethyl chloroformate, followed by identification and quantification, using GC-MS.

Even though they constitute only 1–2% of alkylated amino acids, the N-terminal valine adducts were useful for subsequent quantification efforts. N-alkylated N-terminal valine could be selectively cleaved, using pentafluorophenyl isothiocyanate as a reagent in a modified Edman procedure. The product of this reaction, pentafluorophenyl thiohydantoin, was derivatized with heptafluorobutyric anhydride and quantified by negative ion GC-MS/MS. This method was sensitive enough to confirm an *in vivo* exposure of guinea pigs 48 h after i.v. administration of 0.5 mg/kg; i.e., 6% of the LD_{50} (Fidder et al., 1996b). Recently, an improved method to detect the N-terminal valine adduct was presented by Nie et al. (2011) with a lowered limit of detection for identification of the adduct after a 20 nmol/L *in vitro* sulfur mustard exposure of human blood.

Noort et al. (1999) again used pronase to digest human serum albumin that had been incubated with sulfur mustard. The cleavage product, *S*-[2-[(hydroxyethyl)thio] ethyl]-Cys-Pro-Phe, was analyzed by micro LC-tandem mass spectroscopy.

Carol-Visser et al. (2008) had developed a method for online pepsin digestion-liquid chromatography-tandem

mass spectrometry configuration for rapid analysis of protein adducts of CWAs and demonstrated the detection of specific adducts of sulfur mustard in human serum albumin (along with sarin adducts to human butyryl cholinesterase).

Yeo et al. (2008) presented a verification method for the detection of albumin adducts of sulfur mustard by pronase digestion, followed by LC-MS-MS/MRM analysis. A blood concentration of 50 nM sulfur mustard resulted in formation of adducts that were successfully detected. Adducts of nitrogen mustards could also be detected after exposure to 100 nM HN-2 or 200 nM of HN-1 or HN-3, respectively.

Incubation of lewisite–protein adducts with BAL is capable of transferring its product 2-CVAA into a BAL-CVAA derivative. This derivative can be quantified using GC-MS. The method is able to detect a 1-nM lewisite exposure of human blood *in vitro* (Fidder et al., 2000).

A specific β-globin adduct to Cys93 and Cys112 was identified by the use of electrospray tandem mass spectrometry, as well as by chemical transformation with the cysteine-selective reagent vinylpyridine and derivatization by S-carbamylation (Fidder et al., 2000). No method for quantification of this adduct was described. As of mid-2008, no specific adducts other than the abovementioned ones have been described in literature.

Despite 90 years of research on the effects of vesicant agents and on medical countermeasures, only very limited data on toxicokinetics is available. However, technological advances in analytical chemistry have contributed to a better understanding of toxicokinetic properties of both sulfur mustard and lewisite, particularly in the last two decades. Knowledge gaps exist, but research efforts continue. The Chemical Weapons Convention (CWC) constitutes enormous progress in efforts to completely rule out future incidents of chemical warfare, and the risk of chemical weapons being used by state parties is probably lower than in any decade since 1910. On the other hand, there is a worldwide asymmetric threat, and the risk of terrorist attacks using chemical weapons cannot be ruled out. Defense experts, including medical chemical defense researchers, will be concerned about these issues for the foreseeable future. It is to be hoped that more comprehensive knowledge on both toxicodynamics and toxicokinetics will contribute to development of more effective, and possibly causative, therapies of vesicant poisoning. At the same time, the capability to unanimously prove vesicant exposure, even long after the incident, does certainly increase the possibility that any use of these CWAs will ultimately be detected and sanctioned. State-of-the-art analytical methods may play an important role in deterring and preventing acts of chemical warfare.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Measurement of toxicokinetic profiles is primarily motivated to improve in-depth understanding of the poison's fate in an organism, and thus of pathophysiological consequences. Both are important to optimize antidote treatment and therapy regimens for poisoned humans that intend to boost poison elimination and reverse toxic effects; or at least minimize harm by causal and symptomatic approaches. As outlined in this chapter, the fate of poison is regulated by multifactorial processes characterized by degradation and enzymatic biotransformation, as well as elimination by protein or DNA binding. With respect to state-of-the-art techniques and study design, only a few animal models are being investigated comprehensively for nerve agents, whereas almost no data on sulfur mustard and lewisite are available in literature.

To make matters worse, almost no human toxicity or toxicokinetic data for CWAs is available due to ethical reasons, hindering specific and tailored medical approaches. Therefore, data are to be obtained from animal studies requiring extrapolation of relevant characteristics to human conditions. As is obvious from the tabular compilations on acute lethal doses of nerve agents in different laboratory animals and different routes of exposure (Table 56.2), especially under consideration of stereoisomeric differences (Table 56.4) and underlying kinetic characteristics in terms of rate constants for serine esterase inhibition and catalytic constants for PTE-mediated hydrolysis (Table 56.3), such data are still incomplete. In addition, interspecies and intraspecies variations are enormous, limiting comparability and transferability of experimental results. There are only a limited amount of data considering specific differences between lethal and sublethal exposure scenarios. Data on tissue distribution in animals are rare and correlation to human organisms is not clarified conclusively to explain, for example, the larger persistence of nerve agents in the human body. This lack of information indicates future demands of medical defense research. There is also a rising need for combined toxicokinetic and pharmacokinetic data obtained from poisoned organisms under therapeutic treatment. This will help to find the most valuable reactivators of OP-inhibited cholinesterases, which differ dramatically in efficacy in a poison- and species-dependent manner. Taken together, results of future studies should (i) support development of mathematical models to describe and predict poison and antidote behavior *in vivo* in humans; (ii) unravel novel targets of poison on the molecular or compartmental level, which are useful as biomarkers or to identify additional pathophysiological situations; and (iii) establish novel bioanalytical methods and techniques, which allow very low quantification

limits and sufficient selectivity for stereoisomers in various tissues and compartments. Whereas a number of studies have been performed using commonly known nerve agents, there remains much more demand for further work with respect to vesicants.

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CHAPTER

57

Toxicokinetics and Toxicodynamics of DFP

Miguel Sogorb, Jorge Estevez and Eugenio Vilanova

INTRODUCTION

Diisopropylfluorophosphate (DFP) is an organophosphorus compound (OP) that has not been used as a pesticide, chemical warfare agent or plasticizer. However, a search of the PubMed database yielded 3,514 results searching for different synonyms in titles and abstracts, as indicated in Table 57.1.

In other searches, using only one of the four MeSH terms (isofluorate, DFP, diisopropylphosphofluoridate, or diisopropylphosphoro-fluoridate), 3,085 results were found for those terms. Therefore, the PubMed database uses these four terms as synonyms and the same references are found in any cases. They represent most, but not all, the publications involving DFP. It should be noted that with the abbreviation of "DFP" some nonrelated publications are referred to (i.e., "bacterial DFP-flavoproteins"). Unfortunately, there is not an established tradition of indicating the CAS number for a unique reference of the chemicals in scientific publications, and standard chemical nomenclature is not always used. The statistics of the distribution of publications over time is shown in Figure 57.1.

More specifically, searching only for references mentioning DFP in the title, 597 results were produced. Although these references do not represent all the relevant references, this could be considered an indication of the number of publications for which the compound is of direct interest to the authors. Such a substantial number of publications studying or using DFP demonstrates that it is a compound that has produced a high interest in research activities.

There are several reasons to justify why DFP has been extensively used:

1. It was one of the first synthesized anticholinesterase OPs. On the basis of the ideas of a report by Lange (1930), a preparation of DFP in excellent yield was described by Saunders and Stacey in Cambridge, United Kingdom, in 1948. A US patent was filed in 1949 and proposed that DFP should be used as a chemical warfare agent. It had been previously synthesized by Schrader et al. (1937) and a patent was filed in 1938, but it was not published until 1951.
2. DFP is commercially available through suppliers of laboratory chemical reagents.
3. Radiolabeled DFP with ^{3}H or ^{32}P radioisotopes are commercially available.
4. Other fluorophosphates were synthesized and produced as chemical warfare agents (sarin and soman). However, DFP is less toxic than the G-series nerve agents and, therefore, easier to handle. DFP has been extensively used as a model compound for understanding the toxicity, mechanisms, and therapeutic strategy of the other fluoride-containing OPs.
5. DFP is able to bind to many proteins with serine, tyrosine, and other residues; therefore, it is an inhibitor of proteases and other esterases different from cholinesterases. This capacity has triggered interest in using it for toxicological, pharmacological, and biomedical research. As a consequence, DFP is accessible and has been used in several fields as follows:
   a. Inhibition of cholinesterases *in vitro* and *in vivo* for studying kinetic and molecular mechanism of inhibition and interaction of OPs.
   b. Model compound for anticholinesterase toxicity of OP and, in particular, of chemical warfare agents.
   c. Model compound of study of neurotoxicity and pharmacological studies related to cholinesterase inhibition.
   d. Model compound for development and mechanistic studies of reactivators (oximes and

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TABLE 57.1 Identification of DFP

Molecular formula	C_6-H_{14}-F-O3-P
	$(C_3H_7)_2$-P(O)-F
Cas number	55-91-4
Synonyms	Diisopropylfluorophosphate
	Isoflurophate (MeSH, Medical Subject Headings File)
	Isoflurophate (USP, USPDDN, United States Pharmacopeia Dictionary of Drug Names)
Other synonyms	PF-3; Diisopropylphosphorofluoridate; Phosphorofluoridic acid, bis(1-methylethyl) ester; Isofluorphate; Diflupyl; Diflurophate; Diisopropoxyphosphoryl fluoride; Diisopropylphosphofluoridate; *O,O′*-Diisopropylphosphoryl fluoride; *O,O*-Diisopropylfluorophosphate; fluorophosphoric acid, diisopropyl ester;
List of synonyms found in publications referred in PubMed data base	DFP; diisopropylfluorophosphatase; diisopropylfluorophosphatases; diisopropylfluorophosphate; diisopropylfluorophosphatetreated; diisopropylphosphorofluoridate; diisopropylphosphorofluoridates; diisopropylphosphorofluoride; diisopropylphosphorofluorofluoridate; diisopropylfluorophosphatase; diisopropylfluorophosphate; diisopropyl fluorophosphates diisopropylphosphorofluoridate; diisopropylfluorophosphate; diisopropylphosphorodiamidate; diisopropylphosphorodiamidofluoridate; diisopropylphosphorodithioato; diisopropylphosphorofluoridate; diisopropylphosphorothioate; diisopropylphosphorothioic; di isopropylfluorophosphonate; di iso propyl fluoro phosphate; isoflurophate
MeSH terms used as synonyms in PubMed	isoflourate; diisopropylfluorophosphate; diisopropylphosphofluoridate; diisopropylphosphorofluoridate
Molecular weight	184.1456

Source: ChemIDPlusin ToxNet databases, US National Library of Medicine (http://toxnet.nlm.nih.gov) and PubMed data base National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed).

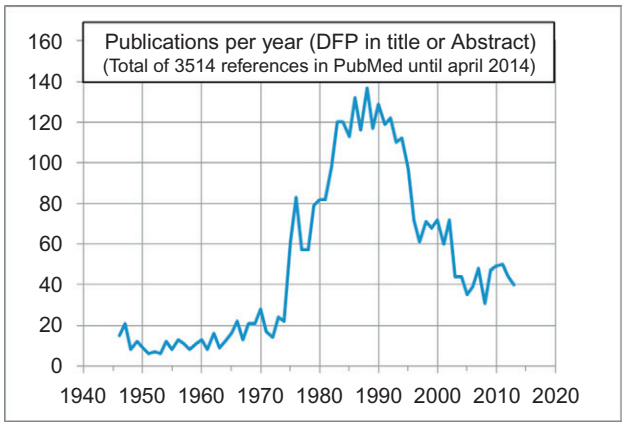

FIGURE 57.1 Statistic of publication referred to in PubMed containing DFP synonyms in the title or abstract. Data until 2013. Indicated publications per year.

others) for therapy and prophylaxis against intoxications of OPs.

e. As a tool in studies of the role of proteases in biological processes as an irreversible serine-protease inhibitor.

f. In studies related to several biological processes when esterases or other DFP-binding proteins were thought to be involved (i.e., coagulation and thrombin and prothrombin inhibition, role of lysozymes in some cardiovascular process, skin sensitization processes, neurodevelopment, neurobehavioral alterations, and others).

g. In ophthalmological studies related to glaucoma and other vision-related pathological conditions.

h. Use for labeling in study and clinical diagnosis of the turnover and life span of some human cells, such as erythrocytes, platelets, and leucocytes–granulocytes.

i. In the detection of the target of the organophosphorus-induced delayed neuropathy (OPIDN) and the so-called neuropathy target esterase (NTE), and in the understanding of the molecular mechanism of induction, including the role of the so-called aging reaction.

j. Detection of phosphorylable sites in proteins and binding to protein in serine or in tyrosine residues on esterases, proteases, and other proteins.

k. Other biological and biotechnological applications.

In brief, there are many studies in which DFP is used as a model OP compound of toxicity caused by binding to esterases (cholinesterases, NTE, others), as a tool for understanding some biological and toxicological processes, and for developing and studying therapeutic drugs and therapeutic strategies. Some of these aspects are further discussed in the next sections.

PHYSICO-CHEMICAL PROPERTIES AND CHEMICAL IDENTIFICATION OF DFP

Chemical Structure and Analogy with Other Nerve Agents

DFP is a O,O′-dialkylfluorophosphate (CAS #55-91-4) synthesized in the 1930s by procedures patented for insecticides, mold control, warfare agents, and fluorine compounds for dental protection. However, it has not been used as a warfare agent.

DFP has other names (Table 57.1), although it is most commonly called diisopropylfluorophosphate or diisopropylphosphorofluoridate, but it is also referred to as isofluorophate in some databases. It contains the oxon group (P═O) of the oxophosphates and therefore does not need a toxic activating reaction like that needed by the thiophosphates (P═S).

The two organic substitutions with isopropyl groups are bonded to the phosphorus atom through an oxygen atom (alkoxy groups), which is a third substitution of the acidic group fluorine (F), the most labile group against possible reactions of nucleophilic substitution and is considered the "leaving group" or "X-group (Figure 57.2). Therefore, DFP may be considered as F-containing oxo-organophosphate, with the expression of "phosphate" indicating the two O-substitution through oxygen atoms.

Other well-known G-nerve agents contain fluor (i.e., sarin, soman, and cyclosarin), whereas tabun contains —CN as a leaving group (Table 57.2, Figure 57.3). However, sarin and soman have one substitution by a P—O—bond, and another has a P═C bond, so they are usually named phosphonates. Phosphoroamidate compounds have also been synthesized with a non-substituted amido group (P-NH_2, i.e., methamidophos) or monosubstituted (P-NH-R) or di-substituted (P-N-(R_1,R_2)) groups. An example is mipafox (*N*,*N*′ diisopropylphosphorodiamidoflouridate) (Table 57.2).

Mipafox was developed for insecticidal use but was withdrawn for that application because of its potential for causing delayed neuropathy. It is currently used as a selective inhibitor of NTE. DFP has a common property with mipafox for inducing delayed neuropathy.

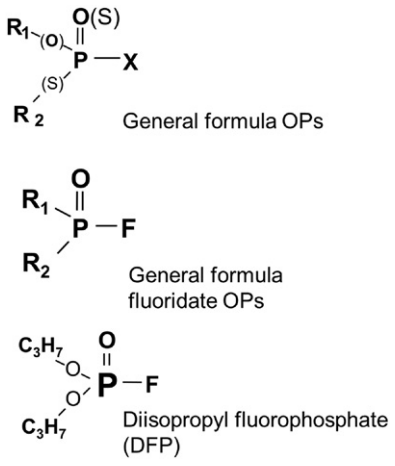

FIGURE 57.2 General structure of organophosphorus compounds and fluorophosphates. R is an alkyl or aryl group. X is the so-called leaving group.

Physico-Chemical Properties

Table 57.2 shows some basic physico-chemical properties of DFP, some nerve agents, and mipafox. DFP is a liquid (m.p. = 82°C) with a clear or slightly yellow color. It was described that dialkyl fluorophosphates produce a pleasant odor by a researcher who inhaled fumes in the process of synthesis and became intoxicated after a few minutes. Melting and boiling points are in the same range as for other nerve agents. The vapor pressure is 0.570 mmHg, which is higher than tabun but lower than sarin. We can use vapor pressure as a criterion of the facility of inhalation exposure when handling the pure liquid compound in the laboratory. It is low when compared with other liquids such as water (25 mmHg) or organic solvents (i.e., 28 mmHg toluene, 151 mmHg *n*-hexane). Therefore, handling small amounts in the laboratory for experimental studies is viable if the appropriate protective measures are used.

HISTORY OF DFP SYNTHESIS AND ITS RELATIONSHIP WITH DEVELOPMENT OF WARFARE NERVE AGENTS

DFP is used in research for understanding mechanisms of toxicity, and for therapy and prophylaxis of chemical warfare OP agents, because of its relatively low toxicity as compared with G-series nerve agents tabun (GA), sarin (GB), soman (GD), or cyclosarin (GF).

A history of monofluorophosphates has been reported by Peter Meiers (Meiers, 2014). Inorganic fluorophosphates (sodium monofluorophosphate) and organic aryl or alkyl substitutes were researched, and patents of methods of synthesis for substances intended for insecticides, warfare chemicals, and fluorophosphoric acid were filed. They are also associated with fluorine compounds for dental protection.

Willy Lange described the synthesis of some fluorophosphates, such as difluorophosphoric acid, and patented the synthesis of aryl fluorosulfonates and proposed their use as insecticides (Lange, 1930). In 1932, Gerda von Krueher, one of Lange's students, was involved in fluorophosphates research for the laboratory work performed for her PhD thesis. By heating silver monofluorophosphate with methyl or ethyl iodide, she prepared

TABLE 57.2 Some Basic Physical Properties of DFP Compared with Other Nerve Agents and F-Containing Ops

Compound	DFP	Soman	Sarin	Tabun	Mipafox
CAS number	(55-91-4)	(96-63-0)	(107-44-8)	(77-81-6)	(371-86-8)
Molecular formula	$C_6H_{14}FO_3P$	$C_7H_{16}FO_2P$	$C4H_{10}FO_2P$	$C_5H_{11}N_2O_2P$	$C_6H_{16}FN_2OP$
Physical state	Liquid (oil, clear-yellow liquid)	Liquid	Liquid	Liquid	Solid
MW	184.2	182.2	140.1	162.1	182.2
Melting point	−82.1°C	−42,1°C	−57°C	−50°C	65
Boiling point	183°C	198°C	147°C	240°C	125°C (2 mmHg)
	61.9°C (9 mmHg)				
Log P	1.17	1.78	0.3	0.38	0.29
Vapor pressure (at 20°C)	0.579 mmHg	0.4 mmHg	2.86 mmHg	0.07 mmHg	0.105 mmHg
Solubility water (at 25°C)	15 g/L (very unstable (at pH 7.5, half-life = 1 h); decomposed by alkali.)	21 g/L	1103 g/L	98 g/L	80 g/L
Solubility other	Isopropanol: 0.1–0.5 M (stable for months at −70°C)				
Density	1.06 g/mL (25°C)	1.02	1.09		
Recommended temperature	2–8°C				

Source: ChemIDPlusin ToxNet databases, US National Library of Medicine (http://toxnet.nlm.nih.gov); Sigma Chemicals Co, Products information DFP: (http://www.sigmaaldrich.com); Merk Millipore (http://www.merckmillipore.com).

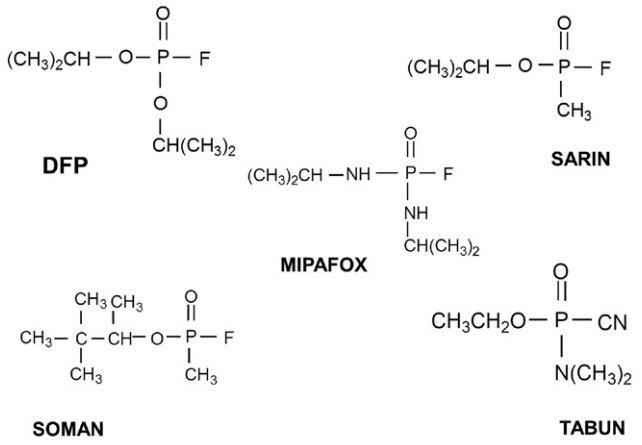

FIGURE 57.3 DFP structure and fluoro and ciano organophosphate analogues.

dialkyl monofluorophosphates. She described that initially the fumes had a pleasant odor but within a few minutes she and her colleagues began to suffer pressure in the larynx, difficulty in breathing, disturbances of consciousness, blurred vision, and painful sensitivity to light. After several hours, these symptoms resolved. Von Krueher described that the effects were not due to the acidic effects but rather from the small amount of esters. Other homologues were prepared as di-*n*-propyl and di-*n*-butyl esters. Lange considered that these compounds might be useful for pest control and offered them to I. G. Farbenindustrie, but the company had no interest at that time. Later, in 1935 at I. G. Farbenindustrie, Gerhard Schrader, Han Kükenthal, and Otto Bayer (Director of Research at Farben) patented alkylsulfonates as insecticides (Schrader et al., 1935), claiming that they were more effective than the aryl analogues proposed by Lange in 1930. They also patented dialkylaminophosphorofluorides for insecticide applications (Schrader and Bayer, 1935).

In 1937, Schrader and Kükenthal prepared several compounds and tested them for insecticidal activity. They observed that the most effective were the di-esters with an acidic group such as Cl, F, SCN, VNO, and others (Schrader et al., 1937). The highly toxic compound in the CN group with an *O*-ethyl and an *N*-dimethyl substitution was developed and is now known as tabun. Schrader also described other methods to synthesize esters of fluorophosphoric acid that were more effective than those described by Lange and Krueger, and synthesized other homologues, among them DFP. A patent was filed in 1938, but it was kept secret and not published until 1951. This patent does not refer to sarin and sarin-type compounds.

Bernard Charles Saunders (University of Cambridge, United Kingdom), considering the earlier 1932 report of Lange, prepared several new monofluorophosphoric esters and tested them for possible use as warfare agents. They were reported to the government in 1941 (Saunders, 1957), and a patent for synthesis of esters of fluorophosphoric acid was filed in 1943 (McCombie et al., 1944). The method was based on treatment of the corresponding chloro compound with a metallic fluoride such as NaF.

Willy Lange, who emigrated to the United States, worked for Procter and Gamble and at the University of Cincinnati. He performed work supported by Ozark Chemical Company. In 1943, he filed a patent for preparing anhydrous monofluorophosphoric acid and claimed that it would be used for synthesis of ester for use as insecticides (Lange and Livingston, 1943). Another related patent was registered in 1944 that also claimed the application would react with the alcohol for preparing the corresponding dialkyl esters (Lange, 1946).

In 1944, Hardy and Kosolapoff from the Monsanto Chemical Company patented the production of dialkylfluorophosphates as DFP by a procedure based on the reaction of alcohol with PCl_3 given dialkylphosphite, then chlorinating and finally fluorinating with NaF (Hardy and Kosolapoff, 1944).

In 1947, Lange and Livingstone (Monsanto) published a series of reports about fluorophoshoric acids and their derivatives (Lange and Livingston, 1947). They reported, "It has recently been shown that the esters of monofluorophosphoric acid, H_2PO_3F, which were previously known to be highly toxic, have a probable usefulness in the treatment of glaucoma and myasthenia gravis, the diisopropyl ester being especially suited for this purpose. Unpublished observations by the senior author also showed that the esters possess insecticidal properties and may act as fumigants" (Lang and Livingston, 1947).

In 1948, a preparation of DFP was described by Saunders and Stacey of Cambridge, United Kingdom (Saunders and Stacey, 1948), and a US patent was filed (McCombie et al., 1949). In 1949, another patent was filed for production of mono and dialkyl fluorophosphates. These were produced by treating alkyl polyphosphates with HF and were called dialkoxy phosphoryl monofluoride (Lange, 1949). The monoesters were purported to have low toxicity in mammals but showed fungicidal properties. For that time period in the open literature, preparation of DFP by other complicated methods has been reported, as well as studies of its effects on the nervous systems and as a potential treatment of human diseases. Further efforts regarding the application of fluorophosphoric acid and its salts were focused on the prevention of dental disease (i.e., as an additive in toothpaste).

DFP was synthesized by B. C. Saunders when he was looking for warfare agents, and it was called PF-3. It appears that he based his research on the previous study by Willy Lange and his PhD student Gerda von Krueger, who synthesized the series of mono-*o*-alkyl flourophosphoric acid with C1, C2, C3, and C4 alkyl groups. However, it was less effective than the G-series agents (sarin, tabun, soman).

DFP may be synthesized by reactions similar to that used for other OPs, for example, by causing the alcohol (isopropyl alcohol) to react with phosphorus trichloride (PCl_3) to form diisopropryl phosphite. Chlorinated and chlorine-substituted fluorine were patented by Hardy and Kosoloapoff (1944). Therefore, the process of synthesizing DFP is closely related to the development of the synthesis of organophosphates for nerve agents, insecticides, and other fluorine compounds for dental applications.

DFP has been commercially available since the 1970s from chemical suppliers, such as Sigma. Radiolabeled as [^{3}H]DFP and as [^{32}P]DFP, it has been used for labeling proteins and for toxicokinetic studies. Therefore, it has been available and used either for understanding its toxicity or for use as a tool for toxicological or pharmacological research activities.

TOXICOKINETIC AND BIOTRANSFORMATION OF DFP AND STUDIES ON DFPASE

Absorption, Distribution, and Toxicokinetic Studies

Radiolabeled [^{3}H]DFP has been used for studying its absorption and biodisposition by inhalation in the guinea pig (Scimeca and Martin, 1988) and mice (Scimeca et al., 1985), and by intravenous administration in mice (Martin, 1985). Skin penetration has been studied in pigs and humans *in vitro* (Vallet et al., 2007).

Distribution after Inhalation Exposure

The tissue disposition of radiolabeled [^{3}H]DFP and its metabolites was studied in guinea pigs after inhalation exposure from 5 min to 24 h after treatment (Scimeca and Martin, 1988). [^{3}H]DFP was rapidly distributed in all tissues. The product of hydrolysis, metabolite diisopropryl phosphate (DIP), was covalently bound to the tissue biphasic curve, with an initial phase representing a very rapid decrease in tissue concentrations, followed by a slower phase of tissue clearance for bound [^{3}H]DFP and free [^{3}H]DIP.

After 4 h, the higher proportion of radioactivity in all the tissues was in the form of bound [^{3}H]DIP. Bound [^{3}H]DIP levels did not follow a biphasic clearance curve and declined at a slower rate than [^{3}H]DFP and free [^{3}H]DIP tissue levels. By 5 min, the greatest accumulation of bound [^{3}H]DIP occurred in the liver (nearly 20% of the total body burden), with a noticeably small amount in the brain (0.1%). Total cholinesterase activity in the brain and red blood cells was inhibited by approximately 90%, with plasma pseudo cholinesterase activity and true cholinesterase activity inhibited by 99% and 97%, respectively.

Distribution After Intravenous Administration

Intravenous administration of [^{3}H]-DFP was studied in mice, and the disposition of [^{3}H]DFP in selected tissues

and cholinesterase activity and recovery were studied (Martin, 1985). After 1 min, [^{3}H]DFP had penetrated tissues and was irreversibly bound. The tissue concentrations decreased quickly and after 2 h all concentrations were less than 50 pg/mg tissue. Most radioactivity was bound to tissue as [^{3}H]DIP but decreased with time in all tissues except liver, kidneys, and fat, which reached a maximum 30 min before declining. Only in liver and kidney did appreciable quantities of [^{3}H]DIP remain after 3 days.

[^{3}H]DFP was rapidly hydrolyzed to free [^{3}H]DIP, which was found in all tissues within 1 min of [^{3}H]DFP administration. [^{3}H]DIP concentrations were equivalent to or exceeded those of [^{3}H]DFP in all tissues except brain.

Cholinesterase inhibition in plasma, diaphragm, and brain after DFP treatment (1 mg/kg, intravenous) was temporarily correlated with the concentrations of bound [^{3}H]DIP in these same tissues between 1 h and 3 days. Cholinesterase inhibition in brain and diaphragm did not correlate well with bound [^{3}H]DIP, suggesting binding to sites different from cholinesterase.

DFP treatment (1 mg/kg) induced motor hypoactivity up to 6 h after intravenous administration with a time course that did not correlate with free [^{3}H]DFP, bound [^{3}H]DIP concentrations in the brain, or with cholinesterase inhibition in the brain, which suggested that another noncholinesterase interaction was responsible.

Other older studies have been reported regarding the distribution in oxygenated blood and atrial tissue (Schuh, 1970), excretion in cats (Hansen et al., 1968a), and distribution and metabolism in the guinea pig (Hansen et al., 1968b).

In summary, DFP is rapidly distributed, bound to tissues in the form of the bound diisopropryl phosphoryl group to proteins, released as metabolite (DIP), and finally excreted, mainly in the urine. There is evidence that a high proportion of binding is to other sites different from cholinesterases. Moreover, the time course of CNS effects suggests that other noncholinesterase interactions are involved in DFP neurotoxicity.

Skin Penetration

Percutaneous penetration of [^{3}H]DFP was tested *in vitro* with human and pig skin. In the test using full skin, the absorbed dose 24 h after depot was 15.6 ± 1% in pig skin and 9.4 ± 1.5% in human skin, with a ratio of 1.7 pig:human. The penetration rates between 0.5 and 4 h were 3.22% and 1.63% dose per hour, with a ratio of 1.7 for pig:human skin. Similar behavior was observed using split-thickness skin.

The radioactivity quantified in the receptor fluid corresponds not only to intact DFP, but also to its metabolites, both tritiated. In preliminary findings, it was also found that at 24 h after exposure more than 99% of DFP reaching the receptor fluid was hydrolyzed into the skin. It was not clear if this was attributable to degradation by phosphotriesterases, by binding and dephosphorylation to proteins/esterases, or by spontaneous degradation.

Physiologically Based Pharmacokinetic/ Pharmacodynamic Studies

Two studies with physiologically based pharmacokinetic (PBPK) and pharmacodynamic (PD) models have been reported (Gearhart et al., 1990; Chen et al., 2009). Gearhart et al. (1990) developed a model for mammals in which the following factors were considered: DFP tissue/blood partition coefficients; rates of DFP hydrolysis by esterases; and DFP-esterase bimolecular inhibition rate constants determined in rat tissue homogenates. Other model parameters were scaled for rats and mice using standard allometric relationships. These DFP-specific parameter values were used to simulate *in vivo* pharmacokinetic data from mice and rats. DFP concentrations in mouse plasma and brain were successfully simulated as compared with data of single intravenous injection reported by Martin (1985) as well as AChE inhibition and its reactivation. Effects of repeated subcutaneous DFP dosing on AChE activity in rat plasma and brain (Michalek et al., 1982; Traina and Serpietri, 1984) were also well-simulated, but the return of brain AChE activity to basal levels after cessation of repeated dosing was not well-described. The model returned brain AChE activity to the original level, but in laboratory studies brain AChE never returned to basal levels, even after 35 days. These data suggest modulation of AChE synthesis with prolonged DFP exposure. This study demonstrated the possibility of using a model based on mammalian physiology and biochemistry to simulate *in vivo* data regarding DFP pharmacokinetics and AChE inhibition. Scaling of the model between rats and mice was also successful.

Another study developed a model using mice and rats (Chen et al., 2009) with the following steps. First, the influence of the variability of the rate constants for synthesis [*K*(syn)] and degradation [*K*(deg)] of AChE, and regeneration [*K*(reg)] and aging [*K*(age)] of inhibited AChE on the variability of AChE activity in venous blood and brain was calculated by a global sensitivity analysis. Second, the mouse PBPK/PD model was calibrated by optimizing the values of *K*(syn), *K*(deg), *K*(reg), and *K*(age). Third, scale-up of the D FP-induced AChE activity was performed from mouse to rat. Validation of the rat model was performed by comparing the time course of venous blood and brain AChE activities from a Monte Carlo analysis with those obtained *in vivo*. Sensitivity analysis of the verified models showed that *K*(reg) and *K*(syn) were the most influential factors of AChE activity in shorter and longer durations, respectively, after

DFP challenge. Finally, scale-up of the AChE dynamics from mouse to rat was successfully evidenced by significant overlapping between the predicted 95th percentile confidence intervals and the experimental data. These approaches hold promise for predictive simulation of organophosphate-mediated AChE inhibition in humans.

Biotransformation of DFP

DFP is a phosphoric triester; consequently, it is not subjected to bioactivation through desulfuration, as in the case of thiophosphates such as chlorpyrifos, malathion, and other OP compounds used as insecticides. DFP can be enzymatically hydrolyzed by the enzymes called phosphotriesterases or phosphoric triester hydrolases according to the nomenclature of the International Union of Biochemistry and Molecular Biology (Table 57.3). The hydrolysis of DFP releases the fluoride ion and the acidic moiety of DFP, the DIP. This hydrolysis must be considered a detoxication reaction (Sogorb and Vilanova, 2010).

There are two types of phosphotriesterases, the aryldialkylphosphatase (EC 3.1.8.1) and the diisopropylfluorophosphatase (EC 3.1.8.2). The group EC 3.1.8.1 includes paraoxonases, whereas the group EC 3.1.8.2 includes the enzymes dialkylfluorophosphatases (also known as DFPases) (Table 57.3). The enzymes belonging to both families are metal-dependent (calcium in the case of paraoxonases and magnesium in the case of DFPases) and are inhibited by chelating agents (Vilanova and Sogorb, 1999). All these enzymes also contain active centers with discrete binding pockets to accommodate the three ester moieties (Bigley and Raushel, 2013). Although there are mechanistic differences among different phosphotriesterases, in all cases the divalent metal is needed for activation of phosphoryl oxygen that is further attacked by a hydroxide group causing hydrolysis and the release of the leaving group (fluoride in the case of DFP) (Bigley and Raushel, 2013).

Paraoxonases are expressed mainly in mammals, display a broad range of substrate specificity, and are able to hydrolyze P–F bonds. Consequently, they may also hydrolyze DFP and other analogous warfare agents. DFPases are enzymes with a specific capability to break the P–F bonds and have higher efficacy than paraoxonases for the hydrolysis of DFP and related compounds. However, DFPases are not expressed in mammals. The usual biological source for studying DFPases is the squid (*Loligo vulgaris*) (Hartleib and Rüterjans, 2001; Scharff et al., 2001; Katsemi et al., 2005; Wymore et al., 2014).

DFPases are highly specific for hydrolysis of P–F bonds in DFP. These enzymes can enhance the hydrolysis of DFP and analogous warfare agents. As a result, these enzymes can be used either for the deactivation of chemical warfare agents or as prophylactic and therapeutic agents against exposures to these chemicals (Sogorb et al., 2004a). However, because DFPases are not expressed in humans, the hydrolysis of DFP by paraoxonases, despite the low specificity, is the only defense against this compound in mammals based on enzymatic hydrolysis. This book contains other chapters dealing with phosphotriesterases (both DFPases and paraoxonases) and readers are referred to those chapters for more detailed information.

TABLE 57.3 DFPase in the IUPAC Classifications of Enzyme

3.1 ESTERASES Hydrolases that act upon *ester* links
- 3.1.1 Carboxylester hydrolases
  - EC 3.1.1.1 carboxylesterase
  - EC 3.1.1.2 arylesterase …
  - EC 3.1.1.5 lysophospholipase
  - EC 3.1.1.7 acetylcholinesterase
  - EC 3.1.1.8 cholinesterase …
  - EC 3.1.1.84 cocaine esterase ….
  - EC 3.1.1.90
- 3.1.2 Thiolic esters hydrolases
- 3.1.3 Phosphoric monoester hydrolases
- 3.1.4 Phosphoric diester hydrolases
- 3.1.5 Triphosphoric monoester hydrolases
- 3.1.6 Sulfuric ester hydrolases
- 3.1.7 Diphosphoric monoester hydrolases
- 3.1.8 Phosphoric triester hydrolases (PHOSPHOTRIESTERASES)
  - EC 3.1.8.1 aryldialkylphosphatase
  - *EC 3.1.8.2 diisopropyl-fluorophosphatase (DFPase)*

EC 3.1.8.2. *DIISOPROPYL-FLUOROPHOSPHATASE* (DFPASE)

Reaction: Diisopropyl fluorophosphate + H_2O = diisopropyl phosphate + fluoride

Other name(s): DFPase; tabunase; somanase; organophosphorus acid anhydrolase; organophosphate acid anhydrase; OPA anhydrase; diisopropylphosphofluoridase; dialkylfluorophosphatase; diisopropyl phosphorofluoridate hydrolase; isopropylphosphorofluoridase; diisopropylfluorophosphonate dehalogenase

Systematic name: Diisopropyl-fluorophosphate fluorohydrolase

Comments: Acts on phosphorus anhydride bonds (such as phosphorus-halide and phosphorus-cyanide) in organophosphorus compounds (including "nerve gases"). Inhibited by chelating agents; requires divalent cations. Related to *EC 3.1.8.1* aryldialkylphosphatase. Previously listed as EC 3.8.2.1.

Source: Nomenclature Committee of the International Union of Biochemistry and Molecular.
Biology (NC-IUBMB). In consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN). Enzyme Nomenclature. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse http://www.chem.qmul.ac.uk/iubmb/enzyme/ (Last update February 17, 2014).

Detoxication of DFP via Protein Binding

In the distribution studies described, it was observed that after administration of radiolabeled DFP, most radioactivity is bound DIP and after it is released it is free DIP.

It is then excreted mainly via urine. This suggests that, in mammals, binding to protein by phosphorylation is a main mechanism of withdrawal and detoxication. The mechanism involved in binding to proteins also explains the mechanism involved in toxicity. In the next chapter, examples of binding to protein are described either as detoxicating mechanisms (i.e., binding to albumin) or as binding to the target esterases causing toxicological consequences (i.e., acetylcholinesterase (AChE) and NTE).

Role of Albumin in the Detoxication of DFP

Albumins exhibit esterase-like activities for carboxylesters, as reported years ago (Tildon and Ogilvie, 1972; Means and Bender, 1975; Kokubo et al., 1982). These esterase activities are based in the reversible acylation of a tyrosine residue of the sequence Arg-Tyr-Thr-Arg (position 410 in bovine serum albumin and 411 in human serum albumin) (Peters, 1996a). However, it has also been suggested that this activity, at least in human serum albumin, is the result of the irreversible acetylation of 82 different amino acid residues rather than reversible acylations (Lockridge et al., 2008).

Serum albumin also displays other esterase activities against carbamates, such as carbaryl (Sogorb et al., 2004b, 2007), based on the Tyr-411 residue as the main target of the active center. The Tyr has also been proposed as a target of phosphorylation of OP compounds, such as phosphoramidates (Sogorb et al., 1998), azamethaphos, chlorfenvinphos, chlorpyrifos–oxon, diazoxon, dichlorvos, malaoxon, pirimiphos-methyl (Carter et al., 2007; Tarhoni et al., 2008), and paraoxon (Sogorb et al., 2008). Of course, it is necessary to add DFP to this list. It has been demonstrated that 1 h of *in vitro* exposure of rat and human serum albumin to 19 μM DFP causes the binding of 0.011 or 0.039 mol of DFP/mol of albumin, respectively (Tarhoni et al., 2008). In addition, this binding is prevented by exposure to most of these OP compounds (Carter et al., 2007; Tarhoni et al., 2008), which suggests that DFP and others are in competition for the same amino acid residue. Other analogous structures to DFP, such as soman, sarin, cyclosarin, and tabun, have been shown to bind to albumin via phosphorylation (Williams et al., 2007; Li et al., 2008; Read et al., 2010).

Mass spectrometry assays have allowed us to confirm the target amino acid for binding of DFP and analogous nerve agents, as well as other OP compounds such as Tyrosine-411 (Li et al., 2007, 2008). However, other target amino acids for phosphorylation of albumin have also been suggested, specifically four additional Tyr, two Ser (Ding et al., 2008), and several Lys (Grigoryan et al., 2009).

The binding of DFP to albumin, despite the fact that it can be considered an enzymatic reaction or a scavenging process with an undetermined number of target amino acids, causes the release of the fluoride ion and generates an adduct between albumin and the diisopropyl moiety. The immediate consequence is that a molecule of DFP is removed from the media and cannot reach the nervous system, where it would cause inhibition of esterases, thereby triggering the neurotoxic effects. Thus, this binding is a detoxication mechanism.

The detoxication of DFP through binding to albumin might seem irrelevant because of its low efficacy in comparison with the detoxication capability of DFPase and paraoxonase. However, the efficacy of this process is based on the high concentration of albumin circulating in the blood (40 mg/mL or 600 μM) (Peters, 1996b) that is higher than the systemic DFP concentrations compatible with the survival of the individual, but not in high turnover of the phosphorylated albumin residues. The binding of paraoxon to albumin has proven to be responsible for the detoxication of physiologically relevant paraoxon concentrations in mammals (Sogorb et al., 2008).

All these considerations allow us to predict that the binding of DFP to albumin, in addition to forming adducts with the capability to act as a biomarker of exposure, might play a relevant role in the detoxication of DFP, especially considering that paraoxonases are not very efficient in the hydrolysis of OPs with the fluoride leaving group. DFPases are not expressed in mammals and paraoxonases exhibit a double genetic polymorphism in certain individuals who are more susceptible because they express low amounts of paraoxonase with very low specific activity.

ACUTE TOXICITY OF DFP AND INTERACTION WITH ACHE

Soon after DFP was discovered, it was demonstrated that it had the capacity of inhibiting AChE irreversibly. One of the earliest published reports of a systematic study of the inhibitory properties of DFP was that of Koelle and Gilman (1946). *In vitro* log pI_{50} was reported to be 6.5 for serum BuChE and 4.2, 4.5, 4.8 for AChE from rat erythrocyte, brain, and muscle, respectively, with similar values in dogs. *In vivo* studies after intramuscular injection in rats, dogs, and monkeys were performed and inhibition, toxic effects and AChE regenerations were evaluated. Levels in the range of 0.05–1 mg/kg showed significant inhibition of serum ChE and doses of 1–5 mg/kg caused a total inhibition of erythrocyte cholinesterase. Human exposure to a dose of 0.5–2 mg/60 kg caused marked inhibition of serum BuChE, without any inhibition of erythrocyte AChE and/or toxic symptoms.

Other studies of administration of DFP to humans in the range of 0.07–0.5 mg/kg were reported (Table 57.4) with moderate ChE inhibition and some moderate alterations (Grob and Lilienthal, 1947).

TABLE 57.4 The Effect of DFP (4.0 mg/kg) and Sarin (0.3 mg/kg) on Cholinesterases and CaE activities. Atropine Sulfate (37.5 mg/kg) Administered Subcutaneously Immediately After the Organophosphate

Enzyme Control Activity nmol/min × mg Protein	Compound	Male	Female
BrainAChE (nmol/min × mg protein)	DFP	10.2 ± 2.5%	10.1 + 0.9%
M:152.0 ± 12.4	Sarin	10.3 ± 4.5%	10.8 + 2.9%
F:145.9 + 10.7			
BloodAChE (nmol/min × mL)	DFP	48.9 + 9.8%	57.0 + 8.5%
1434.9 + 207.5	Sarin	55.0 + 8.6%	53.9 ± 7.2%
1485.7 + 46.0			
Plasma BChE (nmol/min × mL)	DFP	3.5 + 0.5%	1.7 + 0.1″%
3429.9 + 394.6	Sarin	13.8 + 5.4%	33.6 + 10.5″%
7115.5 + 728.2*			
Plasma CaE (nmol/min × mL)	DFP	15.2 + 3.1%	13.0 + 1.5%
2081.4 + 265.6	Sarin	22.8 + 6.3%	25.6 + 2.2%
2825.6 + 276.0*			

Source: Data obtained from Tuovinen et al. (1997).
Means ± SD are given (n=5–7); *P<0.05 compared to male mice.

Since then, a number of studies have shown AChE inhibition, including gender difference (Table 57.4) (Tuovinen et al., 1997) and effects on the different AChE forms in mice, rats, and chickens (Cisson et al., 1981; Michalek et al., 1981; Sung and Ruff, 1987).

Several routes of acute exposure have been tested, including oral, intramuscular, subcutaneous, intravenous, and intraperitoneal. LD_{50} doses are expressed in terms of mg/kg body weight and are shown in Table 57.5.

The main observed affects are those expected from cholinesterase inhibition:

- Respiratory stimulation, cyanosis, and dyspnea
- Gastrointestinal tract hypermotility, diarrhea, and hypersalivation
- Behavioral changes, such as tremors, convulsions, seizures, coma, muscle weakness, alterations in sleep time, and righting reflex
- Alterations in peripheral nerves with neuromuscular blockage and flaccid paralysis and ataxia
- Fasciculations
- Tremors

There is very little reported information regarding accidental acute toxicity in humans. It has been noted that intramuscular and oral doses in the range

TABLE 57.5 DFP Acute Toxicity in Experimental Animals

Species—Route	LD_{50}/LC_{50}	Reference
ORAL		
Rat	5 mg/kg	(1)
	10 mg/kg	(10)
Mouse	2 mg/kg	(1)
Rabbit	4 mg/kg	(2)
Dog		
DERMAL		
Mouse	72 mg/kg	(2)
Rabbit	>117 mg/kg	(2)
INHALATION		
Rat	360 mg/m^3/10 M	(3)
Mouse	440 mg/m^3/10 M	(4)
SUBCUTANEOUS		
Rat	1.44 mg/kg	(5)
Mouse	3 mg/kg	(6)
Dog	3 mg/kg	(2)
Monkey	1 mg/kg	(3)
Rabbit	1 mg/kg	(2)
INTRAPERITONEAL		
Rat	1.28 mg/kg	(7)
Mouse	2.45 mg/kg	(8)
INTRAVENOUS		
Mouse	3.2 mg/kg	(9)
Dog	3.43 mg/kg	(2)
Monkey	0.1 mg/kg	(1)
Cat	1.7 mg/kg	(2)
Rabbit	0.3 mg/kg	(1)
INTRAMUSCULAR		
Rat	1.8 mg/kg	(10)
Rabbit	0.75 mg/kg	(1)
Dog	3 mg/kg	
OCULAR		
Rabbit	1.15 mg/kg	(2)

Data obtained from the database ChemIDPlus: Isoflurophate [USP]US NLM (http://chem.sis.nlm.nih.gov/chemidplus/rn/55-91-4/), and referred as: (1) *National Technical Information Service*. Vol. PB158-508. (2) *Journal of Pharmacology and Experimental Therapeutics*. Vol. 87, pg. 414, 1946. (3) *Deutsche Gesundheitswesen*. Vol. 15, pg. 2179, 1960. (4) *Nature*. Vol. 157, pg. 287, 1946. (5) *Archives Internationales de Pharmacodynamieet de Therapie*. Vol. 226, pg. 302, 1977. (6) *Journal of Pharmacy and Pharmacology*. Vol. 34, pg. 603, 1982. (7) *Arzneimittel-Forschung. Drug Research*. Vol. 14, pg. 85, 1964. (8) *Archivum Immunologiaeet Therapiae Experimentalis*. Vol. 23, pg. 769, 1975. (9) *Biochemical Pharmacology*. Vol. 15, pg. 169, 1966. (10) *Journal of Clinical Investigation*. Vol. 37, pg. 350, 1958.

TABLE 57.6 Effect of DFP in Humans

Dose (mg/kg)	Effect	Reference
0.07 mg/kg (i.m.) 0.1 mg/kg (oral)	Reported as dose causing 50% depression RBC ChE in man *in vivo*	Grob (1957)
0.083 mg/kg (i.a.) 0.32 mg/kg (oral)	Reported as dose producing moderate symptoms	Grob (1957)
0.48 mg/kg (i.m.) 2.1 mg/kg (oral)	Estimated lethal dose	Grob (1957)
8.2 mg/m^3/10 min	Headache miosis (pupillary constriction): eye	*National Technical Information Service*. Vol. PB158-508

Source: Data from Grob and Harvey (1957).

TABLE 57.7 Comparison of Inhibition of Human Cholinesterases by DFP in Comparison with Other Nerve Agents and Their LD_{50} for Acute Toxicity

Chemical	Plasma ChE (nM)	Erythrocyte ChE (nM)	Brain ChE (nM)	Muscle ChE (nM)	LD_{50} (i.m.) in Rats (mg/kg)	LD_{50} (Oral) in Rats (mg/kg)
Sarin	4.2	3	3.3	3.6	0.17	0.6
Tabun	13	15	15	20	0.80	3.7
DFP	9.5	400	300	250	1.80	6.0

Source: From Grob and Harvey (1957).
ChE was measured using acetylcholine as substrate. Based on the method used in brain and muscle, it cannot discriminate BuChE activity from AChE, as no specific inhibitors were used. It is indicated the I_{50} (nM) for 45 min of preincubation with the inhibitor.

of 0.07–0.1 mg/kg body weight can produce a 50% decrease of erythrocyte cholinesterase and moderate clinical symptoms (Grob and Harvey, 1957) (Table 57.6).

The acute toxicity of DFP can be well-correlated with a rapid and high degree of AChE inhibition. Table 57.7 shows the I_{50} for 45 min as reported for human cholinesterases for DFP in comparison with other nerve agents. There is a correlation between AChE inhibition and acute toxicity.

Other data regarding I_{50} for chicken brain AChE are shown in the section about delayed neuropathy (later in this chapter).

DFP IN STUDIES ON NEUROTOXICITY AND THERAPY WITH REACTIVATORS

Neuropharmacological Studies of Cholinergic System

DFP is used in studies of neurotoxicity and neuropharmacology. In most studies, DFP is used as a model compound causing depression of cholinesterase activity and an increased level of acetylcholine (ACh). They include examples of studies of rats surviving status epilepticus induced by DFP (Deshpande et al., 2010), a comparison of strain rats in their susceptibility to neurotoxicity (Gordon and MacPhail, 1993), age-related differences in rate recovery of cholinesterase and receptors (Michalek et al., 1990), inhibition of the motor system (Chemnitius et al., 1989), and alterations in energy homeostasis in skeletal muscles (Gupta et al., 1986) and brain (Gupta et al., 2001a,b).

Pharmacological effects and interactions have been a matter of many publications in which DFP is used for inducing cholinergic effects and the modulation, agonist, or antagonist, of drugs. Examples include: alteration of cannabinoid signaling and its relationship with cholinergic toxicity (Nallapaneni et al., 2008); role of opioid receptors in development of tolerance to DFP toxicity (Tien et al., 2005); preventive effect of galantamine (Saghafi et al., 2013); alteration in the patterns of GFAP upregulation (Liu et al., 2012); alteration of the muscarinic receptor density and its regional adaptation (Yamada et al., 1983a,b); and genetic and sex differences in the development of tolerance (Russell et al., 1983). The alteration of electrophysiology, nerve conduction, and action potential has been described since the early toxicological studies with DFP (Couteaux et al., 1946; Toman et al., 1947), for which the use of the squid giant axon is a frequent model (Hoskin and Prusch, 1983).

Neurobehavior and Neurodevelopment

DFP has been used as a model compound for studying neurobehavioral alterations related to the increased cholinergic effects caused by AChE inhibition. In some studies these alterations are compared with those caused by other OPs and are also related to oxidative stress (Dettbarn et al., 2006; Milatovic et al., 2006, 2014; Gupta and Milatovic, 2012, 2014; López-Granero et al., 2013).

Chronic persistent alterations in spatial learning and memory have been described after exposure to DFP (Terry et al., 2012).

Long-term changes in behavior, such as anxiety, have been reported in adult mice exposed to DFP during the pre-weanling period (Kofman and Ben-Bashat, 2006). The relationship between behavioral changes and decreased brain cholinesterase activity has been described after intracerebral injections in studies since the 1950s (White, 1956). Since then, many studies have been performed with DFP and other OPs and their neurobehavioral effects. Some of the neurobehavioral studies have been related to effects developing as a result of exposure during the prenatal and neonatal periods (Gupta et al., 1985; Das Gupta et al., 1988; Ahlbom et al., 1995; Bushnell and Moser, 2006; Slotkin, 2006).

The alteration of circadian patterns (Raslear et al., 1986) and the involvement of nitric oxide in mitotoxicity in muscle and brain hyperactivity induced by DFP have been investigated (Gupta et al., 2001a,b, 2002; Zaja-Milatovic et al., 2009).

Therapy Against Anticholinesterase Toxicity

Therapy for cholinergic symptoms continues to be a topic of interest for many researchers. DFP has been used extensively as a model compound for inducing cholinesterase inhibition as well as other nervous system proteins. Studies about kinetic mechanisms of action with oximes and other therapies continue to be an issue of current research. In experimental studies, some of the compounds tested against DFP include bipyridinum oximes (Theirmann et al., 2009), tubocuarine (Barstad, 1956), meptazinol (Galli and Mazri, 1988), 2-PAM (Tuovinen et al., 1996), and quaternary ammonium compounds (Funke et al., 1955).

DFP in Other Biological Studies

During the 1950s and 1960s, DFP (isofluorate) was used in ophthalmological studies, including studies of esotropia. Its potential therapeutic application in glaucoma and myasthenia gravis was described in 1947 (Quilliam, 1947), and other studies were later published in relation to ocular pressure (Zekman and Snydacker, 1953) and retinal detachment (Weekers and Lavergne, 1955).

During the 1960s and 1970s, studies of the mechanism of coagulation were performed using DFP as an agent for blocking endogenous thrombin (Baskova et al., 1970). The interaction of DFP with human blood coagulating factors has been studied, including factor VII (Osterud et al., 1979), factor V and factor Va (Bartlett et al., 1978), and the Hageman factor (factor XII) (Becker, 1960).

The interaction of anticholinesterase effects of DFP with cardiovascular and respiratory reflexes and cardiac failure was also the subject of some studies (Heymans and Pannier, 1946; Wolthuis and Meeter, 1968). During the 1970s, a methodology was developed and applied based on labeling proteins with [^{32}P]DFP for studying the turnover, life span, and survival of human blood cells (Matsuda, 1969). These approaches have also been applied for studying the kinetics of platelets (Leeksma, 1963; Ebbe et al., 1966, 1970; Ginsburg and Aster, 1973–1974), the survival of erythrocytes (Hjort and Paputchis, 1960; Cline and Berlin, 1963; Manunta and Cancedda, 1975; Saito and Sakai, 1977; Derelanko, 1987), and the kinetics of granulocytes and neutrophils (Dresch et al., 1971; Vorob'eva, 1976; Nowotny et al., 1978).

INTERACTION OF DFP WITH OTHER ESTERASES

Serine Proteases and Albumin: Role of Tyrosine Residues

Serine proteases are proteins containing a serine residue in their active catalytic center which has a molecular mechanism similar to esterases. Therefore, they are susceptible to phosphorylation by OPs in general and by DFP in particular. This has been the basis for DFP use in biochemical studies for blocking serine containing esterases, and thereby for stabilizing proteins and avoiding proteolysis in tissue homogenates. Moreover, DFP has been used as a tool for specific studies either for understanding the mode of action of proteases or for biological studies for testing the role of proteases in some biological processes. DFP-inhibited chymotrypsin and chymotrypsinogen were studied using P^{31} NMR (Reeck et al., 1977). DFP induced inactivation of bovine trypsinogen and chymotrypsinogen (Morgan et al., 1972). The substilisin serine protease has been analyzed by NMR (Chen et al., 2008). The involvement of DFP-sensitive proteasomes in the control of oocyte maturation has been evaluated (Takahashi et al., 1994). The evidence of the role of tryptophan residue in the interaction of DFP with alpha-chymotrypsin has been reported (Wooton and Hess, 1960). Effects on sulfhydryl proteases have also been demonstrated (Heinicke and Mori, 1959).

Human serum albumin has been demonstrated to show arylacylamidase activity sensitive to DFP (Manoharan and Boopathy, 2006). A tyrosine residue has been characterized as a target of the binding of DFP to albumin (Means and Wu, 1979). Evidence has been reported that the tyrosine residue 411 is responsible for *p*-nitrophenyl acetate catalytic hydrolysis (Tildon and Ogilvie, 1972) and that it has a critical role in detoxication of OPs, such as paraoxon and diazoxon, and carbamates, such as carbaryl (Sogorb et al., 1998, 2004b, 2007, 2008). Therefore, albumin has been suggested as a biomarker of OP nerve agent exposure (Lockridge et al., 2008; Read et al., 2010).

Inhibition of Soluble PVases of Peripheral Nerve by DFP

In the peripheral nerves, soluble carboxylesterases measured with the substrate phenylvalerate (PV) are found to have high sensitivity to DFP. Tissue preparations were preincubated with DFP at nanomolar concentrations (up to 10 nM) for different inhibition times (up to 120 min) (Figure 57.4) and with DFP at nanomolar concentrations (up to 1,000 nM) for 30 min of fixed inhibition time (Figure 57.5). Inhibition data were analyzed with model equations of one, two, and three sensitive

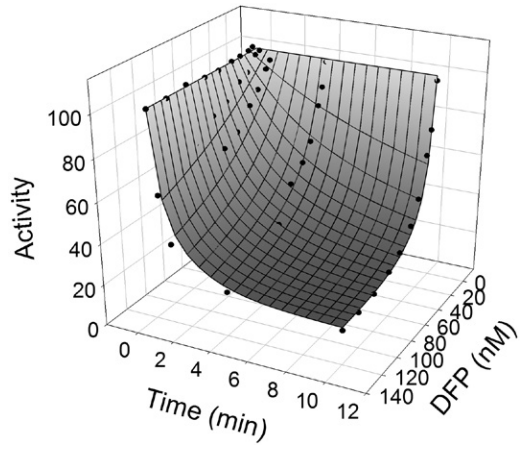

FIGURE 57.4 Kinetic inhibition 3D. Representation of the inhibition kinetics of soluble peripheral nerve PV esterase activity PVases by DFP. Inhibitory surface obtained by fitting the 3D model equation to the data corresponding to DFP inhibition. The surface reflects the result of the best model according to the *F* test. It corresponds to a model with two sensitive enzymatic components plus other resistant components.

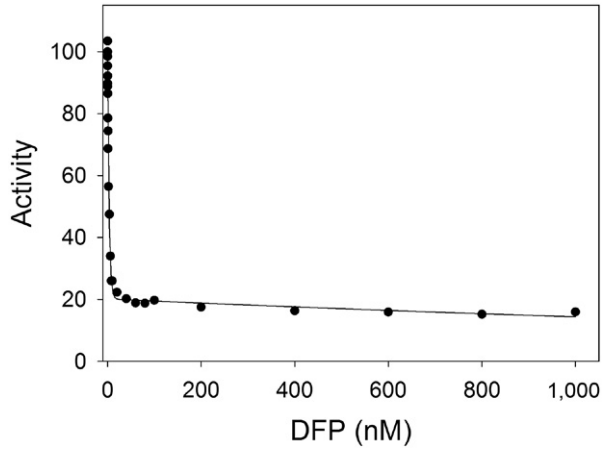

FIGURE 57.5 Fixed time inhibition. DFP 30-min fixed-time inhibition curve of soluble peripheral nerve PV esterase activity (PVase). The curve was fitted to the best model according to the *F* test (two exponential and a resistant component). Each point represents the mean of three replicates (SD <5%).

(exponential) components, with or without resistant components. The best model according to an *F* test in both experiments was a model composed of two sensitive enzymatic components, one resistant to DFP. Approximately 84% of PVase activity was highly sensitive to DFP with I_{50} (30 min) of 0.8–1.2 nM and approximately 16% was resistant (Table 57.8). The number of enzymatic components and proportions are similar to the results obtained in inhibition experiments with mipafox, PMSF, paraoxon, and S9B (Estévez et al., 2004, 2010, 2011, 2012).

DFP, OPIDN and NTE

Phosphorylation Site Identified by Radiolabeled DFP

OPIDN is a neurodegenerative syndrome induced by some but not all OPs (Johnson, 1982). An epidemiological outbreak was first described as a consequence of an adulterated alcoholic drink during Prohibition in the United States (Smith and Elvove, 1930; Smith et al., 1930), and as a consequence of an episode in Morocco in 1959 involving consumption of a lubricant oil (Travers, 1962), for which tri-*o*-cresyl phosphate was identified as the causative agent. It was demonstrated that neither AChE nor BuChE inhibition was involved as a mechanism for inducing this syndrome (Aldridge and Barnes, 1961). Adult hens were demonstrated to be an appropriate animal model for testing this syndrome in a toxicological effect protocol. The search for the target biomolecules triggering the mechanism was approached using radiolabeled [^{32}P]DFP (Johnson, 1969a). Brain tissue treated with [^{32}P]DFP indicated binding of DFP to all sensitive phosphorylable sites in proteins. The fractions of sites that can be protected by a previous incubation with a non-neuropathic OP (TEPP) were considered irrelevant sites. In the remaining sites, the small numbers that can be protected by binding with a neuropathic compound (mipafox) were considered candidates to contain the target of the toxic mechanism. In the total phosphorylable sites (approximately 600 pmol/g fresh tissue), approximately 200 pmol/g was blocked by the non-neuropathic TEPP. The fractions of phosphorylable sites are potential targets for binding with mipafox and were quantified to be approximately 33–39 pmol/g (Johnson, 1969a).

Target Site Identified as an Esterase: NTE

Johnson (1969b, 1970) assumed that phosphorylable sites would be serine-containing proteins. Several carboxylesters were tested to find a selective substrate able to interact in the same site (able to reduce the speed of [^{32}P]DFP labeling on the mipafox binding fraction). Phenyl-phenyl acetate was selected as that substrate. In other studies, PV was observed to be more selective and has been used for decades for testing the target site, the neurotoxic esterase, and was later called the NTE. NTE has been monitored as the PVase activity resistant to 40 µM paraoxon (20 min) ("B" activity) and sensitive to 40 µM paraoxon plus 60–150 µM mipafox ("C" activity), with NTE as the difference between the activity in condition B and condition C.

Protection and Induction of Neuropathy: The Role of the Aging Reaction

Some carbamates or esterase inhibitors, if dosed before DFP, have been described to be able to protect the onset of neuropathy (Johnson and Lauwerys, 1969). A series of

TABLE 57.8 Inhibition by DFP on Esterases Component in Soluble Fraction of Peripheral Nerve

	*E*1 (%)	*k*1 ($nM^{-1}min^{-1}$)	I_{50} (30 min) (nM)	*E*2 (%)	*k*2 ($nM^{-1}min^{-1}$)	I_{50} (30 min) (nM)	*R* (%)
A	38.9	0.0187	1.2	43.3	$4.1 \cdot 10^{-3}$	5.7	17.9
B	40.7	0.0274	0.8	41.7	$5.2 \cdot 10^{-3}$	4.4	17.6

The kinetic constants (ki) and the proportions of obtained esterase components from the different inhibition experiments with DFP. The I_{50} values were calculated from the kinetic constants for each component. (A) Experiment of time-progressive inhibition with different concentrations in a 3D fitting (Fig "Kinetic inhibition 3d"); (B) Experiment of fixed time inhibition with different concentrations (Fig "fixed time inhibition"). The R^2 coefficients were: 0.980 (A); 0.986 (B).

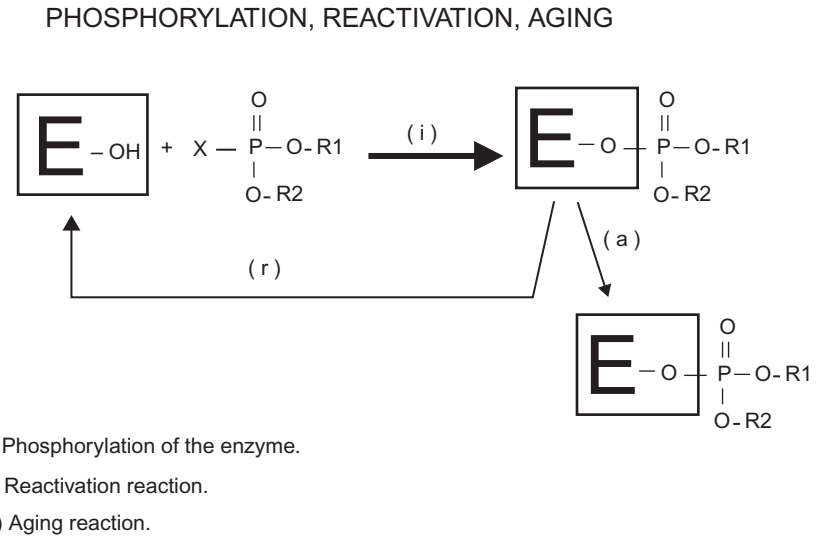

FIGURE 57.6 Reaction of phosphorylation and aging of OPs on esterases. R is an alkyl or aryl group. X is the so-called leaving group.

studies were conducted for understanding the age-related sensitivity, structure activity relationship with many OPs (Johnson, 1975a), motor nerve dysfunction (Lowndes et al., 1974), and protection by carbamates, phosphinates, and sulfonyl fluorides (Johnson, 1975b, 1976).

Although some OPs include neuropathy, phosphinates, carbamates, and sulfonyl fluorides do not; instead, they protect from a further dose of a neuropathic compound. This was hypothesized to be related to the ability of the OP for dealkylating reaction and is called an aging reaction. This was more clearly demonstrated by using radiolabeled DFP (Clothier and Johnson, 1979).

The aging process involves dealkylation of one isopropyl group (Figure 57.6). It was demonstrated by both molecular and enzymatic approaches. The loss of time of the capacity of the inhibited NTE to be reactivated by nucleophilic reagent (fluoride ion) was demonstrated, confirming the aging reaction. In contrast with AChE, the dealkylated isopropyl group in the DFP inhibited NTE remains bound to the other site ("Z") of the protein (Figure 57.7). This group may be released to the medium as isopropyl alcohol by alkaline treatment. This was demonstrated molecularly using DFP labeled with ^{3}H and ^{32}P. The molar ratio (^{3}H-isopropyl)/(^{32}P) is 2:1 in the inhibited enzyme and it is converted to 1:1 in the aged protein, confirming that one of the isopropyl groups has been released.

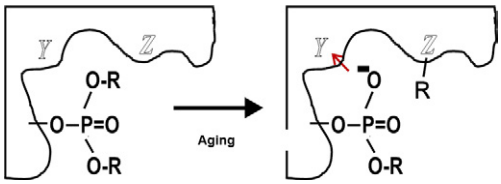

FIGURE 57.7 Aging of the DFP phosphorylated NTE protein. The aging process is a dealkylation reaction. In the case of AChE, the alkyl group is released to the medium as the corresponding alcohol. This represents the case of NTE with DFP in which the isopropyl group remains attached to the protein and is released to the medium only after an alkaline treatment. The nature of the "Z" site remains unknown.

Structure activity relationship and the relationship of the capacity of inducing neuropathy and the capacity of causing the aging reaction have been demonstrated in a wide range of OPs (Johnson, 1982). Therefore, the proposed hypothesis is that blocking the site by itself is not a sufficient requirement, and an additional modification of the function of NTE is needed. The protein alteration after the aging reaction is able to trigger the physiopathological process inducing the neuropathy.

TABLE 57.9 I_{50} on AChE and NTE

	NTE I_{50} (uM)	AChE I_{50} (nM)	AChE/NTE
DFP	0.930	1,050	1.13
Sarin	0.338	1.9	0.0056
Soman	0.377	0.46	0.0012
Tabun	6.650	3.5	0.00053
VX	250.000	0.36	0.0000014

Source: From Gordon et al. (1983).

This pathological process is involved in a number of molecular events, including alteration of neurofilament phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase, and alteration of microtubules has been reported from the laboratory of Abou-Donia et al. (Gupta and Abou-Donia, 1993, 1999; Gupta et al., 2000). Also, using DFP as a model, c-fos mRNA expression has been reported (Damodaran and Abou-Donia, 2000; Damodaran et al., 2000, 2001; Gupta et al., 2000) as well as glyceraldehyde-3-phosphate dehydrogenase mRNA in the CNS of hens treated with DFP (Damodaran et al., 2002). Early alterations are induced by DFP on the PKA/p-CREB pathway, and differential persistence of beta-tubulin subtypes in the CNS of hens has been proposed to contribute to OPIDN (Damodaran et al., 2009).

Testing Delayed Neuropathy

The capacity of inducing neuropathy means that the compound should be able to induce a high degree of inhibition of NTE and that animals survive the acute cholinergic effect. A standard test has been adapted by the OECD as protocols 418 and 419 (OCDE, 1995a,b) and by regulatory agencies. The method involves dosing of animals with a single $5 \times LD_{50}$ dose or repetitive dose in animals protected with atropine. Animals are observed for 21 days, NTE activity in the nervous system is measured, and the animals are sacrificed 24 h after dosing (Table 57.9).

An *in vitro* approach has also been proposed based on the relative inhibition of AChE and NTE in the hen brain homogenate or in human neuroblastoma cells after testing the aging reaction from the loss of capacity or reactivation with nucleophilic reagent (Sogorb et al., 2010).

Molecular and Genomic Characterization of NTE and its Role in Embryonic Development

The molecular identification of the NTE protein and its genomic identification (encoding gene: PNPL6) was the result of research conducted by Glynn et al. (1993). For this process, proteolysis of [^{3}H]DFP-labeled polypeptide was approached. After isolation and by using a specific biotinylated inhibitor, molecular cloning was possible (Glynn et al., 1994). The protein catalytic domain was isolated and used for some studies, and phospholipase activity was demonstrated and proposed to be involved in its biological function in the neuronal maintenance (Atkins et al., 2002; Glynn, 2005; Mühlig-Versen et al., 2005; Read et al., 2009).

The role of NTE in embryonic development was envisaged in studies with NTE-deficient mice (Moser et al., 2004). The expression of NTE in mouse embryonic stem cells in differentiation (Pamies et al., 2010) and the alteration of pathways during differentiation by silencing the mRNA NTE encoding gene (PNPL6) in mouse and human cells (Pamies et al., 2014a,b), but not the inhibition by a neuropathic compound as mipafox (Pamies et al., 2014b), suggested that its role in embryonic development has no relationship with its enzymatic property or with the same mechanism inducing delayed neuropathy in adults.

In summary, the history of understanding delayed neuropathy, the identification of the target protein, and the molecular events including aging reaction in the mechanism inducing the neurodegenerative syndrome, have been very closely related to the use of DFP as a tool for research studies.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

DFP is a dialkylfluorophosphate synthesized in the 1930s for insecticides, warfare agents, and other applications. Although it has never been used for chemical warfare, it has been extensively used in research as a model compound of an AChE inhibitor and in other toxicological, pharmacological, and biomedical studies because of its lower toxicity compared with G-series nerve agents. DFP is detoxified by hydrolysis by A-esterases as the enzyme DFPase (not present in humans) and by binding to B-type esterases as the tyrosine residue of albumin and the serine residue on cholinesterases. The acute toxicity is caused by inhibition of AChE and delayed neuropathy is related to the initial inhibition of the so-called NTE. DFP is known to bind and inhibit serine proteases and other unidentified esterases. DFP has been used and is still used as a tool for many toxicological and pharmacological applications in which the consequence of protease inhibition or cholinergic effects is needed to study a pharmacological or cell biology process.

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CHAPTER

58

Physiologically Based Pharmacokinetic Modeling of Chemical Warfare Agents

Jeffery M. Gearhart, Peter J. Robinson and Edward M. Jakubowski

INTRODUCTION

Pharmacokinetics (PK) is the quantitative determination of drug or chemical movement (time-course concentration) throughout the body. One of the basic principles of pharmacology and toxicology is that the effect of a drug or chemical is directly related to its concentration at some target site or receptor in the different tissues of the body. This premise is the critical reason and continued justification for the development of improved PK descriptions of chemical warfare agents (CWAs). Mathematical models are applied to this type of data to simplify or reduce the description of the basic chemical–biological processes of absorption, distribution, metabolism, and excretion (ADME). These model types are driven by the basic premise that significant pharmacological or toxicological understandings are gained by knowing the internal drug/chemical concentration at the target site. Most often termed "classical" PK models, they reduce the entire body mass to either one or two mathematical compartments that represent the volume of blood, plasma and/or readily accessible extracellular spaces, and a deep compartment storage representing other tissues. The advantage of this analytical approach is the determination of a simple global description of a chemical's behavior in the body, and potentially the concentration at major target receptor sites. The major disadvantage is that these empirical models are poor at interspecies extrapolation, since the parameters do not have a physiological interpretation, and it is difficult to predict how they change when the underlying physiology changes.

Physiologically based pharmacokinetic (PBPK) models are a special type of PK model that attempts to provide more definition to the model analysis by incorporating physiological factors into the model design, like tissue volumes, blood flow rates, and species-specific enzyme characteristics that can more accurately differentiate the dose–response relationship for a chemical or drug in one species from that of another species. The power of this approach is to be able to perform laboratory studies, both *in vitro* and *in vivo*, in common experimental species to develop a complete PBPK model, and then extrapolate these results to predictions in humans based on *in silico* experimentation.

PBPK models have become useful analytical tools to interpret PK data and to interpret data from complex chemical exposure scenarios. These models are mathematical constructs that allow the coordination of species-specific physiology, chemical-specific information, and the experimental protocol for the chemical(s) of concern. The power of PBPK models lie in aiding the ability of scientists and decision makers to simulate the time-course concentration of chemicals in experimental animals and humans and to better determine estimates of actual chemical doses delivered to the target tissue, thereby providing a better prediction of response. Due to the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route to validate the PBPK model, and then this model can be used to simulate and predict the kinetics and pharmacodynamics (PD) in the human by one or multiple exposure routes. This provides decision makers with a fairly rapid method of comparing results from *in vitro* and *in vivo* laboratory studies, to potentially real-world exposure scenarios. Perhaps the most useful application of these models lies in a strongly animal data-based physiologically based pharmacokinetic-pharmacodynamics (PBPK-PD) model being used

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to make predictions about human responses to CWAs, across a broad range of doses and CWA types, in light of not being able to actually conduct human exposure studies.

CWAs are represented by any one of a number of chemicals exhibiting very high toxicity by various mechanisms. This chapter covers CWAs with structures as simple as carbon monoxide (CO) and as complex as botulinum toxin or ricin proteins. While this chapter could address the development of PBPK models of CWAs in general, the focus will primarily be on the organophosphate based nerve agents typically represented by sarin (GB; isopropyl methylfluorophosphonate).

DEVELOPMENT OF PBPK MODELS

Most attempts at describing PK and PD descriptions of CWAs have used classical kinetic models that are often fit to one set of animal experimental data, at lethal doses, with extrapolation to low-dose or repeated-exposure scenarios with limited confidence. This is due to the inherent nonlinearity in high dose to low-dose extrapolations. Also, the classical approach is less adept at addressing multidose and multiroute exposure scenarios, as occurs with agents like *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), where there is pulmonary absorption of agent as well as dermal absorption. PBPK models of chemical warfare nerve agents (CWNAs) provide an analytical approach to address many of these limitations.

There are only a few PBPK models that have been developed to describe the PK and PD of CWNAs. Maxwell et al. (1988) developed a PD model for soman in the rat describing the inhibition of acetylcholinesterase (AChE) in different tissues, with mass balance equations including parameters for blood flow, tissue volumes, soman metabolism and tissue/plasma partition coefficients. This effort resulted in an accurate prediction of AChE activity in eight different tissues after intramuscular soman dosing and was able to reproduce dose–response inhibition from 10% up to 100% of the brain AChE activity. Gearhart et al. (1990) used diisopropylfluorophosphate (DFP) as a model compound for CWNAs, to develop a PBPK-PD model describing the PK of DFP and the inhibition of AChE and BuChE in all the pertinent tissues of the body (Figure 58.1). This model construct was able to predict the PK of DFP and inhibition of AChE and BuChE after acute and repeated doses by three different routes. Recently, the PBPK-PD model for DFP was reparameterized to predict GB kinetics, inhibition of AChE, and regenerated GB from bound AChE sites on red blood cells (RBCs; Gearhart et al., 2005).

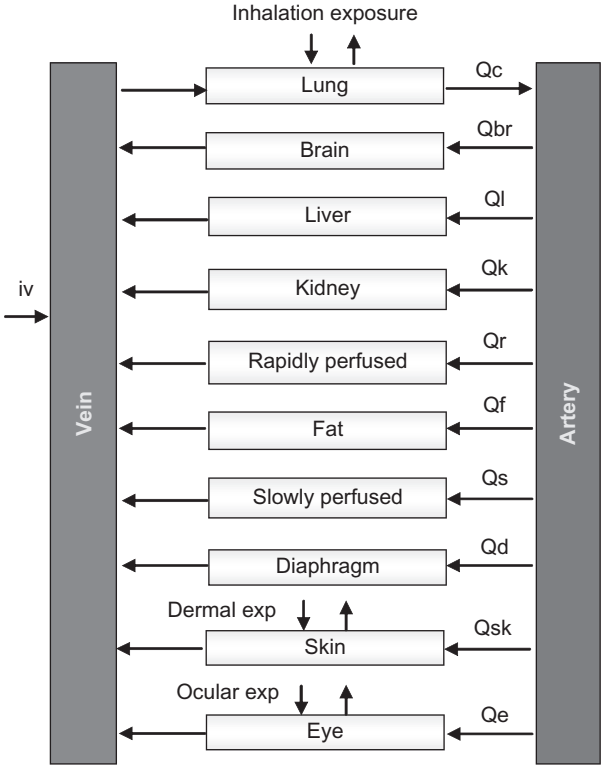

FIGURE 58.1 PBPK-PD model schematic of sarin in the Hartley guinea pig. This model structure allows for the simulation of experimental studies with dosing by IV or SC dosing and IH exposure. This model design was based on Gearhart et al. (1990) and adapted to simulate the PK and PD of sarin in the guinea pig.

The most recently developed PBPK-PD models for CWNAs have continued to focus exclusively on the most potent G agent, soman (GD), due to its rapid aging rate for permanent binding to serine sites on cholinesterase and resultant resistance to therapeutic countermeasures. The main expanded application in these modeling efforts is to multiple species, providing greater confidence in the application of the PBPK-PD method for eventual extrapolation to predictions in the species of concern (namely, humans). Sweeney et al. (2006) augmented the intravenous (IV) exposure model of Langenberg et al. (1997) for GD in guinea pigs, to predict subcutaneous dosing and inhalation exposure, as well as adding the rat and marmoset species. Both of these changes are significant additions, as subcutaneous dosing is the most common experimental method for controlled CWNA challenge to determine efficacy of therapeutic treatments. Inhalation is the primary route of threat exposure for G agents, which tend to readily form vapors, and any of the other CWNAs which, while having very low vapor pressures, still can present an inhalation hazard via aerosol droplets.

Chen and Seng (2012) extended the PBPK model simulation for GD from these previous efforts to include

the pig species, as well as the PD component predicting the inhibition of AChE and carboxylesterase (CarbE) in blood and tissues. One significant area of needed validation from Chen and Seng's model is the assumption that tissue/blood partition coefficients for GD were identical across all species for all tissues, as well as other model parameter assumptions, which could have a critical impact on the broader application to more diverse simulation scenarios (Ruark et al., 2013). For the most significant step of extrapolation to the human species and making predictions about CWNA lethality, few of these parameter values are likely to be available, an area in which Quantitative Structure Activity Relationship (QSAR) analysis can provide a viable impact (Knaak et al., 2004; Ruark et al., 2013).

There are three basic critical components to PBPK models: (i) species-specific physiological parameters, (ii) chemical-specific parameters, and (iii) specific experimental details for the studies to be simulated. Species-specific physiological parameters are the organ weights and blood flow rates for the defined compartments in the PBPK model. These values are most often available in the published literature and when lacking, are derived from the closest like species. Chemical-specific parameters that are unique for each chemical are the tissue solubility (partition coefficient), metabolism of the parent compound, and plasma and tissue-binding characteristics. Tissue solubility is most often measured experimentally for the volatile CWNAs by the vial equilibration method (Gargas, et al., 1989). Existing models for the physiological distribution of CWNAs are based on a limited number of data sets for each agent and typically rely on single exposures at relatively high doses, often in the supra-lethal range (van der Schans, et al., 2003). Mathematical models of these data have been developed, but extrapolation to low-level exposures has proven problematic (Sweeney and Maxwell, 1999). Further, there are very little experimental data and essentially no modeling efforts for repeated low-level exposures (Benschop and De Jong, 1991). Thus, new experimental data at the appropriate dose ranges appear to be required for further refinement and extension of existing PBPK models.

Much of the animal data on the biological effects of low-level exposure to GB and other nerve agents has been collected via the subcutaneous or intramuscular route, particularly in guinea pigs, because of their lack of CarbE. A good deal of data has also been collected by this dosing route or intramuscular dosing in rats, miniature pigs, and nonhuman primates. The primary exposure route of interest for human exposure is almost exclusively inhalation or dermal contact, so the quantitative implications of these laboratory exposure data for human health risk assessment is not straightforward. The most reliable tool for extrapolating across both species and exposure routes is PBPK modeling, in which species differences in physiology are taken directly into account, and equivalent systemic and tissue doses for different routes of exposure can be calculated. Such extrapolation procedures, exemplifying the integration of diverse data sets in both animals and humans, are illustrated in Figure 58.2. Here, the lower pentagon represents the dermal (or subcutaneous) exposure route for different species, while the upper pentagon represents inhalation exposure. The arrow represents the extrapolation of subcutaneous exposure data in the guinea pig to apply them to human inhalation exposure situations. In this example, the relevant biological effects observed in the guinea pig at specific subcutaneous exposures are noted. The guinea pig PBPK model then allows blood and tissue concentration time-courses corresponding to these doses to be calculated. Assuming that similar biological effects occur at similar target-site concentrations across species, the necessary inhalation exposure corresponding to the same blood and tissue concentrations in the humans can then be back-calculated using the human PBPK model for the agent. By using PBPK models in this way, human inhalation exposures are thereby quantitatively linked to the rich animal response and health effects databases.

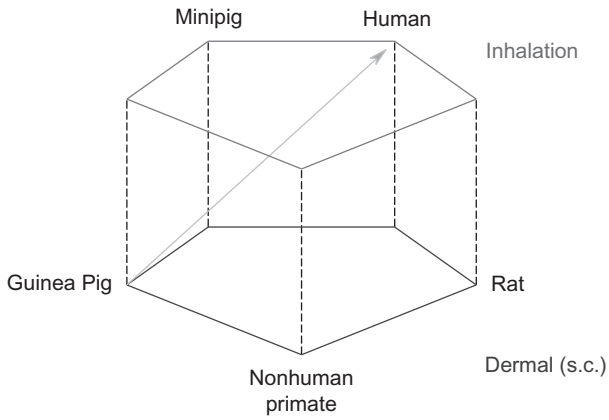

FIGURE 58.2 Schematic illustration of combined interspecies and route-to-route extrapolation. The arrow represents the extrapolation of guinea pig subcutaneous exposure data to human inhalation exposure situations.

NEED FOR IMPROVED MEASURES OF CWNA EXPOSURE—THE USE OF PBPK ANALYSIS OF DATA

Historically, cholinesterase activity has been used to evaluate exposure of humans and animals to nerve agents. Unfortunately, circulating cholinesterase activity is a relatively insensitive and imprecise marker of nerve agent exposure. By contrast, regeneration of nerve

agents from inhibited enzymes using a high fluoride concentration (fluoride regeneration) is well suited to monitoring absorbed doses resulting from low-level exposures to GB, GD, and other CWNAs (Polhuijs et al., 1997; Jakubowski et al., 2001, 2003; Adams et al., 2004). This technique has not yet been applied systematically for kinetic modeling of CWNA exposures in guinea pigs. Recently, a method for regenerating GB from blood and tissue has been developed and validated in rat, guinea pig, and pig models and provides a sensitive and quantitative means for estimating the kinetics of GB in blood (Jakubowski et al., 2004). This method is based on fluoride ion regeneration of the protein-bound agent. Fluoride ion-regenerated sarin (R-GB) was found in blood and tissues of miniature pigs exposed to sarin vapor levels ranging from miosis to lethality. The R-GB in these samples was analyzed by gas chromatography–mass spectrometry (GC–MS) after a C18 solid-phase extraction sample preparation that included fluoride ion addition and pH adjustment. Serial blood samples taken before and during miosis-level GB inhalation exposures resulted in RBC R-GB levels that steadily rose during the exposure. This demonstrated the ability of the R-GB assay as a biometric of exposure. The slopes of the lines created by plotting R-GB versus time closely correlated to the experimental exposure level. Therefore, the rates of GB absorption in these animals were proportional to the GB exposure concentration. In contrast to R-GB, monitoring AChE activity was an ineffective indicator of exposure at miosis levels in these experiments.

RELATIONSHIP BETWEEN REGENERATED SARIN AND ACHE ACTIVITY AND ITS USE AS A DOSE SURROGATE

Regenerated sarin S_R from RBCs is experimentally related to AChE activity A, as shown in Figure 58.3. It is apparent that the relationship can be adequately described in terms of a Michaelis–Menten equation, as follows:

$$A = A_{\max}\left[1 - \frac{S_R}{S_R + K_m^{eff}}\right], \qquad (58.1)$$

where $A_{\max}$ = Amax = 0.623, and K_m^{eff} = 0.006. Note that K_m^{eff} is not the true K_m for GB binding to AChE, which is the concentration of sarin needed to fill half the AChE receptors, since we do not know that the regenerated sarin is the same as the original exposure concentration. (If there were additional sarin binding sites than AChE, for example, the regenerated sarin would underestimate the exposure concentration, and the true K_m would be larger than K_m^{eff} by a certain factor.) The fact that these data fit so well to an M–M type equation strongly suggests that there is a simple proportionality between K_m^{eff} and the "true" K_m, and that sarin regenerated from RBCs is a suitable surrogate for the original exposure to sarin in blood (Figure 58.3).

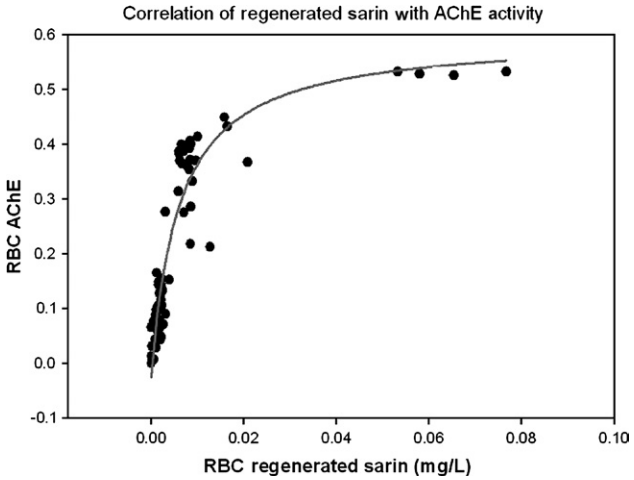

FIGURE 58.3 Plot of RBC AChE activity as a function of regenerated RBC sarin.

Regenerated sarin from RBCs at high doses may exceed the total available binding capacity of the AChE receptors associated with the RBCs. There are, therefore, additional sarin binding sites proposed to be associated with RBCs that continue to bind sarin well after the AChE receptors are filled, and therefore may comprise the bulk of the regenerated sarin at high concentrations. We thus have proposed two competing binding processes: one high-affinity, low-capacity site that dominates the binding of sarin at low concentrations, and which normally releases sarin very slowly, if at all (the AChE receptors); and one relatively low-affinity, high-capacity site, that releases sarin rapidly compared with the circulation time of the blood (Figure 58.4). This latter site would dominate at higher concentrations, and in the absence of any evidence for its saturation even at these high concentrations, we approximate it as a first-order process described with simple association and dissociation rate constants k_{on} and k_{off}.

GENERAL PBPK MODEL STRUCTURE

The basic structure of the PBPK model used to describe GB PK and PD followed that of the PBPK-PD model for DFP (Gearhart et al., 1990). Tissue compartments (Figure 58.2) were added to the previous model structure describing the eye and the skin, where before, these compartments had been lumped together in rapidly perfused or slowly perfused tissues. The eye was added to provide the means of predicting miosis during CW agent exposure, from

the systemic absorption of chemicals, but more importantly, from the amount of chemical absorbed directly to the eye structures via the ocular surface via absorption and diffusion. Adding this compartment also provided a mechanism of addressing the PD effects of sarin on the eye, by correlating inhibition of AChE enzyme levels in the iris with levels of miosis. The skin was added primarily to provide an exposure route for those agents that have a significant dermal absorption potential. Decreases in absorption due to upper respiratory tract (URT) deposition were accommodated by refining the model as shown in Figure 58.5.

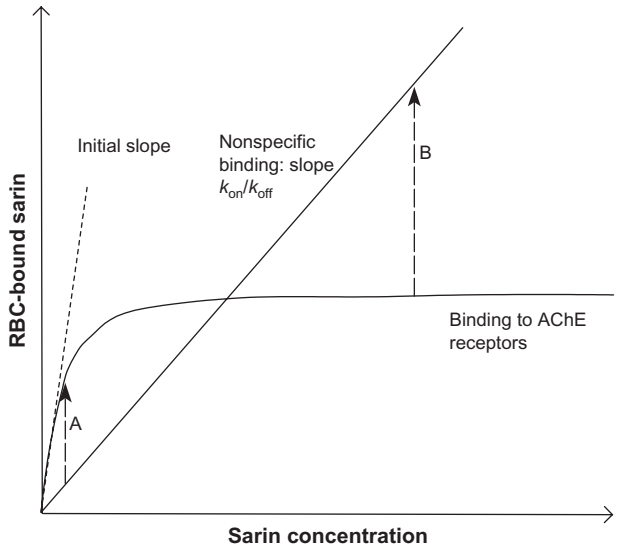

FIGURE 58.4 Hypothetical bound GB versus free sarin concentrations for saturable AChE binding, and linear nonspecific binding. The AChE binding has a higher initial slope, so it will dominate in a competitive situation at low doses (A), but it is saturable, so it will eventually be swamped by the nonspecific binding at higher doses (B).

PBPK parameters for individual organ weights were obtained from Breazile and Brown (1976); Altman and Katz (1979), and Peeters et al. (1980). Blood flows for most organs were obtained from Peeters et al. (1980) or were scaled from other rodent species. The partition coefficients were based on the values used for DFP (Gearhart et al., 1990) and soman (Langenberg et al., 1997), as well as the cholinesterase and sarinase values for tissue and blood.

The description of the interaction of GB with the mammalian system of the guinea pig followed that as described by Gearhart et al. (1990) for the CWA simulant DFP in rodents and Gearhart et al. (2005) in the Göttingen miniature pig. The description of sarin binding to AChE is shown in Figure 58.6. Sarin interaction with AChE is described by a bimolecular rate constant, causing loss of AChE activity. The inhibited AChE sites represent the amount of sarin, which can be regenerated from the RBCs.

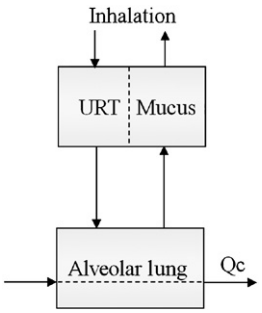

FIGURE 58.5 Detailed schematic for URT interactions for inhalation exposures.

PBPK SIMULATION OF CHOLINESTERASE INHIBITION AND REGENERATED GB

The main focus of this effort was to develop and validate methods to relate and integrate CWNA toxicity data across routes of exposure in a common species. The specific goal is to compare uptake and clearance kinetics of similar sublethal doses of GB in the blood of guinea pigs exposed to the agent by acute intravenous (IV), inhalation (IH), or subcutaneous (SC) injection. GB regenerated from blood was used as the dose metric to

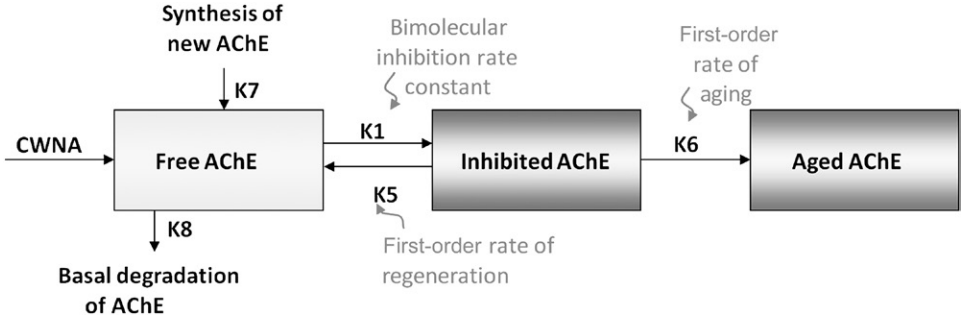

FIGURE 58.6 Schematic for AChE inhibition by sarin.

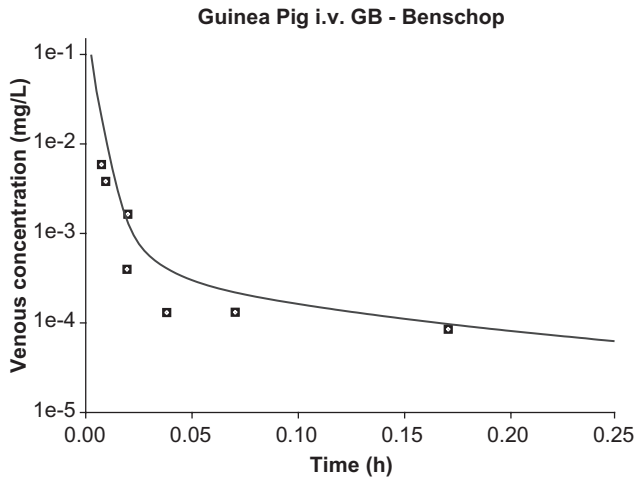

FIGURE 58.7 PBPK-PD simulation of AChE inhibition in the guinea pig after an IV GB dose of 0.8 × LD_{50} (0.0042 mg/kg body weight) (data from Benschop and De Jong, 2001—19.2 μg/kg).

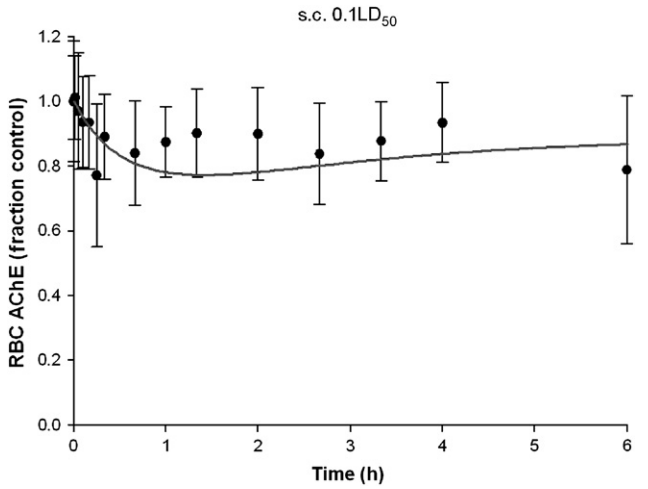

FIGURE 58.8 PBPK-PD simulation of AChE inhibition in the guinea pig after a SC dose of 0.1 × LD_{50} (0.0042 mg/kg body weight).

compare the uptake and clearance kinetics of similar doses of GB administered to guinea pigs by an IV, SC, or IH route of exposure. The resulting database will be used to derive a quantitative ratio (SC/IH) of systemically absorbed agent that will allow predictions of the atmospheric GB concentration relevant to a second species based upon subcutanous injection exposures.

Guinea pigs were exposed to sublethal levels of GB by IV, IH, and SC routes. Serial blood samples were collected in order to determine simple uptake and clearance kinetics using regenerated agent as a dose metric in a single dose (delivered via IV, SC, and IH). The resulting database was used to derive the PBPK model. This model will be used to determine a quantitative ratio (SC/IH) of a systemically absorbed agent that will allow predictions of the atmospheric GB concentration relevant to the increased guinea pig data sets based upon subcutaneous injection exposures.

Simulation of inhibited cholinesterase and regenerated GB versus the plotted data are exhibited in Figures 58.7–58.13. The methods of GB exposures or dosing shown in the figures are for either the IV, SC, or IH route. In most cases, the experimental data was well represented by the PBPK-PD model simulations. Those instances where the greatest deviation occurred between the model and the data occurred most likely because of data variability, such that the trend of the data would not follow a reproducible model behavior (Figure 58.11), or cases where there is still some question about the strength of the model parameters being used. Figures 58.7–58.10 show the model simulation of some of the key data used to determine model behavior. In each of these cases, the simulations provide a good representation of the overall magnitude and tread of the data. In some cases, as in Figures 58.7 and 58.9, there are

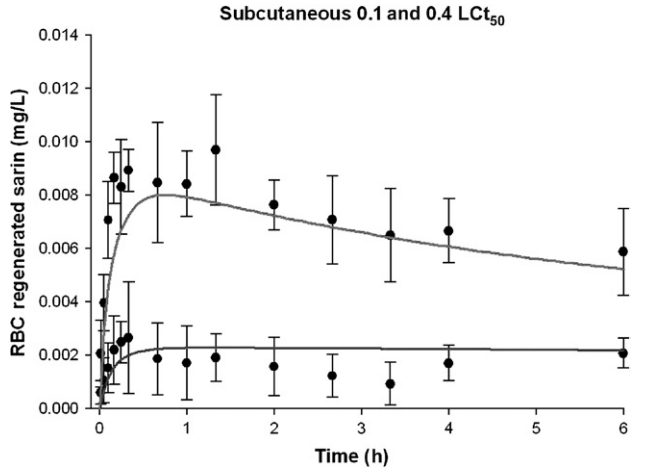

FIGURE 58.9 PBPK-PD simulation of regenerated sarin in the guinea pig after a SC dose of 0.1 × LD_{50} (0.0042 mg/kg body weight) and 0.4 × LD_{50} (0.0168 mg/kg body weight).

points during the simulation when there is either an overestimation or underestimation of the data. This type of variation between PBPK-PD simulations and data is a common occurrence whenever there is an attempt to exactly simulate time-course kinetic data for such toxicologically active a compound as GB. If it appears that significant decreases or increases in the actual experimental values are indications of real mechanisms and not just animal-to-animal variability, then that may be simulated using more sophisticated methods, as with stochastic processes where actual fluctuations in blood flows or shunting of blood could cause changes in represented concentrations outside the normal trend of the data. Certainly one major issue not addressed in this chapter is the effects of GB on its own PK, which in turn has a significant impact on key PBPK parameters such

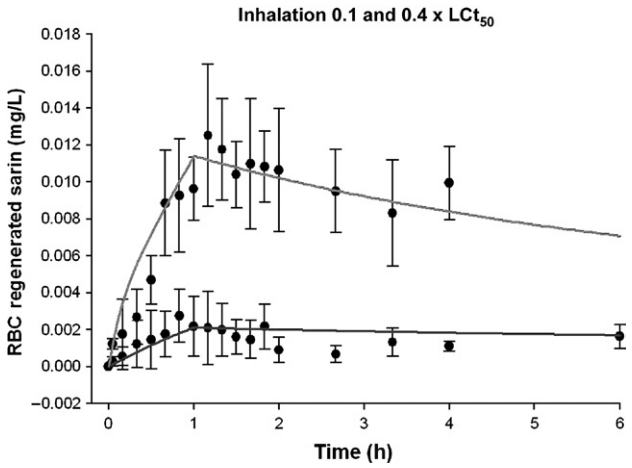

FIGURE 58.10 PBPK-PD simulation of regenerated sarin in the guinea pig after a 1-h IH exposure to sarin vapor at 0.1 × LC_{50} (0.4 mg/m^3) or 0.4 × LC_{50} (1.6 mg/m^3).

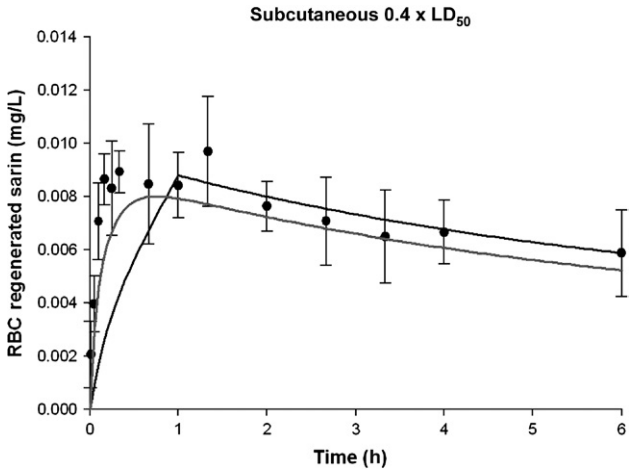

FIGURE 58.12 PBPK-PD simulation of an exposure of guinea pigs to the SC concentration equaling 0.4 of the lethal concentration affecting 50% of the exposure population (LD_{50}).

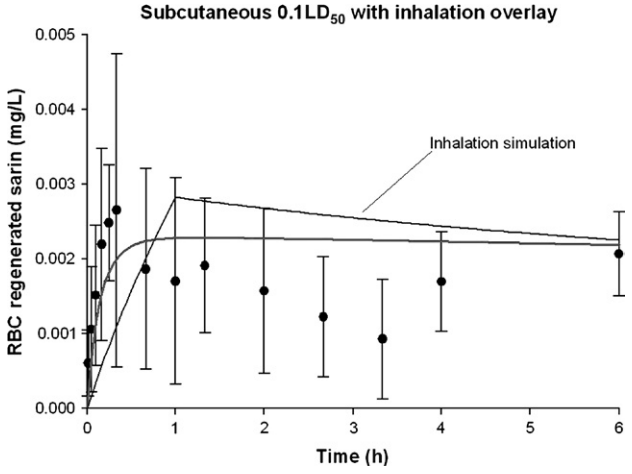

FIGURE 58.11 Overlay of the simulation of regenerated RBC sarin after a 0.1 × LD_{50} SC dose of sarin *vs.* IH dosing simulation at the inhalation concentration required to reproduce the SC data.

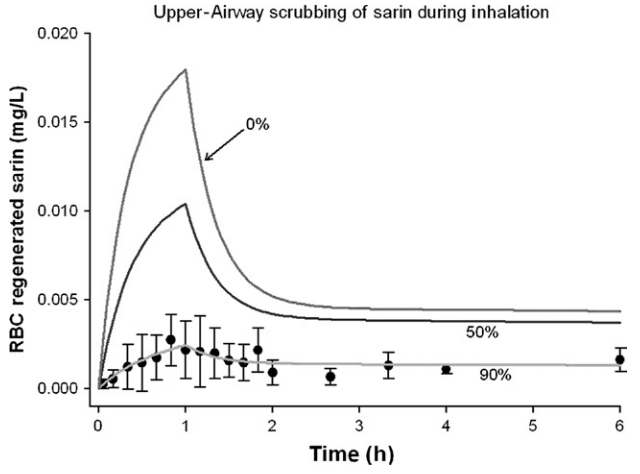

FIGURE 58.13 Upper-airway scrubbing simulations. As was previously seen with the simulation of minipig inhalation exposures, a significant percentage of the inhaled dose was scrubbed by the URT, with the best fit to the data requiring the loss of 90% of the inhaled dose.

as blood flow and respiratory ventilation parameters. A well-known response to severe CWNA exposure is a suppression of the cardiac output and respiratory ventilation, both of which could alter measured PK and PD endpoints, and if not modulated in the PBPK-PD model, lead to overestimation or underestimation of data.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The overall performance of the PBPK-PD model in predicting both the concentration of regenerated GB and the inhibition of RBC AChE was very successful for both the SC and IH routes of exposure at these doses. Previously published studies showing the significant deposition of GB in the URT were confirmed in this analysis of the inhalation of GB in the guinea pig at 0.1 and 0.4 times the LC_{50} for this species. The PBPK-PD model was utilized to equate the delivered dose of GB by SC exposure to what would be obtained after IH exposure. In this analysis, it was determined that at lower GB inhalation concentrations, there is a significant amount of deposition of the agent on the URT (Figure 58.13), material that is both hydrolyzed and resultantly inactivated, or else it is bound by a mechanism such that it presently is hypothesized to have limited activity. This is one aspect of this analysis that requires further study. To determine

the actual IH concentration that would correlate with a particular SC dose, the PBPK-PD model was exercised repeatedly, and the simulation output was overlaid on the SC simulations. This process is shown in Figures 58.11 and 58.12 for the 0.1 and 0.4 SC LD_{50} levels, respectively. This calculation showed that to reproduce the SC $0.1 \times LD_{50}$ of 0.0042 mg/kg (Figure 58.11), required an inhalation concentration of 0.08 ppm for a 1-h inhalation exposure. This value is very close to the experimentally determined value for the inhalation $0.1 \times LC_{50}$ of 0.05 ppm. The SC $0.4 \times LD_{50}$ of 0.0168 mg/kg required an inhalation concentration of 0.28 ppm for a 1-h inhalation exposure (Figure 58.12). Further simulations will need to be carried out to confirm these apparent relationships.

A previously developed PBPK-PD model for the CWNA surrogate DFP was parameterized to simulate the concentration and effects of low-level CWAs in the guinea pig after exposure by IH and SC injection. The model code was written to account for absorption of CWAs from multiple sites (respiratory tract—lower and upper, dermal, and ocular) after vapor or SC exposure. References to guinea pig physiology in the literature were used to determine the majority of organ volumes and blood flows, while some parameter values were scaled from other species. Unique features of this PBPK/PD model structure were physiological compartments for the eyes, as a source of external CWA absorption and as a site of cholinesterase binding, and the skin, as a dermal absorption pathway. One initial PD endpoint developed in this model was CWA inhibition of cholinesterases (such as AChE and BuChE). Covalent binding of sarin to cholinesterases and other proteins was also estimated by a novel parameter based on fluoride ion regeneration of the agent. The PBPK/PD model was used to simulate AChE inhibition after inhalation and subcutaneous injection of CWA and to predict potential PD effects at different tissue target sites. This preliminary model will provide a quantitative tool to predict the physiological consequences of low-level, nonlethal exposure to CWNA exposure.

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CHAPTER

59

Biotransformation of Warfare Nerve Agents

Milan Jokanović

INTRODUCTION

The first organophosphorus (OP) warfare nerve agents (WNAs), tabun (GA) and sarin (GB), were developed in the 1930s by Gerhard Schrader. These, and the even more toxic soman (GD), were developed in 1944 and are members of the so-called G-agents. Together with VX, developed in 1952 in the United Kingdom, these compounds have emerged as the major WNAs known to have been produced and weaponized. The WNAs are alkylphosphonic acid esters. Tabun contains a cyanide group. Sarin, cyclosarin, and soman contain a fluorine acyl radical and are methylphosphonofluoridate esters. These WNAs contain a C-P bond not found in organophosphate pesticides. The C–P bond is very resistant to hydrolysis. VX contains sulfur and is an alkylphosphonothiolate.

The mechanism of extremely high toxicity of these agents is excessive cholinergic stimulation caused by inhibition of acetylcholinesterase (AChE) at nervous system synapses. WNAs are direct AChE inhibitors reacting rapidly with a serine hydroxyl group in the active site of AChE with the formation of a phosphate or phosphonate ester. The phosphorylated enzyme regenerates very slowly, rendering the enzyme inaccessible for its physiological substrate acetylcholine (ACh). The chirality around the phosphorus atom largely influences the toxicity of these agents, as documented in the case of soman whose P(−) isomers are much more toxic than the P(+) isomers (Benschop and De Jong, 1991; van der Schans et al., 2007; Jokanović, 2009).

Signs and symptoms of the cholinergic syndrome occurring in acute poisoning with WNAs and other OP compounds are predictable from their biochemical mechanism of action and are directly related to the levels of AChE activity. After exposure to WNAs, the symptoms of poisoning occur quickly depending on the dose taken, mainly by inhalation (G-agents). These symptoms include the following: miosis (unreactive to light); sweating, rhinorrhea, lacrimation, and salivation; abdominal cramps and other gastrointestinal symptoms; respiratory difficulties and cough; dyspnea, constriction sensation in the chest, and wheezing; twitching of facial muscles and tongue, tremors, and fasciculations; bradycardia and ECG changes, pallor, and cyanosis; and anorexia, nausea, vomiting, diarrhea, and involuntary urination and defecation. These signs and symptoms are accompanied by central effects such as dizziness, tremulousness, confusion, ataxia, headache, fatigability, and paresthesia. Finally, seizures, convulsions, twitching, coma, and respiratory failure may occur. Death usually occurs because of respiratory failure resulting from a combination of central and peripheral effects, paralysis of the respiratory muscles, and depression of the brain respiratory center (World Health Organization, 1986; Marrs and Vale, 2006; Karchmar, 2007; Jokanović, 2010; Jokanović et al., 2011).

In the case of G-agents, the intact agent is present in the organism for only several hours. The dominant metabolic pathway of G-agents is hydrolysis, a process mainly mediated by so-called A-esterases, and the metabolic products formed are corresponding *O*-alkyl methylphosphonic acids in the case of sarin, cyclosarin, and soman (Figure 59.1). VX is a less suitable substrate for A-esterases. There are also reports discussing a possible role of prolidase (EC 3.4.13.9), a naturally occurring enzyme, in hydrolysis of G-agents. In addition to hydrolysis, binding reactions of WNAs to proteins such as AChE, serum cholinesterase (ChE), carboxylesterase (CarbE), albumin, and other macromolecules also occurs. Toxicological importance of binding reactions

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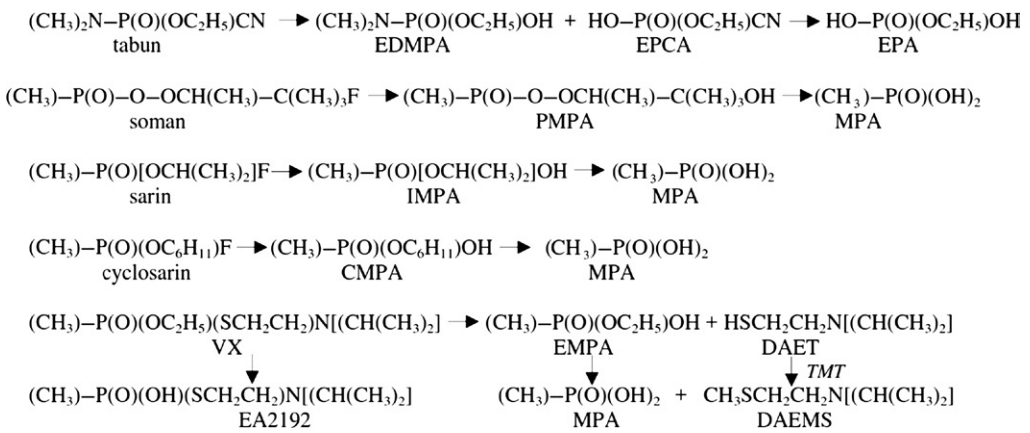

FIGURE 59.1 Metabolic detoxification of tabun, soman, sarin, cyclosarin, and VX. Abbreviations: EDMPA, ethyl dimethylaminophosphoric acid; EPA, ethyl phosphoric acid; EPCA, *O*-ethyl-cyanophosphoric acid; IMPA, isopropyl methylphosphonic acid; PMPA, pinacolyl methylphosphonic acid; CHMPA, cyclohexyl methylphosphonic acid; EMPA, ethyl methylphosphonic acid; MPA, methylphosphonic acid; DAET, 2-(diisopropylamino) ethanethiol; DAEMS, 2-(diisopropylaminoethyl) methylsulphide; TMT, thiol *S*-methyltransferase (EC 2.1.1.9) (Nakajima et al., 1998; Katagi et al., 1999; Jokanović, 2001, 2009; Evans et al., 2008; Black, 2010; Tenberken et al., 2010; Reiter et al., 2011; Black and Read, 2013; Kranawetvogl et al., 2013).

of WNA to these proteins involves decrease of free unbound concentration of WNA in blood, which causes less AChE inhibition in the nervous system. Both WNA and OP pesticides lose their acyl radicals when they react with AChE, ChE, and CarbE, and other proteins. After binding to AChE and ChE, the phosphoryl residues of soman, sarin, cyclosarin, tabun, and VX undergo an intramolecular rearrangement with subsequent loss of one phosphoryl group, known as aging reaction.

In addition, because of the reversibility of the binding reaction of sarin and soman to CarbE, it appears that CarbE are involved in metabolic detoxification of these agents to their corresponding nontoxic metabolites IMPA and PMPA (Jokanović et al., 1996; Jokanović, 2009).

Important proof supporting the significance of detoxification reactions of WNA in the body was presented by Fonnum and Sterri (1981), who reported that only 5% of LD_{50} of soman in rats, or approximately 5 μg/kg, reacts with AChE and causes acute toxic effects, whereas the remaining 95% passes through various metabolic reactions.

In this chapter, the mechanisms involved in biotransformation of WNA are discussed. Mechanisms of biotransformation of OP pesticides are beyond the scope of this chapter and interested readers are referred to other publications (Chambers et al., 2001; Jokanović, 2001, 2009; Tang et al., 2006).

CHEMICAL ASPECTS OF BIOTRANSFORMATION OF WNAS

The G-agents are anticholinesterase substances that, at sufficient concentrations, can be toxic or fatal by any route of exposure. Differences in volatility and water solubility result in varied degrees of persistence and variations in the likelihood of exposure by certain routes. Of the G-agents, tabun gives rise to the greatest number of degradation products. The metabolites of tabun EDMPA and EPCA (Figure 59.1) are apparently unstable, whereas the EPA is an excretion metabolite of tabun as well as of certain pesticides and plasticisers (Tenberken et al., 2010; Black and Read, 2013). The final metabolite of tabun is a phosphate (Black, 2010). Toxicity data are available only for a limited subset of the tabun degradation products. One of the tabun hydrolysis products, dimethylamine, is moderately toxic in terms of acute lethality but causes human irritation of the respiratory tract (Munro et al., 1999).

Sarin is metabolized to IMPA, which slowly undergoes further hydrolysis to the very stable MPA. IMPA also forms in the course of spontaneous reactivation of sarin-inhibited CarbE and ChEs. IMPA had shown low toxicity in rats and mice in all toxicity tests conducted, but has shown mild skin irritation in rabbits. Limited data suggest that MPA is nontoxic in terms of acute oral toxicity in rats and mice, but show irritant effects on human eyes and skin (Munro et al., 1999; Jokanović, 2009).

In the study by Little et al. (1986), a single sublethal dose (80 μg/kg) of radiolabeled sarin was administered intravenously to mice and the tissue distribution was followed for 24 h. Within 1 min, sarin was distributed to the kidneys, lungs, brain, liver, heart, and diaphragm. Thereafter, the concentrations in all tissues rapidly declined, and within 15 min only trace quantities of [^{3}H] sarin were found in the brain. Within the first minute, approximately half of the labeled sarin was in the form of IMPA. The highest concentrations of sarin and its

metabolites were found in the kidneys. Lower concentrations of IMPA and MPA were detected in the liver, suggesting a minor role for the liver in detoxification of sarin.

Shih et al. (1994) injected rats subcutaneously with a single dose of 75 μg/kg of sarin, cyclosarin, and soman, and measured excretion of the hydrolyzed metabolites and the alkylmethylphosphonic acids, including IMPA and corresponding MPAs. MPA was a major and common metabolite of the three compounds. Urinary excretion over the first 24 h accounted for approximately 90% of the administered doses of sarin and cyclosarin. Almost total recoveries of the given doses for sarin and cyclosarin in metabolite form were obtained from the urine. Urinary elimination was found to be rapid and the terminal elimination half-life of sarin metabolites in urine was 3.7 h. Most of the administered dose of sarin was retrieved from the urine in a metabolite form after 2 days. The terminal elimination half-life of cyclosarin in urine was 9.9 h. Soman metabolite showed a biphasic elimination curve with terminal half-lives of 18.5 and 3.6 h. Soman was excreted at a slower rate, with a recovery of only 62%. The first phase of elimination of soman results from enzymatic hydrolysis of the inactive P(+) isomers, and the slower phase is from the active P(−) isomers (Benschop and De Jong, 1991). The elimination study in rats determined IMPA in blood up to 14 h after exposure, CHMPA up to 2 days, and PMPA up to at least 3 days.

Distribution, metabolism, and elimination of sarin in humans appear to resemble findings in animals. Minami et al. (1997, 1998) detected the sarin metabolite IMPA in urine of humans after the terrorist attack in Tokyo in 1995. They found peak levels of IMPA or MPA in urine 10–18 h after exposure. The levels of IMPA in urine correlated with the clinical symptoms. They also found evidence of distribution of sarin to the human brain in 4 of the 12 people who died after exposure. IMPA and MPA were detected in urine of a patient poisoned with sarin in Matsumoto (Nakajima et al., 1998).

Biotransformation products of soman include PMPA and MPA. No biologic data were found for PMPA (Munro et al., 1999; Jokanović, 2009). It has been shown that the toxic C(±)P(−) isomers of soman react rapidly with covalent binding sites. The less toxic C(±)P(+) isomers are hydrolyzed several orders of magnitude faster than the C(±)P(−) isomers. The low toxicity of the C(±)P(+) isomers is primarily due to a low intrinsic reactivity toward AChE and rapid metabolic hydrolysis (van der Schans et al., 2007). The levels of C(±)P(−) isomers remain toxicologically relevant for periods of 50–100 min in rats, guinea pigs, and marmosets at doses of 2–3 LD_{50} (Benschop and De Jong, 1991).

VX is a potent anticholinesterase agent that can act by dermal, oral, and inhalation routes of exposure. There are certain characteristics of VX that make the agent different from G-agents, such as the following: VX is present in blood as protonated amine; it is hydrolyzed at a much slower rate than G-agents; it reacts more slowly with CarbE (because of its positively charged quaternary ammonium group) and A-esterases; and VX can be metabolized by other routes such as oxidation reactions at nitrogen and/or sulfur (Jokanović, 2009; Black, 2010). A few of the metabolic products may retain some anticholinesterase activity (such as EA2192) (Munro et al., 1999), but hydrolysis of one or more alkyl ester bonds of organophosphonic acids results in generally nontoxic alkyl methylphosphonic acids EMPA and MPA. MPA is resistant to further hydrolysis. Munro et al. (1999) discussed the metabolic degradation products of VX in mammals and found that there are approximately 25 such products and each had shown different levels of toxicity.

There are three phosphorus-containing metabolites of VX: EA2192 (which has shown some anticholinesterase activity), EMPA, and MPA (not AChE inhibitors) (Figure 59.1). EMPA appears to be the major metabolite of VX in urine (Black and Read, 2013). The presence of EA2192 metabolite in humans is important because it retains the 2-diisopropylaminoethylthio substituent that confirms exposure to VX (Black and Read, 2013). After intravenous and dermal administration of sublethal doses of VX to swine, both VX and EA2192 could be quantified during 540 min after exposure (Reiter et al., 2011). In addition, there are two nonphosphorus metabolites of VX: DAET and its methylation product DAEMS identified in human serum from a victim of the Osaka VX accident (Tsuchihashi et al., 1998). The conversion from DAET to DAEMS is catalyzed by thiol *S*-methyltransferase (EC 2.1.1.9) (Reiter et al., 2011).

Benschop et al. (2000) and van der Schans et al. (2003) studied the toxicokinetics of VX stereoisomers in hairless guinea pigs and marmosets. After an intravenous dose of 28 μg/kg (marmosets) or 56 μg/kg (guinea pigs), VX was found in the blood at toxicologically relevant levels even after 6 h. Detoxification proceeded at a slower rate in marmosets than in guinea pigs. The VX metabolite EMPA (Figure 59.1) was found in the blood of the exposed animals; however, the metabolite contributed only 5% to the recovery of the phosphonyl moieties related to the VX dose. Metabolites of VX were also evaluated in *in vitro* studies by treating liver homogenates and plasma from hairless guinea pigs, marmosets, and humans with the radio-labeled compound, ^{35}S-VX. The potential toxic metabolite VX-*N*-oxide was not found. Desethyl-VX was found after incubation of VX in plasma of all three species; however, because of its slow rate of formation, Benschop et al. (2000) concluded that it would be unlikely that VX would be present at toxicologically relevant levels after administration of VX *in vivo*. *In vitro* studies with ^{35}S-VX

revealed that a significant part of the thiol-containing leaving group (S-2-(*N*, *N*-diisopropylamino)ethane thiol) was bound to albumin.

Tsuchihashi et al. (1998) developed a method for identification of VX metabolites in serum collected from a victim of the Osaka VX incident. In the serum sample, both EMPA and DAEMS were detected. The techniques using GC-MS and GC-MS-MS were applicable to biological samples such as serum. These results provided the first documented identification of the specific metabolites of VX in a victim's serum and clarified a part of the metabolic pathway of VX in the human body. In addition, methods have been developed for measuring the VX-inhibited AChE hydrolytic product EMPA (Noort et al., 1998, 2002).

ESTERASES INVOLVED IN METABOLISM OF WNAs

Numerous esterases can react with OPs, but in a different way. The first classification was provided by the late Professor Aldridge (1953). In the first group, there were esterases that hydrolyze OPs as substrates, particularly their uncharged esters, that are not inhibited by these compounds. This group of enzymes was named *A-esterases*, although in the literature there are many other names for the same group of enzymes given according to the substrate hydrolyzed (paraoxonase, somanase, sarinase, etc.) or their chemical structure (phosphotriesterases, phosphorylphosphatases, anhydrases of OP compounds). In the second group of enzymes interacting with OPs there were *B-esterases*, which are inhibited with OPs in the progressive reaction that is time-dependent and temperature-dependent. This group of enzymes comprises AChE, ChE, CarbE, trypsin, chymotrypsin, and other enzymes. In the third group were *C-esterases* that do not interact with OP. It is paradoxical, but basically true, that OPs can be substrates for both A-esterases and B-esterases because their concentrations in blood and tissues are decreased in the presence of these enzymes.

The mechanism of interaction of A-esterases and B-esterases with WNA and other OP is similar. B-esterases initially form Michaelis complex with an OP inhibitor producing phosphorylated or inhibited enzymes that either reactivate very slowly or do not reactivate at all. However, after formation of Michaelis complex with OP, *A-esterases* catalyze hydrolysis of OP and their catalytic activity and turnover rate are very high.

A-Esterases

Classified in 1992 (International Union of Biochemistry, 1992), hydrolases of OP were described as a special entity called "phosphoric triester hydrolases," which comprise three groups of enzymes: phosphoric monoester hydrolases (EC 3.1.3); phosphoric diester hydrolases (EC 3.1.4); and phosphoric triester hydrolases (phosphotriesterases) (EC 3.1.8). Phosphoric triester hydrolases are further divided in two similar subgroups: aryldialkylphosphatases (EC 3.1.8.1) and diisopropylfluorophosphatases (EC 3.1.8.2).

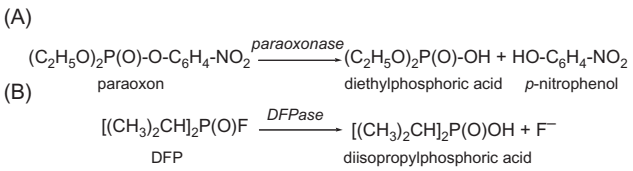

FIGURE 59.2 (A) Hydrolysis of paraoxon with aryldialkylphosphatase (paraoxonase, EC 3.1.8.1) and (B) hydrolysis of diisopropylfluorophosphate (DFP) with diisopropylfluorophosphatase (DFPase) (EC 3.1.8.2).

Aryldialkylphosphatases take part in hydrolysis of aryldialkylphosphates producing dialkylphosphate and aryl alcohol. These enzymes react with substrates such as paraoxon (Figure 59.2), and also with phosphonates and phosphinates. Other names for this group of enzymes are hydrolases of OPC, A-esterases, paraoxonases, (PON1), aryltriphosphatase, and aryltriphosphate dialkylphosphohydrolase. They are inhibited with compounds that form chelates like EDTA because they require the presence of divalent ions, mainly Ca^{2+} (Walker, 1993). Some fractions of the enzyme purified from human serum were able to hydrolyze both paraoxon and phenylacetate and it was thought for a long time that the same enzyme is responsible for both. However, enzymes hydrolyzing aryl esters are classified as arylesterases (EC 3.1.1.2).

Diisopropylfluorophosphatases take part in hydrolysis of DFP and similar compounds producing diisopropylfluorophosphoric acid and fluoride ion (Figure 59.2). These enzymes react with phosphorus anhydride bonds such as those between phosphorus and acyl radical (F^-, Cl^-, CN^-) in highly toxic OPC such as soman, sarin, tabun, and DFP, and they were accordingly named somanase, sarinase, tabunase, DFPase, diisopropylfluorophosphate fluorohydrolase, OPA anhydrase, OPAA, and OP acid anhydrolase. They are also inhibited by chelating agents, and their activity requires presence of divalent ions such as Mg^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+}, but not Ca^{2+} (Walker, 1993; Kondo et al., 2004). They exist in several forms, even in different tissues of the same species, and react differently with substrates.

In the literature, there are articles reporting an important role of senescence marker protein-30 (SM-30) in hydrolysis of DFP and similar substrates (Kondo et al., 2004; diTargiani et al., 2010). SM-30 protein was given the code EC 3.1.8.2, which was previously assigned to DFPase. After checking all available data, it appears

that both SM-30 and DFPase are the same enzymes and that their names are synonyms! Therefore, DFPase is a 34 kDa metalloprotein expressed predominantly in the liver and kidney of mammals. Although the identity of the metal ion is not clearly established, it was shown that the activity of purified DFPase from rat liver was stimulated by $MgCl_2$, $MnCl_2$, $CoCl_2$, and $CdCl_2$ (Kondo et al., 2004). It has been linked with the regulation of calcium homeostasis and the protection of cells from apoptosis. Consistent with these observations, it was found that the sequence of DFPase was identical to that of a Ca^{2+}-binding protein, regucalcin. Rat liver DFPase was shown to hydrolyze DFP, phenyl acetate, and gluconolactones. *In vivo* studies with SMP-30 knockout mice confirmed its role in the hydrolysis of DFP in the liver of these animals (diTargiani et al., 2010). A reduction of DFPase expression might account for the age-associated deterioration of cellular functions and enhanced susceptibility to harmful stimuli in aged tissue (Kondo et al., 2004).

In the literature published during the past 30 years, the term PON1 apparently covers both phosphoric triester hydrolases. In further text, to avoid possible confusion, the term A-esterase will be generally used for enzymes hydrolyzing OPs and with other terms (such as PON1, paraoxonase, etc.) as they appear in original references.

Molecular weight of human A-esterases is between 43 and 45 kDa. Human A-esterase is a protein of 355 amino acids with two polymorph sites in which arginine or glutamine are located at position 192 and methionine or leucine at position 55 (La Du et al., 1993; Li et al., 1995). The former accounts for three genotypes (QQ, RR, and QR) relating to the catalytic properties of two forms of an enzyme (types R and Q allozymes), which hydrolyze certain organophosphates at different rates. Its three-dimensional structure is also known (Benning et al., 1994; Vilanova and Sogorb, 1999). At the active site of A-esterases there are two metal cations connected via common ligand, and most other protein groups are bound to this binuclear site through imidazolium side chains from histidine groups (Benning et al., 1994). Activity of these enzymes largely depends on Ca^{2+}, which represents a necessary factor for maintaining the function of active site, and it is also possible that Ca^{2+} directly participates in catalytic reactions or maintain the conformation of amino acids at the active site. In addition, in the case of paraoxons, Ca^{2+} facilitates the release of diethylphosphate from the active site, probably by polarizing bond P=O, which makes phosphorus atoms much more sensitive to nucleophilic attack of hydroxyl ions (Vitarius and Sultatos, 1995; diTargiani et al., 2010). Human A-esterases can hydrolyze many OPs, including paraoxon, soman, sarin, tabun, chlorpyriphos and chlorpyriphos oxon, DFP, dichlorvos, diazoxon, and pyrimiphos methyl oxon.

The R allozyme (Arg192) hydrolyzes the organophosphate paraoxon at a high rate; however, it has low activity toward sarin, soman, and diazoxon. Lower activity means that more sarin would be bioavailable to exert its anticholinesterase effects. The Q allozyme, however, has high activity toward sarin, soman, and diazoxon (and low activity toward paraoxon). Thus, individuals with the Q allozyme (QQ or QR) are expected to have greater hydrolysis of sarin than individuals homozygous for the R allele (RR) (Costa et al., 1999, 2005). In Caucasian populations, the frequency of the R allele is approximately 0.3, but the frequency is 0.66 in the Japanese population (Yamasaki et al., 1997; Costa et al., 2006). This would make individuals in the Japanese population more sensitive to the toxicity of sarin, a fact that may have contributed to their morbidity and mortality after the terrorist attacks in 1995. The concentration of PON1 in human plasma (approximately 60 mg/L) varies between individuals by as much as 13-fold. PON1 activity level is determined by a combination of complex genetic interactions and environmental/dietary factors, giving rise to a 40-fold variation in PON1 for a single individual (Rochu et al., 2007). The relationship between paraoxonase polymorphisms and toxicity of OP compounds was further discussed in an excellent article by Costa et al. (2006) and other work by his team.

Paraoxonase (PON1) is a member of a family of proteins that also includes PON2 and PON3, the genes of which are placed on the long arm of human chromosome 7 (q21.22). PON2 and PON3 have approximately 65% homology to PON1, but they cannot hydrolyze WNAs and other organophosphates (Draganov et al., 2005; Aviram and Rosenblat, 2008). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoprotein (HDL) particles (Costa et al., 2005). The crystal structure for a recombinant PON1 indicates that it is a six-bladed β-propeller with two calcium ions in the central tunnel, one of which is essential for enzyme activity and the other is essential for enzyme stability (diTargiani et al., 2010). The primary physiological role of PON1 appears to be protection of low-density lipoproteins (LDL) from oxidative modifications (Mackness et al., 1993; Aviram et al., 1998; Vilanova and Sogorb, 1999; Rochu et al., 2007). Human PON1 is apparently involved in drug metabolism and in preventing atherosclerosis (diTargiani et al., 2010). Endogenous PON1 is associated with HDL that represents its main carrier in blood, and this association has been considered a crucial element for increased stability and half-life of PON1 in circulation (Gaidukov et al., 2009; Veliyaveettil et al., 2012). In addition to its role in lipid metabolism, cardiovascular disease, and atherosclerosis, PON1 has been shown to play a role in the metabolism of drugs and xenobiotics containing aromatic carboxyl esters (Costa et al., 2003).

It is also suggested that PON1 hydrolyses various lactones, including naturally occurring lactone metabolites.

The differences in the activity of A-esterases due to polymorphisms may have an important effect on toxicity of OP in humans that are occupationally or accidentally exposed. In this respect, it was proposed that humans expressing lower activity of A-esterases could be more susceptible to toxic effects of OP, and it was proposed that such individuals should not be exposed (Mackness, 1989). Hernandez et al. (2004) have confirmed this hypothesis, suggesting the association of paraoxonase phenotypes with susceptibility of humans to OP pesticides toxicity. However, additional studies are needed to fully understand the effects of A-esterase polymorphism on the capacity of detoxification and toxicity of OP.

Toxicological Relevance of A-Esterases

A-esterases purified from *Pseudomonas diminuta* and given to mice decreased inhibition of AChE in brain and ChE in serum in poisoning with paraoxon and DFP, and this effect was less significant in poisoning with soman and sarin (Tuovinen et al., 1994). Purified A-esterases have shown protective effects when given to mice before poisoning with tabun (Ashani et al., 1991a).

Haley et al. (1999) investigated PON1 genotype and serum enzyme activity in a group of 25 ill Gulf War veterans and 20 controls. Ill veterans were more likely than controls to possess the R allele (QR heterozygotes or R homozygotes) and to exhibit lower enzyme activity. This study raised the possibility that the R genotype (low sarin-hydrolyzing activity) may represent a risk factor for illness in Gulf War veterans. However, because of the very small number of participants included in the study, such findings require confirmation in a larger population (Furlong, 2000). La Du et al. (2001) also found reduced sarinase and somanase levels in plasma obtained from Gulf War veterans. In a similar study, in a group of 152 UK Gulf War veterans, the PON1 activity was lower in veterans than in a control group, but the differences were independent of PON1 genotype (Mackness et al., 2000). However, in both studies there were no indications about the extent of exposure to nerve agents and possible effects of such exposures.

B-Esterases

B-esterases are the group of enzymes that can be inhibited by OP compounds in the progressive reaction that is time-dependent and temperature-dependent. This group of enzymes comprises AChE (EC 3.1.1.7), ChE (EC 3.1.1.8), CarbE (EC 3.1.1.1), chymotrypsin, trypsin, and some other enzymes. A common feature of these enzymes is that they have a serine hydroxyl group at the active site that enables them to react with OP in a similar fashion. AChE, ChE, and CarbE are members of the α/β hydrolase family and have a high degree of overall homology in their amino acid sequences, but they differ in several critical regions that produce differences in their biochemical properties. The most significant biochemical differences in these esterases are related to the extent of aging of the OP-inhibited esterase, the size of the active site, and the ability of the OP-inhibited enzyme to undergo spontaneous or oxime-induced reactivation (Doctor et al., 2001).

Serum ChE

Although the biological role of ChE is still unclear, it is known that soman, sarin, tabun, and VX bind to ChE without any apparent toxic effects, decreasing the amount of free WNA available for inhibition of AChE in the central nervous system and erythrocytes (Jokanović, 2009). Pretreatment with human plasma ChE (hBChE) has protected laboratory mice (Ashani et al., 1991b) and monkeys (Raveh et al., 1997) from lethal and other acute toxic effects of VX exposure. Human BChE is now in an advanced stage of development as a bioscavanger and is close to obtaining marketing approval as pretreatment for OP poisoning (Saxena et al., 2011a; Trovaslet-Leroy et al., 2011). It is estimated that a dose of 200 mg is required to protect a human against $2 \times LD_{50}$ of soman (Saxena et al., 2011a). hBChE reacts with OP to form the 1:1 complex, and an intramuscular or intravenous injection protects animals against 3–5 $\times$ LD_{50} of soman, tabun, and VX (Lenz et al., 2007). A dose up to 250 mg/70 kg of hBChE is able to protect humans from $1 \times LD_{50}$ of OP (Ashani and Pistinner, 2004). Such a dose of hBChE is required because of the stoichiometric and irreversible reaction between hBChE and OP (Trovaslet-Leroy et al., 2011). Animal studies have shown that administration of large doses of hBChE provide protection against up to 5.5 $\times$ LD_{50} of soman or $8 \times LD_{50}$ of VX (Saxena et al., 2011a). Pretreatment with 7.5 mg/kg completely prevents toxicity in minipigs exposed to sarin by inhalation (Saxena et al., 2011b). hBChE derived from plasma is present in the blood of guinea pigs up to 4 days without any signs of toxicity (Saxena et al., 2011a) and without any adverse effect in rats (Nachon et al., 2013). Behavioral and physiological safety of plasma hBChE was established at 21 mg/kg in mice (Ilyushin et al., 2013) and 30 mg/kg in rhesus monkeys (Myers et al., 2012).

ChE is a serine esterase glycoprotein composed of four 85 kDa subunits, synthesized mainly in the liver, and does not have any known physiological function. From the liver, ChE is secreted to plasma and its activity correlates with the physiological and/or pathological condition of the liver. ChE can be found in more than 40 genetic variants, differing in their susceptibility to chemicals (Jokanović and Prostran, 2009). ChE may have roles in cholinergic neurotransmission (Mesulam et al.,

2002), other nervous system functions (cellular proliferation and neurite growth during the development of the nervous system), and in neurodegenerative disorders (Darvesh et al., 2003).

Variability in plasma ChE activity is a parameter of concern for characterization of population susceptibility to nerve agent exposure. Selective inhibition of AChEi ChE had no effect on the acute soman toxicity to mice (Clement, 1984). Because VX reacts with ChE, and because it reacts very slowly with CarbE (due to its positively charged quaternary ammonium group), it appears that ChE may have a significant role in detoxification of VX. Wide variations in ChE activity seen in individuals not exposed to OP but that are caused by genetic, physiological, and pathological conditions, as well as interactions with many drugs, may strongly influence the susceptibility of those individuals to OP compounds (Jokanović and Maksimović, 1997). It is possible that individuals with lower ChE activity may be more susceptible to the effects of OP including nerve agents.

Carboxylesterases (EC 3.1.1.1)

The mammalian CarbE comprise a multigene family whose gene products are located in the endoplasmic reticulum (Hosokawa and Satoh, 2006). CarbE are the enzymes that hydrolyze esters and thioesters or amide groups of carboxylic acids. They were also mentioned in the literature as aliesterases and esterase D. CarbE have a very important role in metabolism of lipids, endogenous fatty acids esters, steroids, and a large number of ester-containing drugs and prodrugs, such as salicylates, clofibrate, procaine, lorazepam, cilazapril, and other angiotensin-converting enzyme inhibitors, narcotics (cocaine, heroin), and capsaicin. CarbE also participate in detoxification of pesticides (carbofuran, pyrethroids, OPs), acrylates, mycotoxins (T2 toxin), and esters of nicotinic acid (Cashman et al., 1996). Certain isoenzymes of hepatic microsomal CarbE are involved in the metabolic activation of some carcinogens and are associated with hepatocarcinogenesis (Hosokawa and Satoh, 2006). Enzymes similar to CarbE are arylesterases (EC 3.1.1.2) that hydrolyze aromatic esters of carboxylic acids. However, this classification is not perfect because CarbE hydrolyze some aromatic esters (i.e., phenyl valerate, phenyl butyrate) and arylesterases hydrolyze certain aliphatic esters. These two enzymes can be clearly differentiated according to their interaction with OP because CarbEs are inhibited by OPs, whereas arylesterases can hydrolyze some OPs that contain aromatic groups, such as paraoxon and chlorpyriphos oxon; because of this, they were sometimes confused with A-esterases.

CarbE profile in humans is not well-known. Although CarbEs were considered to be absent from the blood plasma of humans (Li et al., 2005), they are present in human erythrocytes and monocytes as well as in human liver, kidney, lung, skin, and nasal tissue (Cashman et al., 1996). Additional literature documents the presence of CarbEs in many human tissues and fluids, including brain, milk, mammary gland, pancreas, small intestine, colon, stomach, placenta, and plasma and serum (Kaliste-Korhonen et al., 1996; Chanda et al., 2002). The lung CarbE are associated with alveolar macrophages (Munger et al., 1991). Further, CarbE are present in human tissues and organs, where exposure to nerve agent vapors would likely first occur (nasal tissues and the lung), would be distributed (erythrocytes, monocytes, plasma), and would generate effects (brain, stomach, colon, etc.). Chanda et al. (2002) indicated that full characterization of the OP-protective capabilities of CarbE requires assessment not only of the amount but also of the affinity exhibited by CarbE for the inhibitor as well as the total CarbE activity unlikely to be inhibited. The detoxification potential of CarbE is apparently complex and is an area requiring further studies.

CarbEs are proteins of molecular weight between 47 and 65 kDa that can be found in microsomal fractions of many mammalian tissues (Satoh and Hosokawa, 1998). CarbEs are synthesized in liver and secreted into plasma (via the Golgi apparatus), where they are present in soluble form. Their physicochemical and immunological properties and the sequence of amino acids are very similar, whereas their specificity toward various substrates is different (Hosokawa et al., 1995; Satoh and Hosokawa, 1998). CarbE belongs to the group of esterases with serine at its active site that hydrolyze esters of carboxylic acids in a biphasic reaction. In the first phase, carboxylic ester acylates hydroxyl group of serine at the active site, and in the second phase serine is deacylated in the presence of water (Augustinsson, 1958). The active site of CarbE comprises a peptide isoleucine-phenylalanine-glycine-histidine-serine-methionine-glycine-glycine, with serine and histidine directly participating in biochemical reactions. Physiological substrate for CarbE is probably *O*-acetyl sialic acid (Satoh and Hosokawa, 1998). CarbE can be differentiated from other serine esterases such as AChE (EC 3.1.1.7) and ChE (EC 3.1.1.8) in that AChE and ChE react with positively charged esters (ACh and butyrylcholine, respectively) and can be inhibited with carbamates, whereas CarbE does not react with positively charged esters and inhibition with carbamates occurs only at high concentrations (Gupta and Kadel, 1989). Inhibition of CarbE does not cause any known toxic effects.

The Relationship Between CarbE Activity and Toxicity of WNAs

Several studies have shown that triorthocresyl phosphate (TOCP) and its active metabolite CBDP (2-/*O*-cresyl/-4H-1:3:2-benzodioxaphosphorin oxide) (specific irreversible inhibitor of CarbE and weak

anticholinesterase agent) potentiate toxicity of soman, sarin, and tabun (Bošković, 1978; Clement, 1984; Jokanović, 1989), but not of VX agent (Bošković, 1978), probably because VX in physiological conditions is positively charged and a weak inhibitor of CarbE (Maxwell, 1992). Bošković (1978) found that pretreatment of mice with CBDP increased the subcutaneous toxicity of soman by 19.1-fold, and increased its intraperitoneal toxicity by 17.8-fold. For other nerve agents, he observed an increase of subcutaneous toxicity of sarin, tabun, and VX by 11-fold, 5-fold, and 0.24-fold, respectively. Clement (1984) observed that potentiation of soman toxicity in mice after previous administration of TOCP or CBDP was directly related to plasma CarbE and not to activity of CarbE in liver and other tissues. This effect of TOCP and CBDP was explained by phosphorylation of active sites at CarbE that occupies the binding sites for other OPs increasing their concentration in circulation and therefore their acute toxicity (Jokanović, 2001, 2009). Binding of soman to CarbE in rodents occurs specifically with the most toxic stereoisomer of the agent (Cashman et al., 1996).

The detoxification potential of endogenous CarbE to protect against the lethal effects of WNA exposure was tested by Maxwell (1992), who observed that a wide range in potentiation of toxicity of different OPs *in vivo* cannot be correlated with reactivity of these compounds toward CarbE, showing that soman toxicity in rats with inhibited CarbE was potentiated by sixfold, respectively, despite their similar inhibitory power for CarbE. It was concluded that detoxification of OP via CarbE is very important for highly toxic OPs, such as soman, sarin, tabun, and paraoxon with LD_{50} of <2 μmol/kg, whereas it is less important for less toxic OPs such as DFP (LD_{50} = 9.75 μmol/kg) and dichlorvos (LD_{50} = 98.4 μmol/kg). Keeping in mind that relatively higher concentrations of OP insecticides have to be achieved in circulation and tissues to exert toxicity, dominant factors in detoxification of less toxic OPs are A-esterases because their catalytic activity is proportional to substrate concentration and their K_m value is in the millimolar range (Jokanović, 2009).

Contrary to these findings of decreased CarbE activity increasing toxicity of many OPs, there are also data showing that increased CarbE activity can decrease toxicity of OPs. CarbE activity can be increased by approximately 80% after repeated administration of phenobarbital to rats and mice by mechanism of enzyme induction, which caused a decrease in soman and tabun toxicity by twofold, whereas toxicity of sarin was not affected, probably because plasma CarbE inhibited with sarin spontaneously reactivate very rapidly *in vitro* and *in vivo* with the half-times of 18 and 120 min, respectively (Bošković et al., 1984; Clement, 1984; Jokanović, 1989, 2009; Jokanović et al., 1996).

Various OPs inhibit both CarbE and AChE at similar concentrations ranging from 1 to 1000 nmol/L. CBDP, dichlorvos, DFP, and paraoxon show higher affinity toward CarbE *in vitro* and, as a result, their acute toxicity is lower in contrast to highly toxic OPs soman and sarin that have four- to six-times higher affinity for AChE. This relationship was confirmed *in vivo* after administration of 0.9 × LD_{50} of these compounds (Maxwell, 1992). Rat plasma CarbE appears to be more sensitive for soman and sarin than CarbE in rat liver and brain, and can be completely inhibited at sublethal doses. Significant inhibition of CarbE in liver can be obtained only at multiple lethal doses (Bošković et al., 1984). Even when two-thirds of rat liver was removed by partial hepatectomy, 5 × LD_{50} of soman was not sufficient to achieve significant inhibition of rat liver CarbE (Jokanović, 1990). Somani et al. (1992) found that interspecies variation in response to some nerve agents may be largely due to CarbE binding.

In the study of mechanism of interaction of CarbE with some OPs *in vitro*, it was found that this reaction is not irreversible, but rather reversible because of rapid spontaneous reactivation of inhibited CarbE (Jokanović et al., 1996; Jokanović, 2001, 2009). The highest rate of spontaneous reactivation was obtained for plasma CarbE inhibited with sarin and the half-time of reactivation was 18 min. These results were also confirmed in experiments *in vivo* in which rats were treated with 0.5 × LD_{50} of soman, sarin, and dichlorvos (Jokanović et al., 1996). Calculated half-times of reactivation for plasma CarbE of the rats treated with 0.5 × LD_{50} of dichlorvos, sarin, and soman were 1.2, 2.0, and 2.7 h, respectively. Similar results were reported by Gupta et al. (1987a), who found 50% of spontaneous reactivation of plasma CarbE 24 h after poisoning of rats with 100 μg/kg soman. Gupta et al. (1987b) also reported 94% of reactivation of plasma CarbE in rats treated with 200 μg/kg tabun, but 7 days after poisoning. Spontaneous reactivation of CarbE hydrolyzing phenyl valerate inhibited with paraoxon *in vitro* was observed by Barril et al. (1999).

The Role of CarbE in Detoxification of OP

CarbE participate in detoxification in three different ways. The first is hydrolysis of ester bonds in OP that contain them, such as malathion (WHO, 1986; Fukuto, 1990). The second is binding of OP to CarbE and other proteins that decrease the concentration of free OP in circulation that can react with AChE in vital tissues (Clement, 1984; Jokanović, 1989). The third role is related to all OPs that can phosphorylate CarbE by binding to serine hydroxyl group at its active site (Jokanović et al., 1996; Jokanović, 2001, 2009). During spontaneous reactivation, this phosphoryl residue is separated from the enzyme accepting hydroxyl group from water as its new acyl radical. This newly formed OP (i.e., organophosphoric

acid) is a much less potent (if at all) esterase inhibitor that represents a nontoxic metabolite of the parent OP. In the case of nerve agents, the corresponding metabolites that formed EDMPA (for tabun), IMPA (for sarin), and PMPA (for soman) are shown in Figure 59.1. CarbE activity recovered in this reaction can be inhibited again by other OP molecules. The active role of CarbE in this process is its involvement in metabolic transformation of OP to its nontoxic and biologically inactive metabolites. Because of rapid spontaneous reactivation of CarbE, one active site at the enzyme can metabolize several molecules of OP; this reaction does not occur according to stoichiometric ratio 1:1, which depends only on the stability of the bond between phosphorus from OP and oxygen from serine hydroxyl group. Tissues in which this "turnover" is rapid, such as plasma, has higher capacity for detoxification of OPs than expected because of catalytic activity of CarbE. This reaction can be very important under conditions of repeated (subchronic or chronic) exposure to low doses of nerve agents and other OP that could be inactivated through the reaction with CarbE without any apparent toxic effect.

The role of CarbE as a bioscavanger involved in detoxification of nerve agents and other OPs was investigated. The ideal OP bioscavanger would have a fast rate of reactivity for a broad range of OP compounds, a slow rate of aging, and the ability to reactivate to increase its stoichiometry as a bioscavanger. Evaluation of CarbE on these criteria suggests that it is an important candidate as an OP bioscavanger (Doctor et al., 2001). One of the most important advantages of CarbE is that OP-inhibited CarbE does not undergo the rapid aging that prevents oxime-induced reactivation of OP-inhibited ChEs. This means that OP-inhibited CarbE can be reactivated, yielding an active enzyme involved in further metabolism of OP molecules and an inactive OP metabolite.

Another advantage of CarbE is the much greater size of its active site compared with those of AChE (10-fold difference) and ChE (6-fold difference) (Saxena et al., 1999). The large active site volume of CarbE minimizes steric hindrance effects at the active site and maximizes the potential for reactivation. In a study investigating the structural specificity of AChE, ChE, and CarbE, Maxwell et al. (1998) found that AChE could accommodate OP inhibitors containing only one bulky group (e.g., isopropyl, pinacolyl, phenyl), ChE could accommodate OP inhibitors containing two of the smaller bulky groups (like isopropyl), and the active site of CarbE was sufficiently large to accommodate up to two of the largest bulky groups (e.g., phenyl groups). Therefore, CarbE had the ability to metabolize the broad spectrum of OP inhibitors. The only exception to this observation is, because of fewer aromatic residues in the active site of CarbE in comparison with ChE, reduced affinity of CarbE for positively charged OP inhibitors (such as VX), and this effect apparently has little importance for nerve agents (except VX) and OP pesticides because a few of them are positively charged (Doctor et al., 2001).

Prolidase (EC 3.4.13.9)

There are data in the literature showing that prolidase (EC 3.4.13.9), a naturally occurring enzyme of mammalian and bacterial origin, is involved in hydrolysis of G-type nerve agents. Recombinant HuPON1 and DFPase showed 10-fold lower activity toward sarin compared with recombinant human prolidase (Cheng et al., 1999; diTargiani et al., 2010; Nachon et al., 2013). Constante et al. (2012) reported that partially purified human liver prolidase hydrolyzed DFP and various nerve agents, whereas skin and kidney prolidase presented significantly lower activity against soman, tabun, and VX. Biochemical characteristics of prolidase purified from human erythrocytes, liver, kidney, and fibroblast cells are well-known. However, OP hydrolyzing activity of prolidase is still not well-understood (Wang et al., 2006; diTargiani et al., 2010; Chandrasekaran et al., 2013).

Human prolidase is a 54-kDa binuclear Mn^{+2}-dependent enzyme that breaks the amide bond in dipeptides containing proline or hydroxyproline as the C-terminal amino acid. It plays a crucial role in the recycling of proline. Prolidase is found in most tissues and in several animal species. Deficiency of this enzyme in humans results in a syndrome with a highly variable clinical phenotype, such as chronic recurrent infections, mental retardation, splenomegaly, skin lesion, and the excretion of massive amounts of iminopeptides in urine (Wang et al., 2006; Chandrasekaran et al., 2013).

PROTEIN BINDING

Proteins are amphoteric structures containing anionic and cationic reactive sites. Proteins can also participate in other interactions with xenobiotics through formation of hydrogen bonds, polarity, and electrostatic and van der Waals forces. Many xenobiotics can bind to proteins from blood such as albumin and B-esterases. Easy binding to proteins occurs with substances that are ionized at a physiological pH and those soluble in lipids such as OP. After binding of OP to proteins such as CarbE, ChE, AChE, and other macromolecules, these agents are metabolized because their acyl radical is released and phosphoryl residue remains bound to proteins. This unspecific binding of OP to blood proteins decreases OP concentration in circulation and tissues preserving AChE activity at target sites (Jokanović, 2009). Binding of OP to proteins can be limited by steric hindrance, a protein conformation factor that does not allow OP molecules to access all binding sites at the protein. The involvement of secondary noncholinergic targets (neuropathy target

esterase, fatty acid amide hydrolase, arylformamidase, acylpeptide hydrolase, and other macromolecules) in OP toxicity was reviewed by Casida and Quistad (2004).

Albumin is the most abundant protein found in plasma and has a half-life of approximately 20 days. It is a 67-kDa multifunctional monomer synthesized and secreted by the liver. Albumin has esterase-like and aryl acylamidase activities, but it does not have a catalytic active site serine (Marsillach et al., 2013). The high concentration of albumin in plasma (30–60 g/L) may balance the poor reactivity of this protein with OPs (Jokanović, 2009).

Williams et al. (2007) found that sarin, soman, cyclosarin, and tabun phosphylate a tyrosine residue on albumin in human blood. The tyrosine adducts with soman and tabun were detected in guinea pigs receiving therapy 7 days after subcutaneous administration of 5 × LD_{50} of the respective nerve agent. Bao et al. (2012) reported formation of soman, sarin, and VX adducts with the tyrosine residue of albumin when rats were exposed to sublethal doses of the nerve agents. In blood samples taken from marmosets treated with sarin, soman, cyclosarin, or tabun; tyrosine adducts were detected after 23 or 24 days (Read et al., 2010). Li et al. (2008) have shown that soman covalently binds to albumin at tyrosine 411 and that the adduct was stable ($t_{½}$ = 20 days). However, although the concentration of albumin in plasma is very high, its reactivity with soman was apparently too slow to play a major role in detoxification of the agent. The authors concluded that soman–albumin adducts could be useful for the diagnosis of soman exposure. In addition, when OP pesticides covalently bound to albumin, the adduct was stable for more than 7 days (Tarhoni et al., 2008).

Tyrosine adducts were found in guinea pigs and marmosets poisoned with sarin, soman, cyclosarin, and tabun. VX, which is less reactive than other WNAs, formed an adduct in human plasma *in vitro* only at high concentrations (Williams et al., 2007; Black, 2010; Black and Read, 2013). Tyrosine adducts were less sensitive than ChE as biomarkers with respect to exposure levels but were more stable and did not undergo an aging reaction such as OP binding to serine esterases (Read et al., 2010). However, albumin might have a larger capacity for OP binding compared with ChE and other esterases.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

After more than four decades of research of biotransformation of nerve agents soman, sarin, tabun, and VX, it can be concluded that several enzymes have a significant role in this process. Enzymes capable of hydrolyzing these agents (A-esterase, PON1) were very efficient in the breakdown of the bond between phosphorus and acyl radical, and their activity was proportional to substrate concentration. Esterases such as ChEs and carboxylesterases act by binding of OP molecules to the hydroxyl group placed in their active site, decreasing free concentration of the agents in blood, thus preventing inhibition of AChE at target sites and, subsequently, their toxic effects. In addition, binding of OP to CarbEs is reversible, indicating an active role of the enzyme in metabolic detoxification of nerve agents and other OPs. The importance of prolidase is still unclear and requires further studies. It is necessary to further investigate the role of these enzymes and other macromolecules in detoxification of OP compounds. Important issues for further research in this field should be related to the assessment of detoxification potential of carboxylesterases, the significance of A-esterase polymorphism on the capacity of detoxification, and their possible application in prophylaxis and treatment of OP poisoning in humans.

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SECTION VII

ANALYTICAL METHODS, BIOSENSORS AND BIOMARKERS

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CHAPTER

60

On-Site Detection of Chemical Warfare Agents

Yasuo Seto

INTRODUCTION

Chemical warfare agents (CWAs) were employed in World War I and World War II and during the Cold War, and they continue to be produced and stockpiled even today (Somani, 1992; Gupta, 2009). In the 1980s, Iraq used sarin (GB) and mustard gas (HD) in the Iran–Iraq conflict (Black et al., 1994). In 1992, the Chemical Weapons Convention (CWC), a treaty prohibiting the development, production, stockpiling, and use of chemical weapons and mandating their destruction was ratified, and it came into force in 1997 (Organization for the Prohibition of Chemical Weapons, 2014). However, in the interim, the Japanese doomsday cult group Aum Shinrikyo deployed sarin in the Japanese city of Matsumoto in 1994, and then, more infamously, in the Tokyo subway system in 1995. In both these attacks, many defenseless people were poisoned, some fatally (National Police Agency, 1996; Seto et al., 2000). The Tokyo subway sarin gas attack and the more recent US postal anthrax letter attacks in 2001 (Inglesby et al., 2002) presented a renewed threat of chemical and biological terrorism globally. To realize a safe and secure society, it is highly recommended that authorities at the national levels establish a more strengthened crisis management system for civil defense (Seto, 2006a).

In addition to the known attacks, various CWA stockpiles have been discovered in former Japanese military force facilities during land excavations (Ohashi, 2004). Injuries due to direct contact with CWAs that leaked from containers in Samukawa, Japan, have been reported (Hanaoka, 2004), and some complaints were received in Kamisu, Japan, concerning damage to human health (neurological disorders) caused by drinking water likely contaminated with degradation products from arsenic vomiting agents (Ishii et al., 2004).

In the crisis management of chemical warfare terrorism cases (Society for Countermeasures against Chemical, Biological, Radiological, Nuclear and Explosive Terrorism, 2008) and chemical weapon disposal, CWAs were monitored in public places, security checks at territorial borders, airports, large event venues, executive facilities, and demilitarization spots housing chemical weapons for protection against terrorism and workers' health. With regard to consequence management, on-site detection is initially performed by first responders for personal protection; on-site samples are then transported to laboratories for analysis for investigation and for identification of emergency lifesaving measures. In incident management, laboratory analysis is performed to provide evidence for courts in order to prosecute offenders and prevent future crimes (Figure 60.1). Among these detection schemes, rapid on-site detection is the most important for the minimization of disasters in order to eliminate the prolonged times required for transporting on-site sample specimens to a laboratory for analysis (Harris, 2002; Smith, 2002; Fittch et al., 2003). Various types of measuring technologies have been used for on-site detection and laboratory identification of CWAs and are discussed here.

PROPERTIES OF CWAs

CWAs are low-molecular-weight synthetic compounds that are fast-acting and sometimes lethal, even at low levels (Somani, 1992; Stewart and Sullivan, 1992; Marrs et al., 1996; Ellison, 2000); in terms of physical properties, CWAs can be classified into gaseous blood agents, gaseous choking agents, volatile nerve gases, volatile blister agents, nonvolatile vomiting agents, and nonvolatile lachrymators (tear gases) (Figure 60.2).

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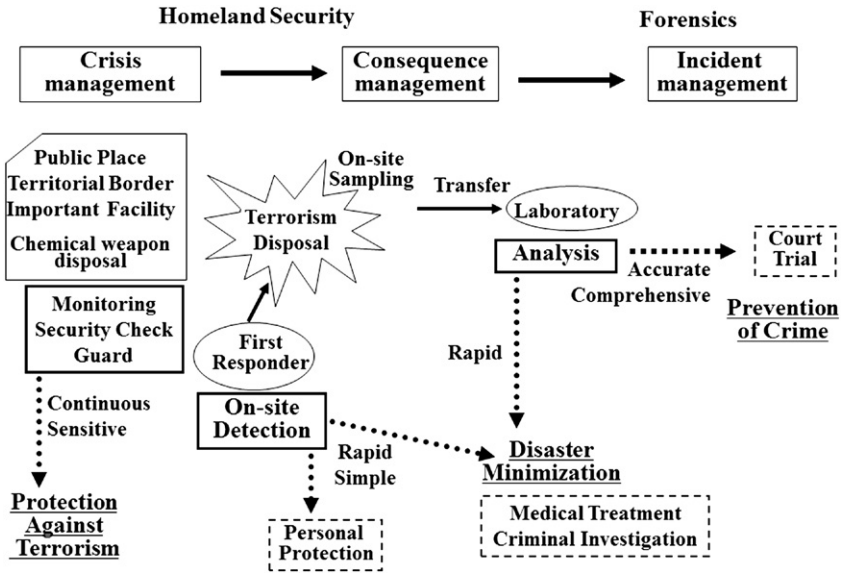

FIGURE 60.1 Detection and identification in chemical terrorism countermeasures and chemical weapon disposal.

The physical and toxicological properties of CWAs vary in many ways; namely in molecular weight, melting point, boiling point (vapor pressure), vapor density, durability in air, lethal concentration, incapacitating concentration, odor, water solubility, stability in water, effect on skin, and antidote prospects. In particular, except for slow-acting agents that manifest toxicity after several hours, such as phosgene (CG) and HD, CWAs are fast-acting. Deadly poisonous organophosphate (OP) nerve gases and toxic blister agents are registered as scheduled compounds in the CWC. Although CWAs show toxicity by skin contact and intake, inhalation toxicity against CWA vapor is mainly considered with respect to chemical warfare/terrorism cases and chemical weapon disposal, and the development of analytical technologies is conducted with the aim of CWA detection in the vapor phase. The parameter used in the description of CWA toxicity is the lethal dose value (LCt_{50}, $mg\,min/m^3$); this value indicates the vapor concentration leading to a 50% incidence of death in human beings with 1 min of inhalation.

CONCEPT OF ON-SITE DETECTION

In chemical warfare terrorism countermeasures, we must consider the dispersion of various types of CWAs, including toxic industrial compounds (TICs), except for overt cases such as previous notices of criminal acts. If clear symptoms of CWA-exposed casualties are manifest, it is possible to narrow down the types of CWAs detected. On the other hand, for chemical weapon disposal, the possible types of dispersed CWAs are obvious. In cases of exposure to CWA vapor and subsequent intoxication, monitoring technology is very important for exposure minimization and effective medical treatment. However, the requirements for the optimal performances of on-site detection technologies are not clear, with most protocols depending only on previous experience. Namely, the on-site monitoring of CWAs is quite different and an unknown field compared to other monitoring situations; therefore, not only researchers, but also first responders, have little idea of how to detect CWAs on site. Some of the required criteria for successful on-site detection are as follows: knowledge of the possible types of CWAs and their limits of alarm (LOAs), response time, time required to start the detector, recovery time after the previous alarm, accuracy (affected by interference and false positives), state of alarm, operational performance (i.e., the required technology and training), maintenance, and cost of introduction and maintenance. In addition, operational conditions, durability, and portability of necessary equipment should be considered.

For considering toxicity manifestation time and vapor dispersion situations in cases of terrorism, it is necessary to initiate the alarm for a vapor concentration of less than the vapor concentration of 1/100 of LCt_{50} within 1 min. In the case of GB, the required detection sensitivity is $1.5\,mg/m^3$; at this level, humans show few signs of intoxication, nor is there a detectable odor. In chemical weapon–disposal situations, because workers are present at the sites for a prolonged time, the time-weighted average values, which are approximately 1/100,000 of LCt_{50}, are adopted as the monitoring target for allowed operational conditions. An alarm time of less than several minutes is desired. There is a trade-off relationship between LOA, alarm time, detection accuracy,

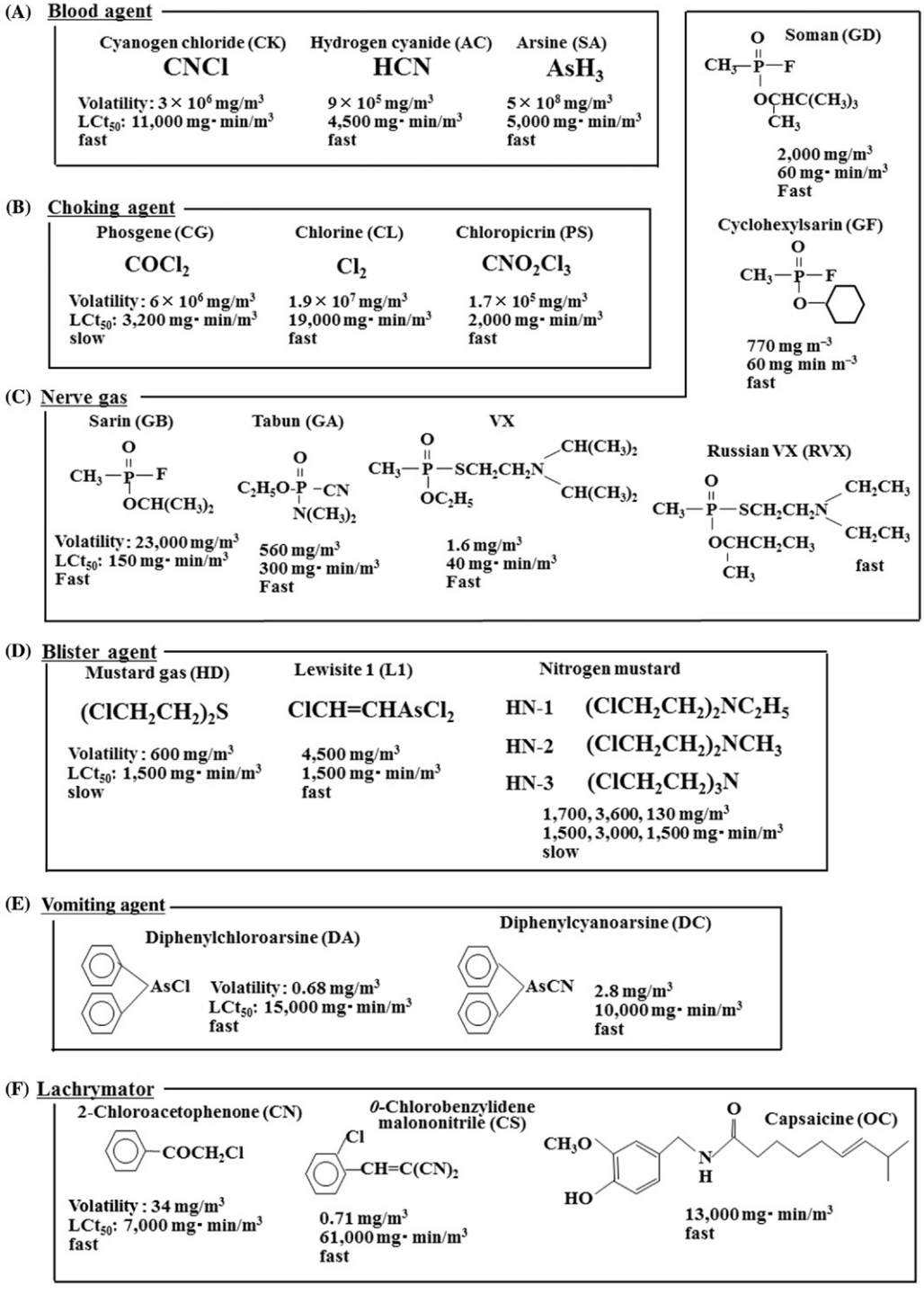

FIGURE 60.2 Chemical structures, volatility (at 25°C) and inhalation toxicity data of CWAs.

and operation; furthermore, for detection equipment employing one detection mechanism, lowering the LOA prolongs the alarm time and increases the frequency of false positives.

In previous instances for which on-site detection technology had not yet been well developed, field damage and injuries to the victims were traditionally observed and understood by using our senses; CWAs were identified through laboratory analysis and diagnostics after transferring the on-site victims' samples to specific laboratories and emergency medical hospitals. To minimize the damage caused by the terrorist activity, it is vital to obtain as much information as possible about the types and concentrations of the dispersed CWAs, which is now

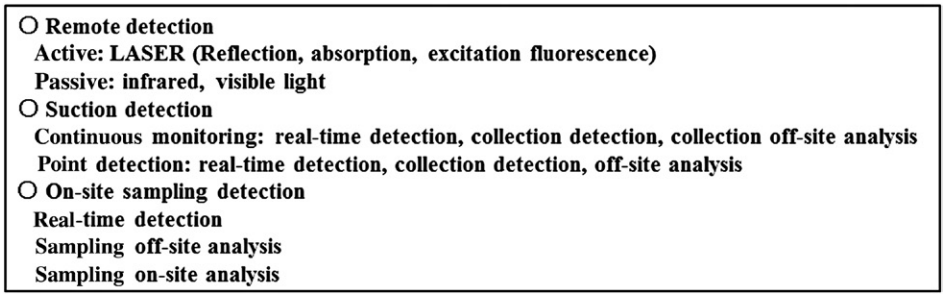

FIGURE 60.3 Concept of on-site detection for CWAs.

possible because of the development of large numbers of laboratory analytical technologies. Figure 60.3 shows the concepts in the determination of CWAs. Remote detection (stand-off detection) is the method to detect CWAs away from the dispersed site using spectroscopic technologies, and can be divided into the following two types: (i) passive detection, which refers to the detection of light emitted from the target and is mostly limited to infrared light; and (ii) active detection, which detects the secondary light (fluorescent or absorbed light—i.e., ultraviolet, visible, or infrared) emitted from the target after the sample is irradiated with primary light using equipment brought to the site, with lasers usually employed as the irradiating light source.

Because the vapor concentrations of CWAs used for terrorism are low and their toxicities are high, and because terrorism is predicted to occur at civilian locations where various types of interfering compounds exist, it is often difficult to fulfill on-site detection requirements. "Suction detection" is the method to detect CWAs by directly measuring CWAs through suction of vapors in the field, and is further divided into the following two types: (i) continuous monitoring, which involves continuous drawing of on-site air samples and detection using fixed or movable equipment; (ii) point detection, which is used to detect CWA vapor by first responders by moving portable devices into the field. In terms of CWA detection, *real-time detection* refers to the alarm signal given under the continuous drawing of air samples, while in collection detection mode, the alarm is given by detection with cycling measurements after air samples have been collected. *Collection off-site analysis* refers to CWA detection in the laboratory using collected samples transferred from the field. For solid or liquid samples and dispersed surfaces, CWAs are detected by direct contact with the detector; alternatively, they are detected by measuring the sampled or stripped specimens by inserting them into the detectors. Finally, large trailers containing mobile chemical laboratories equipped with analytical instruments are used for detection via on-site analysis.

The assessment of dangerous terrorism and chemical weapon-disposal situations by detecting fast-acting CWAs quickly at low-concentration levels must be rapid (Sidell et al., 2003). For people escaping from or arriving on the dangerous sites, injuries and casualties must be taken care of with proper emergency treatment (such as securing aeration and administering antidotes). The roles of the first responders are to install protective masks and gears and perform zoning of the dangerous sites. Field-deployability, rapid alarm capability, and ease of automated operation are most important for on-site detection, with the low frequency of false positives being a secondary requirement; however, if the frequency of false positive alarms is high during a response, it will disturb the proper and timely activities of responders at the terrorism-affected or chemical weapon–disposal sites. Various types of on-site CWA detection devices have been used by military organizations worldwide (Fittch et al., 2003), and some have been introduced for civil defense organizations such as mobile police teams, fire-defense teams, and coast guards for the purposes of counterterrorism.

CWAs can be measured by detecting the chemical constituent; the technologies for chemical detection can adopt different mechanisms depending on the target substances (Paddle, 1996). The discrimination level varies between the screening and identification methods. At present, most field equipment utilizes detection paper, gas detection tubes, ion mobility spectrometry (IMS), flame photometric detection (FPD), photoionization detection (PID), surface acoustic wave (SAW) detection, Fourier transform/infrared spectrometry (FT/IR), gas chromatography (GC), mass spectrometry (MS), or combined GC-MS technologies. Furthermore, chemical sensors, biosensors, and micro total analysis systems (μTAS) have been extensively developed. Except for highly discriminating technologies such as chromatographic or spectrometric measurements, many detectors signal an alarm only for restricted chemical species. The representative species are (as previously described) nerve gases (nerve agent), blister agents, blood agents,

including gaseous choking agents, and TICs. Vomiting agents and lachrymators are not considered because of the mechanical impossibility for detecting such nonvolatile agents and their low toxicity. Nerve gas is the most preferred target, followed by blister and blood agents. So far, CWA detection technology has been developed and utilized for deployment during military missions; thus, it is not guaranteed that such on-site equipment would work properly for real CWAs in civil defense. Considering the present situation of developed terrorism countermeasures and on-site needs, greater emphasis should be placed on the scientific development of on-site detection technology. Several research groups are engaged in the evaluation of commercially available detection equipment and are presently developing new detection technologies. Our laboratory has evaluated some commercially available detection equipment using authentic CWAs. In the following section, the evaluation results obtained in the author's laboratory are discussed (Seto et al., 2005, 2007; Seto, 2006a,b; 2014).

THE CURRRENT SITUATION OF DETECTION TECHNOLOGY

With regard to remote detection technology, passive methods for spectrophotometric measurements of CWA emission (specifically infrared light and the corresponding devices) are partially utilized in military applications. These machines can instantaneously and continuously detect the CWA vapor; however, because of background interference, practical utilization by civilian means is still a far-off prospect. The active method of measuring the reflected light from laser irradiation provides higher sensitivity than the passive method; however, the equipment for this technique is too large (on the ton scale). RAPID (Bruker Corporation, 2014) is a typical detection machine manufactured by Bruker Daltonics (Bremen, Germany). The discriminating capability with respect to analysis of the obtained spectra is significantly raised using this device; furthermore, in addition to ultraviolet, visible, and infrared light, a wide range of other light wavelengths such as milliwave and terahertz light can be detected, apart from laser excitation and Raman scattering.

Classical Manual Method

The classical manual detection tools still in use are detection paper, and gas detection tubes, which can detect the presence of CWAs by visualizing the color changes manifested by the reaction between the reagents and CWAs. Detection paper shows an instant color change in the presence of liquid forms of CWAs with a sensitivity of several $\mu g/cm^2$ (Toyobo, Japan; M8 and M9 paper, US military). Two types of pigments and pH indicators are impregnated in the cellulose paper; the paper turns brown or orange on the addition of a droplet of sarin (GB), soman (GD), or tabun (GA) (G agents in the North Atlantic Treaty Organization (NATO) code); turns red when exposed to a droplet of HD or Lewisite 1 (L1) (H agents); and turns to black or deep green with VX (a V agent). This color reaction is based on the solubility of the agents in organic solvent; thus, almost all organic solvents except water would show a false positive. Dimethyl methylphosphonate, acetone, toluene, and ethyl acetate show a false positive for G agents; 2-mercaptoethanol, carbon tetrachloride, and aniline show a false positive for H agents; and diethylamine shows a false positive for V agents. Because of the high probability of false positives, detection paper seems impractical for civilian defense (Seto et al., 2005).

Gas detection tubes show a color change with vapor CWAs with a sensitivity close to mg/m^3 concentration levels. The specific reagents are impregnated into a silica-gel support in the glass tube. When needed, both sides of the tube are opened by a cutter, the appropriate air sample volume is drawn, and the extent or length of coloring is checked by the naked eye. Commercial industrial-use gas detection tubes (Komyo Kika; Gastec, Japan) are available for the detection of blood and choking agents. However, for special agents such as nerve gases, the Dräger Safety gas detection tube (in Germany) is adequate (Takayama et al., 2007).

For nerve gases, the phosphoric acid ester tube shows a red color, which is based on a sequence of complicated procedures involving butyrylcholinesterase inhibition, substrate butyrylcholine hydrolysis, and pH indication. Positive results are shown for cholinesterase inhibitors. For HD, the thioether tube exhibits a yellow color, which is based on the reaction with silver chloride and chloramines. HD stimulants show false positives. For L1, the arsine and organic arsenicals tube shows a black color; a sequential procedure involving two types of chemical reactions allows the separate detection of both inorganic and organic arsenicals and, based on the reduction by zinc and hydrochloride and subsequent complex formation with gold and mercury, yields a gold-colored colloid. For nitrogen mustard 1 (HN 1), nitrogen mustard 2 (HN 2), and nitrogen mustard 3 (HN 3), the organic alkaline nitrogen compounds tube shows an orange-red color, which is based on the Dragendorf reaction. For hydrogen cyanide (AC), the hydrogen cyanide tube shows a pink color, which is based on oxidation by mercury chloride and subsequent pH indication. For cyanogen chloride (CK), the cyanogen chloride tube also shows a pink color, which is based on the reaction with pyridine and barbituric acid. For CG, the phosgene tube shows a blue-green color, which is based on the chemical reaction with *p*-dimethylbenzaldehyde/*N*,*N*-dimethylaniline. For chlorine (CL), the chlorine tube shows

TABLE 60.1 Detection Performance of Dräger Gas Detection Tubes

Gas Detection Tube	Agent	Limit of Alarm (mg/m^3)	Response Time (min)	Remark
Phosphoric acid ester	Sarin	0.002	5–6	
	Soman	0.02	5–6	
	Tabun	0.5	5–6	
	VX	2	5–6	
	Dichlorvos			False positive at $1\,mg/m^3$
	Methomyl			False positive at $50\,mg/m^3$
	Dimethylmethyl-phosphonate			Negative at $2{,}300\,mg/m^3$
Thioether	Mustard gas	2	2	
	2-Chloroethylethyl sulfide			False positive at $2.4\,mg/m^3$
	1,4-Thioxane			False positive at $2.4\,mg/m^3$
	Diethylether			Negative at $20{,}000\,mg/m^3$
Organic arsenic compound and arsine	Lewisite 1	4	2	
	Diphenylchloro-arsine			Negative at $100\,mg/m^3$
Organic alkaline nitrogen compounds	Nitrogen mustard 1	10	1	
	Nitrogen mustard 2	10	1	
	Nitrogen mustard 3	10	1	
Hydrogen cyanide	Hydrogen cyanide	0.3	1	
	Cyanogen chloride			Negative at $100\,mg/m^3$
Cyanogen chloride	Cyanogen chloride	0.8		
	Hydrogen cyanide			Negative at $100\,mg/m^3$
Phosgene	Phosgene	1	1	
Chlorine	Chlorine	0.8	1	

an orange color, which is based on a chemical reaction with *o*-tolidine. The LOA (i.e., the minimum concentration giving three positive results within three trials), response time, and interference of the stimulants and solvents are shown in Table 60.1. The operation involves a complicated procedure of breaking the inner liquid tube and subsequent incubation in a number of detection tubes. However, the procedure is quite tedious when employing protective gear and gloves, requiring several minutes from the start of the operation until tube coloration is observed. The gas detection tube is recommended as a supplemental means for ascertainment of CWA species after IMS screening.

Photometric Method

The photometric type of detection is based on the photometric or electronic response manifested by the physical-chemical reaction of CWAs. The flame photometric detector (FPD) instrument detects CWAs by measuring the specific phosphorus or sulfur emission line produced via combustion with hydrogen gas. AP2C (PROENGIN, 2014) is a handy portable automated FPD manufactured by Proengin (France). This detector responds rapidly to both vapor and liquid forms of phosphorus containing CWAs in the GV mode and of sulfur-containing CWAs in the H mode. Approximate concentration levels of 0.1 and $1\,mg/m^3$ for nerve gases and HD are detected in the GV and H modes, respectively (Seto et al., 2005, 2007). The simulants containing phosphorus or sulfur show false positivity, and CWAs containing neither phosphorus nor sulfur are not detected. The flame ionization detection (FID) instrument such as MicroFID II (INFICON, 2014a) detects nonspecific combustible CWAs by measuring ion production manifested by combustion with hydrogen; the LOA of this method is at sub-mg/m^3 levels

TABLE 60.2 Detection Performance of Arrayed Surface Acoustic Wave Detector ChemSentry

Agent	Alarm	Limit of Alarm (mg/m^3)	Response Time (s)	Recovery Time (s)
Sarin	NERVE	30	12	235
Soman	NERVE	50	12	234
Tabun	NERVE	100	13	230
Mustard gas	BL	38	8	236
Lewisite 1	NERVE	280	109	272
	BL	57,000	13	394
Hydrogen cyanide	BLOOD	28	100	19
Cyanogen chloride	BLOOD	940	103	27

TABLE 60.3 Response of ChemSentry Against Organic Solvents

Alarm	Compound
NERVE	Methanol, Ethanol, *n*-Propanol, 2-Butanol, *t*-Butyl alcohol, Ethyl acetate, 1,4-Dioxane, Acetonitrile, Acetaldehyde, *N*,*N*-Dimethylformamide, Pyridine
BL	Dichloromethane, 1,2-Dichloroethane, Chlorobenzene
BLOOD	Ammonia, Acetaldehyde
CONFIDENTIAL	*t*-Butyl alcohol, Acetone, Diethylether, Diethylamine, *N*,*N*-Dimethylformamide, Pyridine
Negative	15 solvents

of vapor concentrations. The PID instrument detects numerous types of CWAs in a nonspecific fashion, which is based on the measurement of the produced ion current of charged gas ions by irradiation with ultraviolet light. RAE Systems (San Jose, CA) manufactures the ppbRAE VOC gas monitor, which has an LOA on the level of several tens of mg/m^3 (Seto et al., 2007; RAE Systems, 2014).

Chemical sensor technology adopting acoustic waves (Harris, 2002) is also available for CWA on-site detection. The CWAs are adsorbed reversibly onto the arrayed cells of the specific liquid phase polymer, and the acoustic wave numbers change according to the mass increase due to CWA adsorption. CWAs can be detected and discriminated by analyzing the respective wave number changes (Harris, 2003; Grate, 2000); discriminating power is increased by increasing the number of polymer cells. Portable detectors adopting arrayed SAW devices are commercially available. ChemSentry, manufactured by BAE Systems (London), adopts 10 different polymer cells and identifies CWAs as "NERV" for nerve agents, "BL" for blister agents, and "BLOOD" for blood and choking agents. As shown in Tables 60.2 and 60.3 (Matsushita et al., 2005), the LOA is rather high, the response and recovery time is long, and the number of false positives is high. This chemo-adsorptive sensor technology provides the possibility of increased discrimination by adopting arrayed cells, but because of the strong adsorption of CWAs, disturbs quick and reversible binding on the polymer arrays.

IMS Method

The IMS instrument is most frequently used for military and civil defense missions to detect not only CWAs, but also explosives and illicit drugs (Cottingham, 2003; Eiceman and Stone, 2004; Eiceman and Karpas, 2005). The drawn air sample is ionized by a β emitter or through corona discharge under atmospheric pressure, and the ionized water-cluster molecules (reactant ion peak) react with CWA molecules in the reaction region. The generated cluster ions (positive or negative) periodically traverse through the drift region and are detected on the collector. The ion mobility depends on their charge and molecular mass over cycles of several milliseconds (Figure 60.4). CWAs are recognized according to ion mobility of the resulting peak or peaks, and semiquantified according to the ion peak height (St. Louis and Hill, 1990). In the positive ion mode, a protonated water cluster possibly combines with the targets; in the negative ion mode, a water and carbon dioxide cluster attached to oxygen possibly combines with the targets, and ammonia or organic solvents are introduced as dopants (Puton et al., 2008) into the drift region, which raises sensitivity and discriminating power. Easily ionizable nerve gases show high sensitivity, while weakly ionizable blister agents do not. Low-molecular-weight blood and choking agents do not produce characteristic cluster ions; therefore, detection sensitivity is low. Because of atmospheric pressure ionization, the devices are manufactured to be rather compact, and their response times are fast. However, the resolution of the produced target ions is low compared to MS, leading to frequent false positives by many types of compounds. To raise the sensitivity and accuracy, the ionization mechanism has been improved by using electrospray, laser desorption, matrix-assisted laser desorption, and the development of new hardware (Cottingham, 2003; Kolakowski and Mester, 2007). Field-portable instruments based on high-field asymmetric-waveform IMS and differential mobility spectrometry (Guevremont, 2004) are now commercially available (Chemring Detection Systems, 2014).

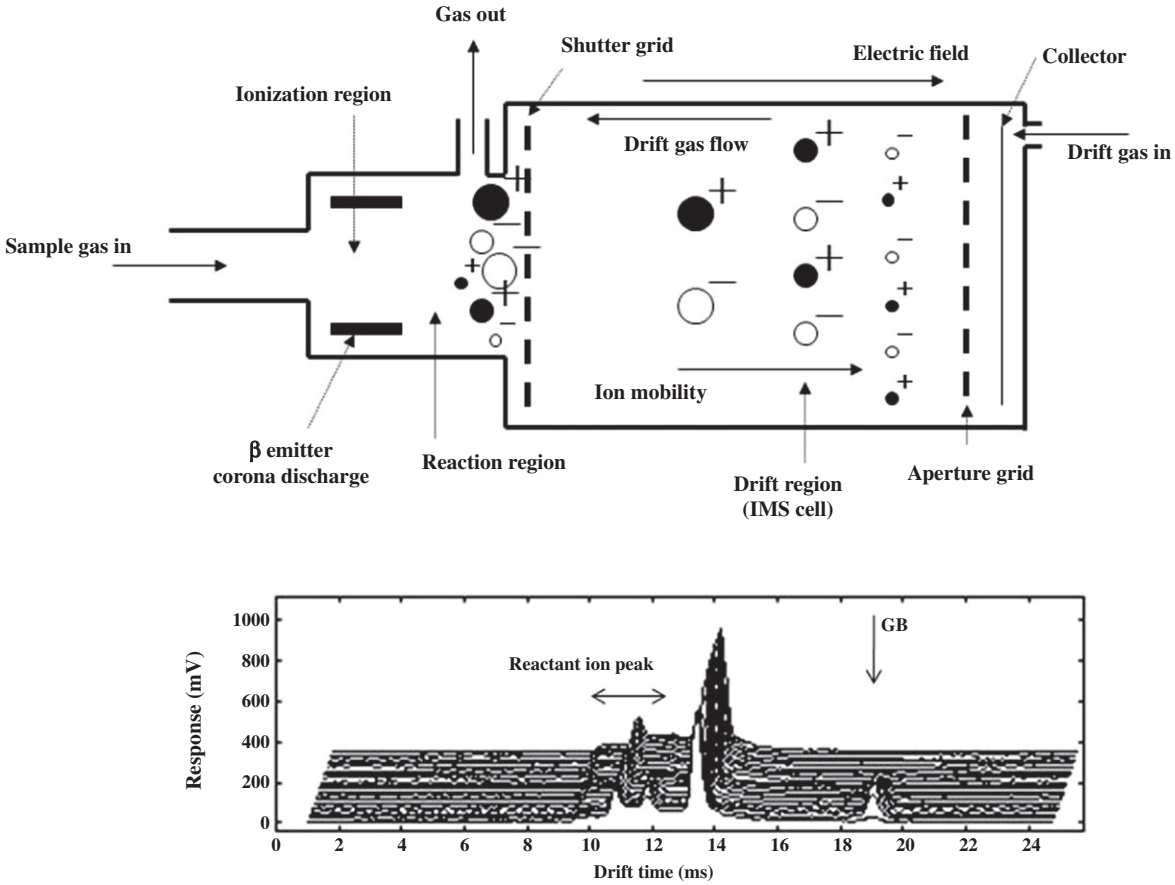

FIGURE 60.4 Ion mobility spectrometry. Detection mechanism and an exemplified spectrum. SABRE4000 (Smiths Detections Ltd, ionization ^{63}N) detected GB.

An aspiration-type device is based on discrimination by the pattern recognition of several IMS cells of different ion mobilities and polarities (Utriainen et al., 2003; Zimmermann et al., 2007). Environics Oy (Mikkeli, Finland) manufactures two types of IMS detectors. M90 (type D1-C), an aspiration-type 160 μCi ^{241}Am-bearing IMS, adopting six IMS cells and one semiconductor cell (Environics Oy, 2014), provides vapor detection of GB, GD, GA, and GF (LOA: sub-mg/m^3) as a NERVE mode alarm with a false-positive alarm against vapors of phosphorus stimulants such as dimethyl methylphosphonate. HD, L1, and HN 3 vapors (LOA: several mg/m^3) cause a BLISTER mode alarm; however, HD stimulants such as 2-chloroethyl ethyl sulfide vapor also cause a false positive alarm. AC or CK gas shows a false BLISTER alarm. CG and CL cause a BLOOD mode alarm. As shown in Tables 60.4 and 60.5, the LOA is rather low for volatile CWAs and high for gaseous CWAs, the response and recovery time is short, and the frequency of false positives is moderate (Kishi et al., 2010). The second, an aspiration-type IMS called ChemPro100 (version 6.2.5), from the same Finnish company, adopts 16 IMS cells and 1 semiconductor cell (Zimmermann et al., 2008), providing similar recognition (Maruko et al., 2006).

LCD-3.2E (Smiths Detection, 2014a), a corona discharge-type short drift tube IMS instrument, developed by Smiths Detection (London), is a useful detector showing two types of alarm: "G," for nerve gases, and H for other CWAs. Tables 60.6 and 60.7 (Sekioka et al., 2007) show the LOA, response and recovery times, and interference toward CWAs. The advanced version (LCD 3.3) provides an agent-name alarm. This instrument adapts an ammonia dopant, raising sensitivity and discriminating power. Maintenance requires that the sieve pack be frequently exchanged for proper operation. SABRE 4000, also manufactured by Smiths Detection, is a long-drift tube (high-resolution) IMS providing an agent-name alarm. As shown in Tables 60.8 and 60.9, the LOA is rather low, the response and recovery time is short, and the frequency of false positives is low (Yamaguchi et al., 2010). AIRSENSE (Schwerin, Germany) manufactures GDA2, a multisensor system consisting of an IMS, a semiconductor, a metal oxide, and a PID, enabling identification and quantification of not only CWAs, but also TICs (AIRSENSE Analytics, 2014).

TABLE 60.4 Detection Performance of Aspiration-Type IMS Detector M90-D1-C

Agent	Alarm	Limit of Alarm (mg/m^3)	Response Time (s)	Recovery Time (s)	Remark
Sarin	NERVE	0.1	7–8	4–5	
Soman	NERVE	0.1	8–9	4–6	
Tabun	NERVE	0.3	7–8	15–17	
Cyclohexylsarin	NERVE	0.1	9–10	7–12	
Dimethylmethyl-phosphonate	NERVE	7	7	43–44	False positive
Trimethylphosphate	NERVE	2.4	7–9	21–38	False positive
Triethylphosphate	NERVE	2.1	8	78–108	False positive
Diisopropylfluorophosphate	NERVE	0.1	8–9	5–8	
Mustard gas	BLISTER	2.4	8–9	4–10	
Lewisite 1	BLISTER	0.9	13–14	2	
Nitrogen mustard 1					Negative at $66\,mg/m^3$
Nitrogen mustard 2					Negative at $23\,mg/m^3$
Nitrogen mustard 3	BLISTER	13	6	68–82	
2-Chloroethylethyl-sulfide	BLISTER	1100	6–7	11–15	False positive
1,4-Thioxane	BLISTER	11000	6	49–53	False positive
2-Mercaptoethanol	BLISTER	2.2	8–9	9–23	False positive
Hydrogen cyanide	BLISTER	420	12–14	1	False alarm
Cyanogen chloride	BLISTER	9500	10–13	4–6	False alarm
Phosgene					Negative at $2{,}300\,mg/m^3$
Chlorine	BLOOD	820	9–10	7–9	
Chloropicrin	BLOOD	17000	8–93	3	

TABLE 60.5 Response of M90-D1-C Against Organic Solvents

Alarm	Compound
NERVE	Ethanol, 1-Propanol, 2-propanol, 1-Butanol, 2-Butanol, Diethyl ether, Formaldehyde, Diethylamine, Triethylamine, *N*,*N*-Dimethylformamide, Aniline, *N*,*N*-Diethylaniline, *N*-Methylaniline, *N*-Ethylaniline
BLISTER	Ethyl acetate, Chloroform, Acetic acid, Hydrogen chloride, *N*-Methylaniline, Nitromethane
Blood	None
Negative	19 solvents

Fourier Transform/Infrared Spectrometry

The FT/IR instrument detects and identifies CWAs by noninvasively and instantly measuring the infrared spectrum of the air sample (Mukhopadhyay, 2004). Considering the interference of water and carbon dioxide, their characteristic absorbance peaks in the low-wave number region are used as a specific marker. Portable FT-IR equipment is commercially available. IGA-1700 (Otsuka Electronics, Osaka, Japan) and DX-4000 (Temet, Helsinki, Finland) (Gasmet Technologies Oy, 2014) show HD ($1{,}200\,cm^{-1}$) and GB ($1{,}000\,cm^{-1}$) detection with a limit of detection (LOD) of $10\,mg/m^3$ after subtracting background water and carbon dioxide absorptions. VIR-9500 (Japan Spectroscopy Co., Tokyo) (JASCO Corporation, 2014) shows L1 detection with an LOD ($815\,cm^{-1}$) of $2\,mg/m^3$ using an 8-m light path gas cell.

Smiths Detection provides a portable FT-IR instrument, HazMatID (Smiths Detection, 2014b), which uses a diamond sensor and an extended onboard spectral library containing spectra for many organic compounds, including CWAs and suspected white powder materials. A handful of Raman spectrometer instruments are commercially available, including First Defender (Thermo Scientific, 2014) and Xantus-2 (Rigaku Corporation, 2014), which allows the quick identification of unknown solid and liquid chemicals from a vast sample library, including explosives, TICs, CWAs, white powders, and narcotics.

TABLE 60.6 Detection Performance of Corona Discharge-Type Short Drift Tube IMS Detector LCD-3.2E

Agent	Alarm	Limit of Alarm (mg/m^3)	Response Time (s)	Recovery Time (s)	Remark
Sarin	G	0.15	4–5	2–3	
Soman	G	0.15	5–8	4	
Tabun	G	0.25	10–30	3	
Dimethylmethyl-phosphonate					Negative at 2,300 mg/m^3
Trimethylphosphate	G	240	3	4	False positive
Triethylphospahte	G	110	4–5	4	False positive
Mustard gas	H	9	2–5	3	
Lewisite 1	H	3.8	5	2	
2-Chloroethylethyl-sulfide					Negative at 2,200 mg/m^3
1,4-Thioxane	G	2200	24–32	3	False positive
2-Mercaptoethanol	H	1100	3–4	4	False positive
Hydrogen cyanide	H	15	4–5	3	
Cyanogen chloride	H	400	12	4	
Chloropicrin	H	13	6–7	4	

TABLE 60.7 Response of LCD 3.2E Against Organic Solvents

Alarm	Compound
G	Trimethylphosphate, Triethylphosphate, *n*-Propanol, Diethylamine, Triethylamine, Tobacco smoke
H	2-Chloroethylethylsulfide, 1,4-Thioxane, Diethyl ether, Acetic acid
Negative	26 solvents

Gas Chromatography

The GC instrument detects CWAs by measuring the peak response appearing on the GC column (Makas and Troshkov, 2004). CWAs are distinguished on the basis of retention time and their detectability by specific detection devices (Henry, 1997). Combined with an automated air collection–thermal desorption system, GC provides a very sensitive detection (lower than $\mu g/m^3$) of nerve gases and blister agents over a 10 min cycle. O. I. Analytical, (College Station, TX), manufactures the movable GC-based MINICAMS (O.I. Analytical, 2014). GC can be hyphenated with other detection systems such as SAW (Williams and Pappas, 1999) and IMS (Buryakov, 2004; GAS, 2014), enabling it to be used to detect CWAs while having a compact body and field portability.

Mass Spectrometry

By miniaturizing the laboratory-type MS instrument and making it portable and resistant to mechanical shock, the machine can be used to detect CWAs in the field (Wise and Guerin, 1997; Smith et al., 2011a). MS provides high-resolution power, but to maintain a vacuum, the device must be large, complicating its operation. Electron ionization is usually used because of the benefit of abundant mass spectra data libraries availability (Virkki et al., 1995). On-site MS adopts a hydrophobic membrane inlet system to eliminate oxygen and nitrogen from the ionization region. The adsorbed CWAs are next introduced to the ionization region. The small molecules and strong adsorptive compounds cannot be detected by this ionization-type MS. A field-portable MS device, which has been developed for environmental TIC measurement (Horiba, Kyoto, Japan) and adopts a time-of-flight mass analyzer and pattern recognition data analysis, detects high vapor levels of GB and HD. The Riken group (Wako, Japan) and the author have developed an elemental analytical system using an electron cyclotron resonance ion source MS to detect CWAs. Phosphorus ions, chloride ions, and hydrogen cyanide fragment ions can be monitored to selectively detect nerve gases, chloride-containing CWAs, and AC and CK, respectively (Kidera et al., 2011; Urabe et al., 2014).

GC-MS technology can also be downsized for field use and is now commercially available. Inficon (East Syracuse, New York) manufactures Hapsite, a field-portable GC-MS instrument (INFICON, 2014b). Vapor is withdrawn for 30 s into the Tenax preconcentration system and thermally desorbed into a nonpolar capillary column with an elevated temperature control; the separated components are finally analyzed using an

TABLE 60.8 Detection Performance of ^{63}Ni-Ionization-Type Long Drift Tube IMS Detector SABRE 4000

Agent	Polarity	Alarm	Limit of Alarm (mg/m^3)	Response Time (s)	Remark
Sarin	+	GB	0.008	4–5	
Soman	+	GD/GF	0.02	2–5	
Cyclohexylsarin	+	GF	0.45	2	
Tabun	+	GA	0.08	4–6	
Dimethylmethyl-phosphonate			2,300		Negative
Trimethylphosphate			2,400		Negative
Triethylphosphate			2,400		Negative
Dichlorvos	–	HD/Phos	14		False positive
Mustard gas	–	HD/Phos	0.48	3	
Lewisite 1	–	Acids	19	15	
Nitrogen mustard 1	+	VERIFIC	0.044	2	
	–	HD/Phos	0.44	8	
Nitrogen mustard 2	1.2	HD/Phos	1.2	2–4	
Nitrogen mustard 3	+	HN3	0.0048	6–9	
	–	HD/Phos	1.2		
2-Chloroethylethyl-sulfide	–	HD/Phos	1.1		False positive
1,4-Thioxane	–	HCN HD/Phos	110		False positive
2-Mercaptoethanol	–	Acids	2,200		
Hydrogen cyanide	–	HCN	0.2	2–4	
Cyanogen chloride	–	HCN	5	1–2	
Phosgene	–	HD/Phos	1.5	2–4	
Chlorine					Negative at 130 mg/m^3
Chloropicrin	–	HD/Phos	0.13	4–5	

TABLE 60.9 Response of SABRE 4000 Against Organic Solvents

Alarm	Compound
HN3	Ethyl acetate
Acids	Diethylamine
Negative	26 solvents

electron ionization quadrupole mass spectrometer. GB, GD, GA, and HD are detected and identified within 12 min, with postulated LOD values of 0.2, 0.5, 8 and 0.3 μg/m^3 (Sekiguchi et al., 2006). Gaseous and weakly volatile (boiling temperature >250°C) CWAs cannot be detected. The new type of the same instrument Hapsite ER, adopting Tri-Bed TA microconcentration, provides more excellent determination against ten CWAs (Figure 60.5). Torion (American Fork, Utah) produces a field-portable GC-toroidal ion-trap MS with a solid-phase microextraction sampling inlet for CWA and related compound determination in water samples (Smith et al., 2005, 2011b; Contreras et al., 2008; Torion Technologies Inc., 2014).

Other Sensor Technologies

As for sensor technologies, chemical sensors can be used for detecting CWAs. Electrochemical sensors composed of multiwalled carbon nanotubes on indium tin oxide surfaces in connection with ferrocene–amino acid conjugates have been developed for detecting nerve gas

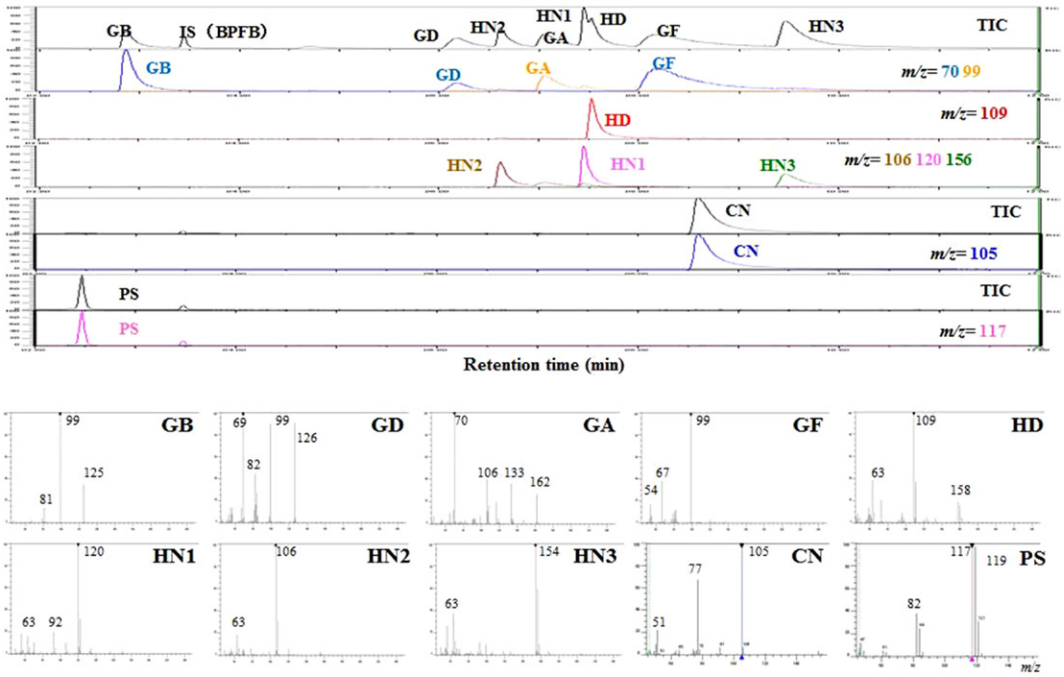

FIGURE 60.5 GC-MS data of portable apparatus, Hapsite ER. Upper: total and extracted ion chromatograms; Lower: Electron ionization mass spectra. CWAs were measured using a HAPSITE ER. GB: 34 mg/m^3; GD: 10 mg/m^3; GA: 22 mg/m^3; GF: 14 mg/m^3; HD: 2.4 mg/m^3; HN1: 6.5 mg/m^3; HN2: 12 mg/m^3; HN3: 7.4 mg/m^3; CN: 2 mg/m^3; PS 3.7 mg/m^3; IS: internal standard (BPFB). Analytical conditions: microtrap desorption, Tri-Bed TA 15 mg, 5 s at 60°C, to 180°C; column, Rtx-1MS (15 m × 0.25 mm, 1.0 µm thickness); oven: 60°C (1 min), 6°C/min to 80°C, 12°C/min to 120°C, 26°C/min to 180°C (2 min); temperatures, membrane 80°C, valve oven 70°C, probe 60°C, NEG 400°C; MS, electron ionization, m/z = 45–300, 70 eV, 300 µA, 0.79 s/scan.

degradation products and related compounds in water (Khan et al., 2008). Electrochemical sensors developed by Riken Keiki Ltd provide sensitive and specific detection of gaseous CWAs (Riken Keiki Co., Ltd, 2014), and newly developed gold nanoparticle dispersed carbon fiber electrodes to selectively detect blistering agents in corporation with Kumamoto University (Kumamoto, Japan) and our group (Matsuura et al., 2010). The field-portable instrument (SC-90, 1.9 kg) is able to detect HD vapor with a LOA of 1.5 mg/m^3.

Biosensors utilizing CWA target enzymes have been developed over the past decades (Zayats et al., 2003; Guerrieri et al., 2005; Walker and Asher, 2005). Acetylcholinesterase (AChE) is fixed on the sensor chip for nerve gas detection; by reaction with nerve gases, substrate hydrolysis velocity is lowered because of enzyme inhibition (Anzai, 2009), and the resulting electric response from a reaction, such as the formation of hydrogen peroxide by choline oxidase, can be monitored (Palleschi et al., 1992). A biosensor device utilizing organophosphorus hydrolase (Russell et al., 1999; Karnati et al., 2007) and a chemical sensor utilizing nerve gas-reactive fluorescent reagent (Zhang and Swager, 2003) have also been developed. Sanders et al. (2001) developed a unique, remote, tissue-based biosensor for the field detection of GB, GD, and HD vapors by photosynthetic fluorescence induction measurements with target Cyanobacteria (Sanders et al., 2001). However, these types of sensors are still not available for practical use. To detect AC, tyrosinase is fixed onto glassy carbon; after increasing the concentration of AC on the colloidal mineral, its inhibition is monitored by electrochemically measuring polyphenol hydrolysis (Shan et al., 2004). Nerve gases and blister agents easily hydrolyze to form characteristic compounds, and the hydrolyzed CWAs can be analyzed in wet systems utilizing µTAS capillary electrophoresis (Wang, 2004).

COMPARISON OF EXISTING ON-SITE DETECTION TECHNOLOGIES

In terms of required detection performance criteria for use by first responders, the detection sensitivity, detection accuracy, response time, recovery time, and operation are selected; results of the detection equipment examined are compared in Table 60.10. The numerical data are derived from our experiments. The evaluations are

TABLE 60.10 Comparison of Detection Performances of Various CWA Detectors

	Gaseous Agent	Nerve Gas	Blister Agent	Vomiting Agent Lachrymator	False Alarm	Response Time	Recovery Time	Operation
Gas detection tube	OK 1 mg/m^3	OK 0.3–2 mg/m^3	OK 0.01–2 mg/m^3	X	Δ	X 1–7 min	–	X
IMS aspiration	X >300 mg/m^3	OK 0.1–0.3 mg/m^3	OK 1–10 mg/m^3	X	Δ	OK 7–11 s	OK Seconds–minutes	OK Portable
IMS short drift	Δ 10–500 mg/m^3	OK 0.3 mg/m^3	OK 0.5–10 mg/m^3	Δ	Δ	OK 3–20 s	OK Seconds–minutes	OK Portable
IMS Long drift	OK 0.1–10 mg/m^3	OK 0.01–10 mg/m^3	OK 0.1–20 mg/m^3	Δ	Δ	OK 3–30 s	OK Seconds–minutes	OK Portable
FPD	X ND	OK 0.1 mg/m^3	Δ (nonAs) 1 mg/m^3	Δ	Δ	OK 2–5 s	OK Seconds	OK Portable
PID	Δ 100 mg/m^3	X 100 mg/m^3	Δ 100 mg/m^3	Δ	X	Δ 5–10 s	Δ Seconds	OK Portable
SAW	Δ 50 mg/m^3	X 50 mg/m^3	Δ 100 mg/m^3	Δ	X	Δ 5–13 s	X 4–5 min	OK Portable
FT/IR	Δ 50 mg/m^3	Δ 50 mg/m^3	Δ 50 mg/m^3	Δ	Δ	Δ Minutes	Δ –	Δ Portable
GC-FID	X	OK	OK	Δ	Δ	X 5–10 min	Δ Minutes	Δ Portable
GC with trap	X	OK	OK	Δ	OK	X	Δ	Δ Fixed
GC-MS with trap	Δ	Δ 0.1 mg/m^3	Δ 0.1 mg/m^3	Δ	OK	X 10–15 min	Δ Minutes	Δ Portable
MS EI	X	Δ	Δ	X	Δ	OK Minutes	OK Minutes	OK Portable

grouped into three categories; OK means acceptable, Δ means that improvement is required, and X means not acceptable. From our laboratory's experiments, a perfect device cannot be assigned that meets all the CWA detection requirements. Low sensitivity, false alarms, and strong adsorption of CWAs on devices are particularly serious problems. The gas detection tube system, which permits the detection of a wide range of CWA vapors, suffers from tedious operation and a slow response. The IMS-based detectors, which permit rapid and sensitive detection of nerve and blistering agents, show low sensitivity toward gaseous agents and false positives in response to some compounds. The FPD-based detector, which permits rapid and sensitive detection of nerve gases and HD, gives false negatives when exposed to nonphosphorus and sulfur CWAs. The PID-based detector shows nonspecific and low detection sensitivity. The SAW-based detector does not permit sensitive detection of CWAs. The FT-IR instrument, which permits noninvasive and constant detection, shows inadequate sensitivity. The GC-based detector with a concentration system, which permits sensitive detection of nerve gases and blister agents, suffers from troublesome operation and a slow response. The GC-MS-based detector, which permits sensitive identification of only volatile nerve gases and blister agents, suffers from difficult operation and slow response. Nonvolatile vomiting agents and lachrymators cannot be detected using any of this equipment.

DEVELOPMENT OF NEW ON-SITE DETECTION TECHNOLOGIES

Figure 60.6 shows a performance map of on-site CWA detection equipment distributed by physical properties of the agents. CWAs are represented in terms of volatility and toxicity, and the target agent territories and drawbacks of the detection equipment can be noted. This figure appears to show that the detection of gaseous agents and nonvolatile CWAs should be improved. For achieving a more sensitive and continuous monitoring of both volatile and nonvolatile CWAs, our laboratory, in coordination with Hitachi Ltd, has developed on-site detection methods utilizing counter-flow introduction

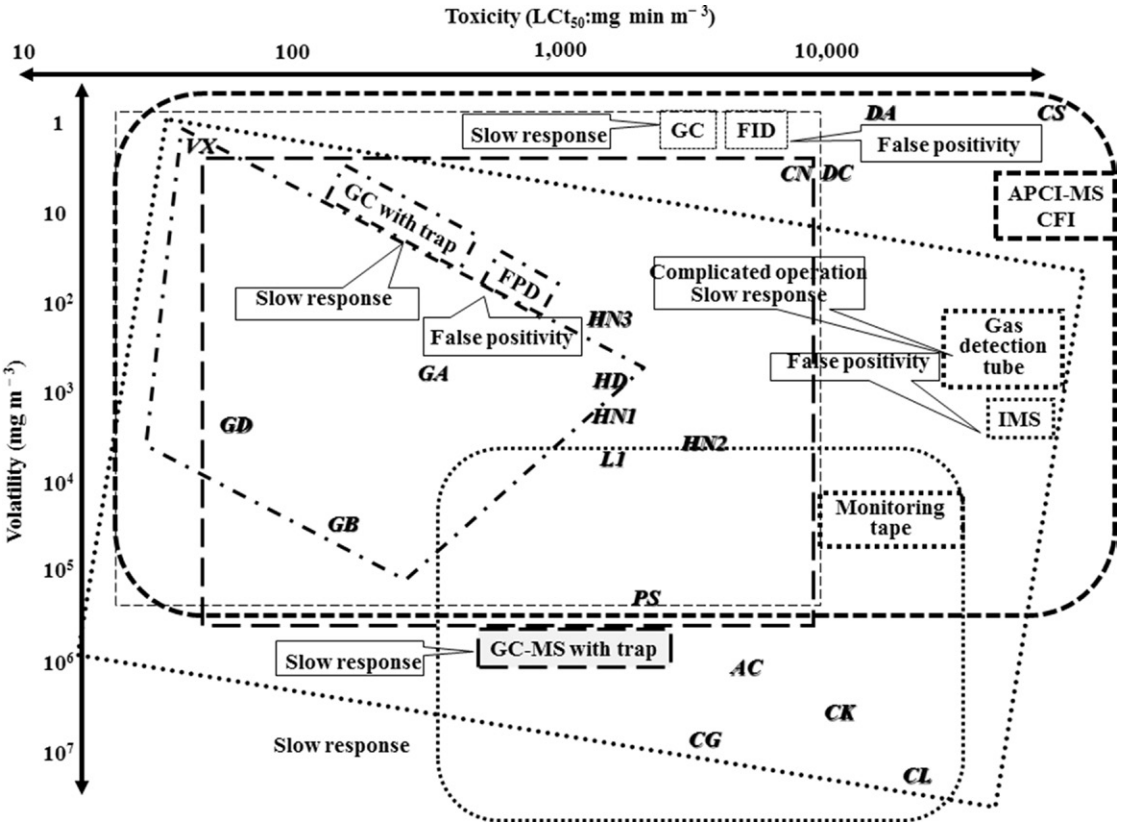

FIGURE 60.6 Performance of on-site CWA detection equipment in terms of target toxicities and volatilities. CWAs are shown in italics. The acceptable detection boundaries achieved for appropriate technologies are shown. Particular drawbacks for certain technologies are also described. *Source: Reproduced with permission from Seto et al. (2014).*

atmospheric pressure chemical ionization MS (CFI-APCI-MS; see Figure 60.7). CFI-APCI-MS technology provides soft ionization of the suctioned CWAs by corona discharge and introduction of only the produced primary target ions into the mass analyzer; the secondary interfering ions are excluded from the ionization region. The CFI-APCI technology is superior in terms of noise ion reduction, realizing ultrasensitive detection. Adopting an ion-trap mass analyzer (MS^n function), many types of nerve gases, blistering agents, vomiting agents, and lachrymators are detected within several seconds with the LOD in the sub-μg/m^3 area (Seto et al., 2013, 2014) (DS-1000, Figure 60.7).

The monitoring tape method (Nakano and Nagashima, 2001) detects hazardous gases by spectrophotometrically measuring the color change on the tape or tab impregnated with specific reagents after reacting with the suctioned air sample (Figure 60.8). The instruments using this technology, developed by Riken Keiki Ltd, are commercially available. A diffusion-type apparatus is used as the portable equipment, and by selecting the appropriate tab, the instrument can monitor the desired gases. A transmission-type apparatus is used as the fixed monitor equipment, and provides more sensitive detection. Riken Keiki and the author have developed a three tab-arrayed detector (FP-100), whereby three types of gaseous CWAs can be detected simultaneously and specifically with LODs lower than 1 mg/m^3 within 1 min. Blood agents (such as AC, CK, and arsine) and choking agents (such as CL and GC) and L1 can be detected.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A recommended on-site detection system for considering on-site CWA detection requirements and the status of present detection technologies is shown in Figure 60.9. For portable detectors utilized by first responders, a combination of IMS instruments, equipment employing the arrayed monitoring tape method, and an electrochemical sensor is desirable. This combination encompasses the detection of CWA forms from the gaseous to the volatile state. GC-MS and gas detection tubes further assist in identifying the detected CWAs. Nonvolatile CWAs are still out of the detection range of portable equipment. For movable detectors, highly sensitive detection equipment is necessary; the combination

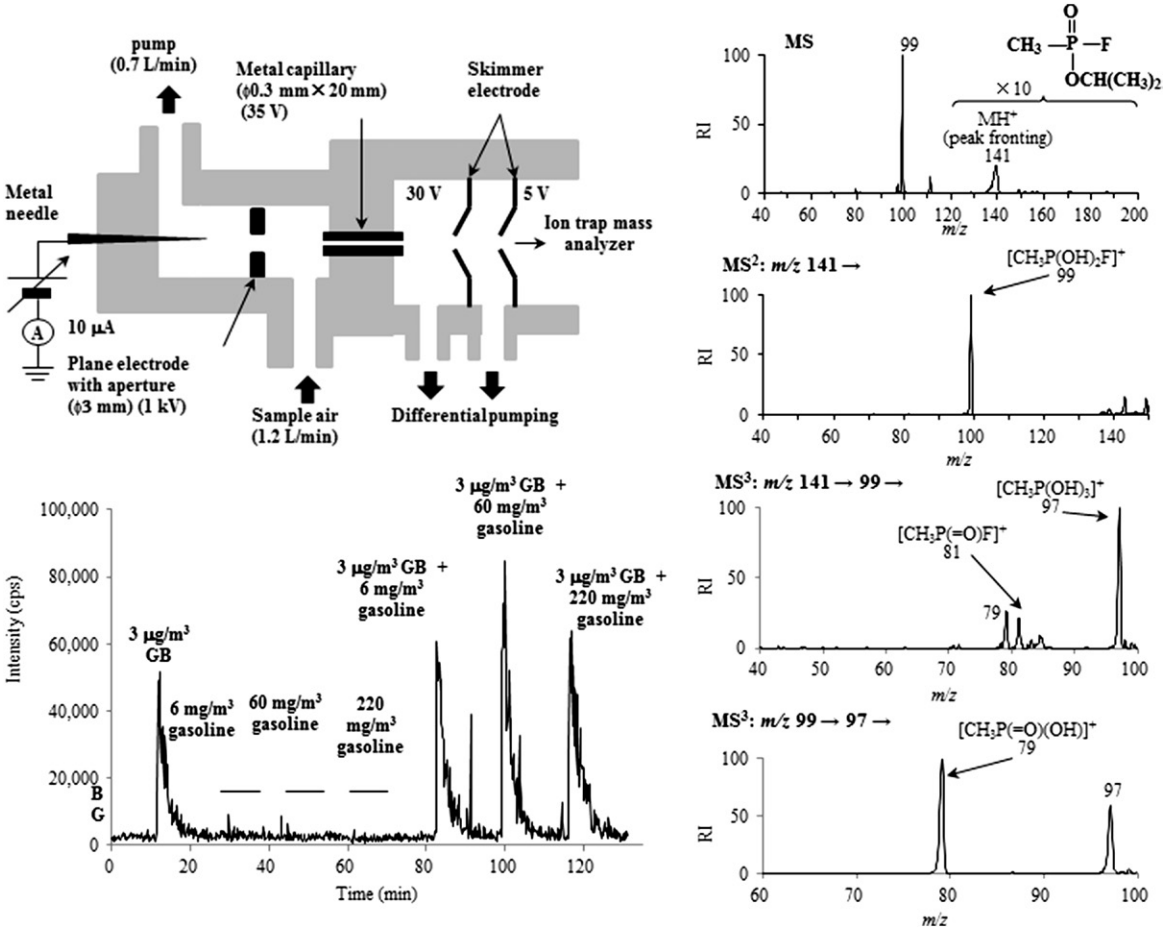

FIGURE 60.7 Counter-flow introduction/atmospheric pressure chemical ionization mass spectrometer. Left upper: structure of ionization chamber; Left lower: Signal time response for sarin by MS^3 ($MH^+ \rightarrow m/z$ 99$\rightarrow m/z$ 97) for sample air introduction with and without sarin and gasoline vapors; Right: MS^n mass spectra for sarin.

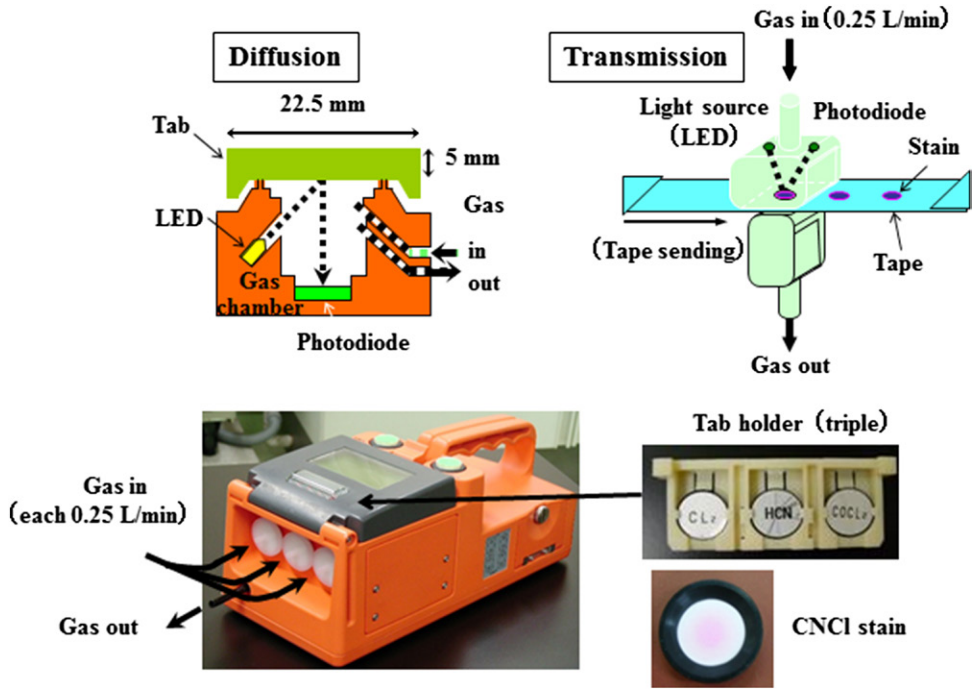

FIGURE 60.8 Monitoring tape method. Schematic detection mechanism of diffusion (upper left) and transmission (upper right) types of apparatus, and arrayed diffusion-type detector (lower left) and related tab (lower right).

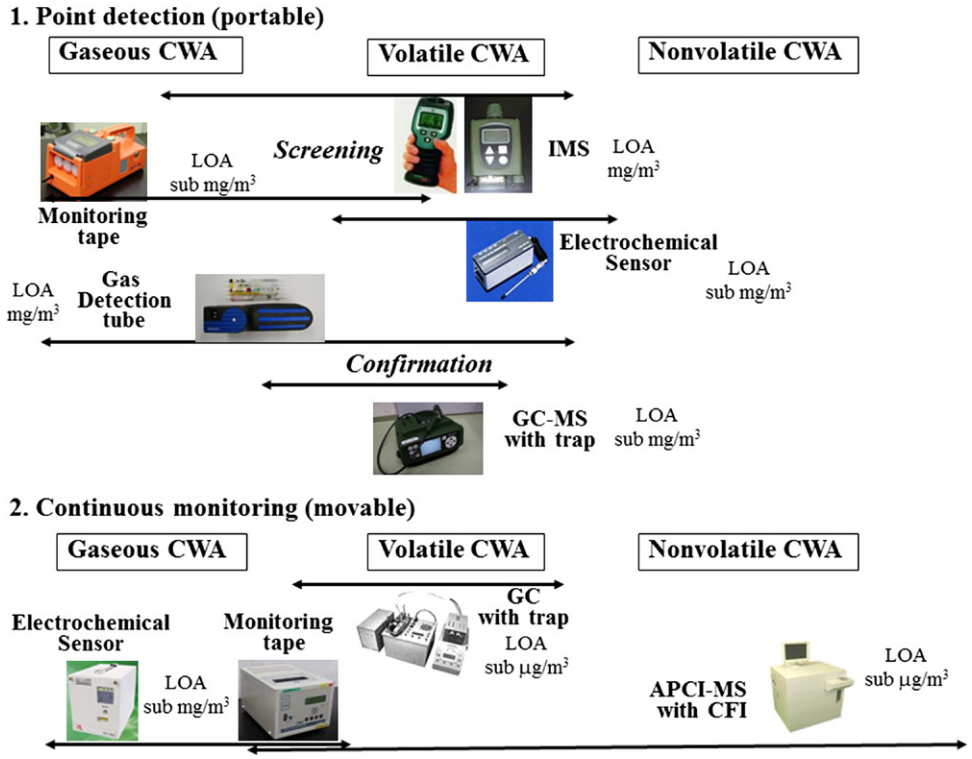

FIGURE 60.9 Recommended combinations of on-site detection equipment for (1) point detection and (2) continuous monitoring equipment for gaseous CWAs (blood agents, choking agents), volatile CWAs (nerve gases, blistering agents), and nonvolatile CWAs (vomiting agents, lachrymators). The acceptable detection boundaries for the technologies are shown, along with the LOA values.

of CFI-APCI-MS and a lined set of instruments using the transmission-type monitoring tape method or electrochemical sensor is almost ideal, handling all the CWAs with the required sensitivity. It is anticipated that new sensing technologies will be developed in the future that will overcome the problems of large size, false positives, low sensitivity, and narrow range of detection.

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CHAPTER

61

Laboratory Analysis of Chemical Warfare Agents, Adducts, and Metabolites in Biomedical Samples

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INTRODUCTION

Chemical warfare agents (CWAs) are the most toxic compounds ever produced. To develop medical countermeasures against the effects of these agents, analytical procedures to analyze these agents in biological matrices are essential for a better understanding of the toxicological process. The need for the analysis of biomedical samples can have several purposes. First, the agents themselves may be detected in their intact form in the case of toxicokinetic studies. Toxicokinetic studies provide a quantitative basis for the development of new strategies for prophylaxis and therapy against intoxication with CWAs. Second, verification of exposure to CWAs is another goal that requires analytical methodology for biomedical samples. Verification of exposure is needed for several reasons:

1. In the case of chemical warfare, military personnel who are exposed need a fast diagnosis to ascertain the level of exposure and the identity of the agent to give victims adequate treatment.
2. Low-level exposures to nerve agents might be associated with unexplainable phenomena, such as the Gulf War syndrome (CIA, 1997).
3. Despite the Chemical Weapon Convention treaty, large stockpiles of intact agents still exist, waiting for destruction. Personnel in such destruction facilities might have an elevated risk of exposure. Careful biomonitoring could minimize the risks associated with these activities.
4. In the case of a terrorist event, unambiguous verification is needed to verify the exposure. In case of an investigation of alleged use, analyses of biomedical samples can also be used as evidence in a court of justice.

In the summer of 2013, the Organisation for the Prohibition of Chemical Weapons (OPCW), commissioned by the United Nations, collected environmental and biomedical samples (UN Report, 2013). The analysis of the biomedical samples provided evidence that sarin was used in the suburbs of Damascus in August 2013. Methodologies for the verification of exposure to CWAs have been published in special issues of the *Journal of Analytical Toxicology* (Barr, 2004, 2008; Black, 2008). Methodology of the clinical methods has also been reviewed by Noort et al. (2002), Black and Noort (2007), and Capacio et al. (2008). This chapter is not meant as a duplicate for these references. It briefly discusses the analytical techniques that are needed for the analysis of biomedical samples. Most of the analytical methods still rely on chromatographic techniques like gas and liquid chromatography. The progress in instrument development on the detection site has been tremendous in the past few decades. Analyses based on mass spectrometry are now more or less routine. Single and triple quadrupole mass spectrometers have become affordable and automated standard configurations with mass spectrometers can now fulfill the analytical need for most type of analyses. In the past few years, mass spectrometric analyses further improved by providing analyses with precise and accurate masses and faster scan speeds that provide further gains in sensitivity.

Analyses of CWAs might need some additional requirements. For example, it might be necessary to

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measure an intact agent at extremely low concentrations, because only these levels are relevant in view of the high toxicity of the agents. In that case, utilizing a large-volume sample introduction should be considered. This puts higher demands on the analytical configurations because the introduction of larger sample volumes also increases the matrix effect, which puts extra demands on the selectivity of the analysis. In the special case of nerve agents, it might be desirable to distinguish the stereoisomers of these compounds from each other, which requires a rather complicated analytical configuration. In this chapter, the methods for the bioanalysis of CWAs or their biomarkers are briefly described; in a case when the instrumentation for a particular analysis is more sophisticated than a standard configuration, it is discussed in more detail.

NERVE AGENTS

Nerve agents are organophosphorus (OP) compounds that rapidly inhibit acetylcholinesterase (AChE), resulting in an accumulation of acetylcholine (ACh), leading to muscle fasciculations and paralysis, and finally resulting in death (Dacre, 1984). There are several strategies available to verify exposure to nerve agents. It is not feasible to measure the intact reagent, because the half-life of these agents is only a few hours, which means that they disappear within 1 day after exposure (Benschop and de Jong, 2001; van der Schans et al., 2008a). Metabolites, often alkyl-methylphosphonic acids, are better biomarkers because they circulate for a longer period of time and are gradually excreted in urine (Shih et al., 1994; Fredriksson et al., 1995; Nagao et al., 1997; Noort et al., 1998). Several methodologies have been published to analyze these metabolites, which were found in plasma and urine (see Table 61.1 for an overview).

Most methods for determination of these compounds are based on liquid chromatography or gas chromatography, which require derivatization (Black and Muir, 2003). Selective MS–MS techniques facilitated by triple quad instruments or ion trap instruments enable the detection of hydrolysis products to the range of pg/mL (Barr et al., 2004; Riches et al., 2005). This concentration is so low that hydrolysis products can be detected in urine up to 1 week after exposure (Riches et al., 2005). Nerve agents bind to proteins such as AChE and butyrylcholinesterase (BuChE). These proteins are not excreted or metabolized rapidly (typical half-life is 12 days for BuChE) (Hall et al., 1984), which means that adducts to proteins can serve as retrospective biomarkers for exposure to nerve agents (Fidder et al., 2002; van der Schans et al., 2004). The enzymatic measurement of AChE activity, known as the Ellman assay, is the easiest method to determine nerve agent exposure (Ellman et al., 1961; Halbrook et al., 1992). The method is based on the enzymatic cleavage of the substrate acetylthiocholine into nitrobenzoate, a yellow product that can easily be measured with a low-cost colorimeter. A major drawback of the method is that the identity of the nerve agents cannot be elucidated from this measurement. Second, the intrapersonal and interpersonal variation of the ChE activity implies that a decrease of ChE activity must be relatively large to be significant (Brock, 1991). Third, the *de novo* synthesis of the enzyme restores the enzyme activity within several days within the range of the control values. Low-level exposures to nerve agents or exposures that took place several weeks before the biosample could be taken cannot be detected using this method. Instead of looking for a decrease in AChE activity, it is more efficient to detect the ChE fraction that is inhibited by the nerve agent. In that case, it is also better to look at BuChE, which has several advantages over AChE. First, BuChE is a protein present in plasma, which is an easier sample matrix to process than whole blood. Second, the concentration of BuChE is approximately 80 nM, which is approximately 10-times higher than the concentration of AChE in blood (Myers, 1952; de Jong and Wolring, 1984). This automatically means that the concentration of that biomarker is higher and therefore easier to detect. The most straightforward method to detect the adduct of the nerve agent is the fluoride reactivation method (Polhuijs et al., 1997). During incubation of a plasma sample with fluoride ions, the nerve agent adduct is released from the protein and can be extracted in a GC-compatible solvent and subsequently analyzed with GC. The lowest detectable degree of inhibition that can be determined with this method depends on the type of GC detector. Typical reasonably priced detectors like the nitrogen phosphorus detector (NPD), flame photometric detector (FPD), and mass selective detector show absolute detection limits of approximately 1 pg, which means that a concentration of 1 ng/mL can be detected using an injection volume of 1 µL. A concentration of 1 ng/mL of nerve agent is equivalent to 5–7 nM, which corresponds with approximately 10% BuChE inhibition. To be able to determine lower degrees of inhibition, higher sample volumes have to be injected, which puts higher demands on the selectivity of the separation and detection method. Better chromatographic selectivity can be obtained by two-dimensional chromatography based on the heart-cutting method, which is discussed later (see *Analysis of Nerve Agents*). Another option is to improve the selectivity of the detector. Chemical ionization with ammonia as the reaction gas is a relatively soft ionization mode, which ensures a more selective detection of only compounds with sufficient proton affinity (Degenhardt et al., 2004; Jakubowski et al., 2004; Holland et al., 2008; Solano et al., 2008). Another more expensive but recently affordable option is to use a high-resolution R–MS or MS–MS instrument, which can

TABLE 61.1 Major Biomarkers for Nerve Agent Exposure

Agent	Matrix	Biomarker	Comment	Analytical Technique	References
Tabun	Urine	Me_2N-P(O)(OEt)OH	No stable biomarker	GC-MS-MS	Driskell et al. (2002)
Tabun	Blood	Adduct to BuChE	Fluoride reactivation	GC-NPD, FPD, MS	van der Schans et al. (2004)
Tabun	Blood	Adduct to BuChE	Peptic digest of BuChE	LC-MS-MS	Fidder et al. (2002) and Sporty et al. (2010)
Tabun	Blood	Adduct to albumin	Tyrosine adduct	LC-MS-MS	Williams et al. (2007)
Sarin	Urine	IMPA	Detected in human exposures	GC-NPD, FPD, MS; LC-MS-MS	Barr (2004), Riches et al. (2005), and Noort et al. (1998)
Sarin	Blood	Adduct to BuChE	Fluoride reactivation	GC-NPD, FPD, MS	Polhuijs et al. (1997) and Degenhardt et al. (2004)
Sarin	Blood	Adduct to BuChE	Peptic digest of BuChE	LC-MS-MS	Fidder et al. (2002) and Sporty et al. (2010)
Soman	Urine	PMPA	Detected in urine of rhesus monkeys	GC-NPD, FPD, MS; LC-MS	Riches et al. (2005)
Soman	Blood	Adduct to BuChE	Not found in human exposures because of rapid aging	GC-NPD, FPD, MS	van der Schans et al. (2004)
Soman	Blood	Adduct to BuChE	Peptic digest of BuChE	LC-MS-MS	Fidder et al. (2002) and Sporty et al. (2010)
Soman	Blood	Adduct to albumin	Tyrosine adduct	LC-MS-MS	Williams et al. (2007)
Cyclohexylsarin	Urine	ChMPA		GC-NPD, FPD, MS; LC-MS	Evans et al. (2008)
Cyclohexylsarin	Blood	Adduct to BuChE	Fluoride reactivation	GC-NPD, FPD, MS	van der Schans et al. (2004)
Cyclohexylsarin	Blood	Adduct to BuChE	Peptic digest of BuChE	LC-MS-MS	Fidder et al. (2002) and Sporty et al. (2010)
VX	Urine	EMPA		GC-NPD, FPD, MS; LC-MS-MS	Barr (2004)
VX	Blood	Adduct to BuChE	Fluoride reactivation	GC-NPD, FPD, MS	Polhuijs et al. (1997) and Degenhardt et al. (2004)
VX	Blood	Diisopropylaminoethyl-methylsulfide	Detected in serum after exposure to VX	GC-NPD	Bonierbale et al. (1997)
VX	Blood	Adduct to BuChE	Peptic digest of BuChE	LC-MS-MS	Fidder et al. (2002) and Sporty et al. (2010)
VX	Blood	Adduct to albumin	Tyrosine adduct	LC-MS-MS	Williams et al. (2007)

provide sensitivity and selectivity for the sensitive detection of low-degree inhibition (Degenhardt et al., 2004; Solano et al., 2008). When the large volume technique is utilized, low degrees of inhibition as small as 0.1% can be confirmed. A major drawback of the fluoride reactivation method is that not all nerve agent adducts are amenable to fluoride reactivation; the best-known example is the aged adduct of soman. This problem can be solved by looking at the BuChE enzyme itself. Fidder et al. (2002) published a method based on the LC–MS analysis of nerve agent phosphylated nonapeptide (FGESAGAAS) derived after pepsin digestion of inhibited BuChE. The authors presented a procedure to extract BuChE from plasma using home-fabricated procainamide gels. The phosphylated nonapeptide is best analyzed with an LC–MS–MS instrument using the single-reaction monitoring (SRM) mode. The mass of the parent ion depends on the mass of the nerve agent that is conjugated to the peptide. During the fragmentation process, the phosphyl moiety of the nerve agent is removed first, and the characteristic daughter ions are 778, 673, and 602, which are all fragments of the native peptide.

Figure 61.1 shows the mass spectrum of the nonapeptide with methylphosphonic acid adduct that resulted after exposure to a rapid aging nerve agent such as soman. This mass spectrum has been acquired on the QTOF instrument with accurate mass determination. The spectrum shows that the mass difference between the protonated parent ion and the highest fragment corresponds with the theoretical mass of methylphosphonic

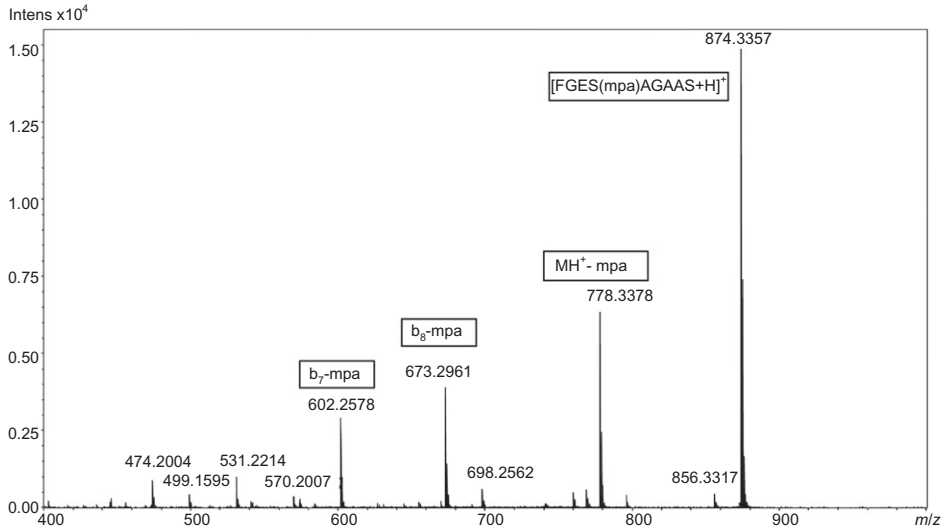

FIGURE 61.1 MS–MS spectrum of protonated ion $[MH]^+$, m/z = 874.3357, representing the nonapeptide FGESAGAAS with methylphosphonic acid adduct. The mass difference between $[MH]^+$ and "$[MH]^+$-mpa" (m/z 95.9979) is identical to the theoretical mass of methylphosphonic acid (m/z 95.9976).

acid. With this result, high-resolution mass spectrometers show their added value in unambiguous identification of CWA adducts. The analysis of the phosphylated nonapeptide and the fluoride reactivation method were both successfully applied on plasma samples that were taken from victims who had been exposed to sarin in the Tokyo subway in 1995 (Polhuijs et al., 1997; Fidder et al., 2002). In the past few years, the sample preparation procedure for the isolation of BuChE from plasma has been further improved. Sporty et al. (2010) described the use of antibody-loaded magnetic beads to isolate BuChE from plasma, which resulted in a better sample clean-up and better possibilities for automation of the method. The analysis of FGESAGAAS nonapeptide adducts is also used for the biomonitoring for exposure to other cholinesterase inhibitors. For example, Schopfer et al. (2010) used the same peptide for diagnosis of 2-(ortho-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP) exposure.

Because of the low concentration of the biomarkers, the analyses are target-directed because a full scan would be at the cost of sensitivity, which means that low-level exposures cannot be detected. However, this is a serious problem in the case when the identity of the nerve agent is not known beforehand. Some progress has been achieved to solve this problem. The number of different OPs exceeds several thousand, but the number of different masses is only 170. Because the OPCW Schedule 1 nerve agents consist only of saturated alkyl groups with mass increments of 14 units, the number of mass possibilities can be further reduced to 36 masses, which means that only 36 multiple reaction monitoring (MRM) transitions have to be recorded (van der Schans et al., 2008a). It is anticipated that the newest mass spectrometers will be able to acquire all MRM transitions, relevant to all nerve agents.

Another approach was recently published by Noort et al. (2006). The phosphylated serine residue of nerve agent–inhibited BuChE can be converted under alkaline conditions into a dehydroalanine residue that can subsequently react with a generic tag, yielding a mutual product, whatever the identity of the nerve agent. The resulting product can be detected in the most sensitive SRM mode of the mass spectrometer.

Recently, it became recognized that albumin is also a target for nerve agents but with a lower affinity than BuChE. Therefore, nerve agent adducts to albumin are also a valuable biomarker for nerve agent and pesticide exposure (Williams et al., 2007; Read et al., 2010; van der Schans et al., 2013; Sogorb et al., 2014).

Analysis of Nerve Agents

G-Agents

The requirements for the analytical equipment of the analysis of nerve agents become more sophisticated when the fate of the nerve agents are analyzed. The toxicokinetic studies create insight into the distribution and elimination of the agents and indicate when toxicologically relevant concentrations are still present in the circulation (Benschop and de Jong, 2001; van der Schans et al., 2008b). In view of the high toxicity of nerve agents, these toxicologically relevant concentrations are in the range of pg/mL. Using gas chromatography, these levels can only be measured when large-volume samples can be introduced on the column. With higher sample

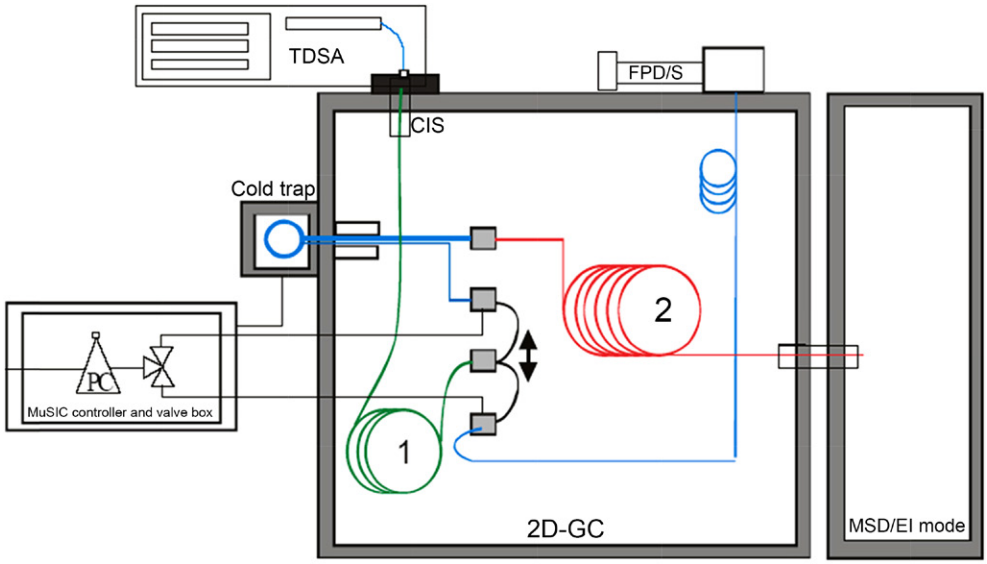

FIGURE 61.2 Large-volume (200–400 µL) thermal desorption from Tenax (300 mg, TA 60–80 mesh) followed by 2D-GC and MS detection in EI mode. (1) Precolumn. (2) Analytical column. Small part of the chromatogram of the first column is re-injected on the second analytical column. *Source: Trap and van der Schans (2007).*

volumes, the matrix effect is increased as well and higher requirements for the selectivity of the analysis are necessary. Another challenging factor is that nerve agents are chiral compounds with an asymmetrical phosphorus atom (Benschop, 1975; Benschop and de Jong, 1988). The difference in toxicity of the two isomers is several orders of magnitude, with the P(−)-isomers being most toxic (Benschop et al., 1984; Benschop and de Jong, 1988). In case of toxicokinetic studies, it is important to differentiate between the two isomers because it is essential to know which isomer is still present in the circulation. Chiral gas chromatography can fulfill this requirement for G-agents like sarin and soman. Soman has two asymmetric atoms, phosphorus and carbon, in the pinacolyl group. Therefore, the compound consists of four different stereoisomers. An optical active stationary phase like beta-cyclodextrin can be used to separate the isomers of sarin (Spruit et al., 2001). Chirasil-Val columns can be used to separate the four isomers of soman (Benschop et al., 1981, 1985). The Chirasil-Val stationary phases were synthesized at the TNO Prins Maurits Laboratory. Smith and Schlager (1996) reported the use of commercially available gamma-cyclodextrin–based columns that are also capable of separating the four isomers of soman.

In the case of toxicokinetic studies, the chiral columns were installed in a two-dimensional GC configuration according to the heart-cutting method (see Figure 61.2 for an outline). First, the introduction of large-volume samples requires additional selectivity from the chromatographic system to alleviate the matrix effect. Second, the fragile optical active phase needs to be protected from the "dirty" extracts of the biosamples and the condensating solvent in the column. Samples are introduced by thermodesorption tubes filled with TENAX material. The components are cryo-focused in a cold trap and, after a certain desorption time, flash-injected into the first column. According to the heart-cutting technique, one small section of the effluent of the first column is collected in a cold trap and then re-injected on the second analytical column with the chiral selective phase. The isomers of soman could be detected in the blood of guinea pigs at levels as small as 10 pg/mL blood (Benschop and de Jong, 1991).

Figure 61.3 shows a chromatogram of the analysis of the four isomers of soman together with one isomer of deuterated soman [d13-C(−)P(−)-soman], which was added as an internal standard.

VX

The chiral separation of VX isomers was achieved using normal phase liquid chromatography with a Chiracel OD-H column. The separation of the enantiomers of VX was first described by Kientz et al. (1994). They used a thermo-ionic detector for the selective detection of phosphorus compounds. Unfortunately, this technique was not robust enough. The same separation was also described by van der Schans et al. (2003) using electrochemical detection. The mobile phase (hexane:ethanol 95:5) was mixed after the column procedure with 0.1 M lithium perchlorate solution to ensure conductivity necessary for electrochemical detection. The system could be used to study the stereo-selective degradation of VX in *in vitro* samples such as liver homogenates and plasma. Although the electrochemical detection was

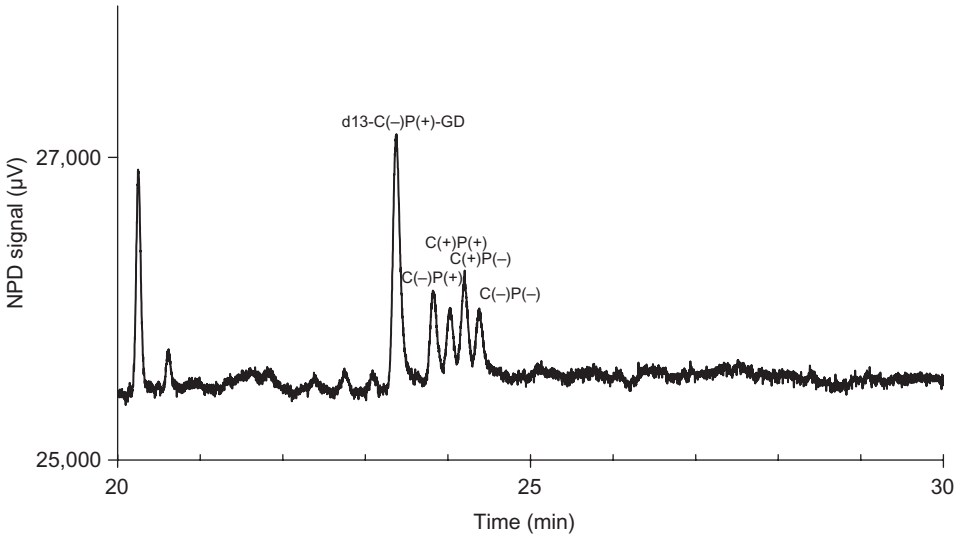

FIGURE 61.3 Typical separation of the stereoisomers of soman and internal standard. d_{13}-C(−)P(+)-soman using the 2D-GC configuration. *Source: Trap and van der Schans (2007).*

rather sensitive (absolute detection limit, 25 pg), the method was not selective enough to measure low levels of VX in extracts of biological matrices at a relevant level (<10 ng/mL). Relevant levels of VX in blood could be detected to 1 ng/mL using two-dimensional off-line LC–GC separation (van der Schans et al., 2003). Smith (2004) published the same separation using a single quadrupole mass spectrometer using atmospheric pressure chemical ionization. In that article, he focused on the separation and detection of VX to study the stereoselective degradation of VX in plasma. Reiter et al. (2008) published the chiral separation of VX isomers with gas chromatography using a HYDRODEX-β-TBDAc column. The same authors reported the chiral separation of VX isomers using LC-MS with Chiral AGP column. Using this analytical method, they were able to study the percutaneous toxicokinetics of VX in pigs.

SULFUR MUSTARD AND LEWISITE

There is a variety of biomarkers that can verify an exposure to sulfur mustard. Table 61.2 shows an overview of these biomarkers. Analogous to the nerve agents, biomarkers can be distinguished in metabolites that are excreted in urine and adducts to proteins. A disadvantage of the major metabolite, thiodiglycol, is that this compound is also present in subjects not exposed to sulfur mustard (Black and Read, 1995; Boyer et al., 2004). In that respect, the protein adducts are more reliable biomarkers for sulfur mustard exposure because these biomarkers point unambiguously to an exposure to sulfur mustard. The sulfur mustard adduct to the N-terminal valine of globin can be analyzed after a sample preparation procedure known as the modified Edman degradation (Fidder et al., 1996a,b; Noort et al., 2004a,b). Sulfur mustard also binds to cystine in albumin; this adduct can be analyzed as a tripeptide Cys-Pro-Phe with LC–MS–MS after digestion with pronase (Noort et al., 1999, 2004a,b). As an analog to the fluoride reactivation method, the sulfur mustard adduct can also be released from the protein by alkaline hydrolysis and measured as thiodiglycol. A precipitation step during the sample preparation removes endogenous thiodiglycol before it is released from the protein (Capacio et al., 2004; Lawrence et al., 2008). Unlike the nerve agents, the analyses can be target-directed and the mass spectrometer can be operated in the most sensitive SIM or SRM mode.

Table 61.2 shows that most methods rely on analytical techniques as gas and liquid chromatography combined with mass spectrometry. Most of the analyses can be performed with standard analytical configurations, without the utilization of large-volume sample introduction. The LC–MS technique is already equipped to handle sample volumes up to 100 μL, and the quality of the LC separation and tandem MS detection is normally sufficient. The concentrations of the biomarkers are normally so high that they can be detected with normal configurations. Smith et al. (2008) showed that after an accidental exposure to sulfur mustard, the albumin adduct could be measured up to several weeks after exposure. Typical detection limits using GC–MS configurations were in the pg range, which means that concentrations as small as 1 ng/mL can be detected. This level is also sufficient to detect the levels several weeks after exposure. Noort et al. (2008) demonstrated that the sulfur mustard adduct on N-terminal valine in

TABLE 61.2 Biomarkers for Sulfur Mustard Exposure

Matrix	Biomarker	Comment	Analytical Technique	References
Urine	Thiodiglycol	Compound also present in non exposed subjects (<2 ng/mL)	GC-MS	Black and Read (1988) and Riches et al.(2007)
Urine	Thiodiglycol sulfoxide	Compound also present in non exposed subjects (<10 ng/mL)	GC-MS	Black and Read (1995) and Riches et al. (2007)
Urine	1,1′-sulfonylbis[2-*S*-(*N*-acetylcysteinyl)ethane]	Minor excretion product in humans	GC-MS	Black et al. (1991)
Blood	Hemoglobin adduct, N-terminal valine	Detected in human samples	GC-MS	Fidder et al. (1996a,b) and Noort et al. (2004a,b)
Blood	Hemoglobin adduct, histidine N-HETE adduct	Detected in human samples	LC-MS	Black et al. (1997)
Blood	Albumin adduct, cysteine adduct	Digestion with pronase	LC-MS/MS	Noort et al. (1999, 2004a,b)
Blood	Albumin adduct	Detected as thiodigylcol after alkaline hydrolysis	GC-MS	Capacio et al. (2004) and Lawrence et al. (2008)
Blood/skin	DNA adduct		Immunoslotblot assay, LC-MS	Fidder et al. (1996a,b), Benschop et al. (1997), and van der Schans et al. (2004)

TABLE 61.3 Biomarkers for Lewisite Exposure

Matrix	Biomarker	Comment	Analytical Technique	References
Urine	Chlorovinylarsonous acid (CVAA)		GC-MS	Wooten et al. (2002)
Blood	CVAA bound to hemoglobin		GC-MS	Fidder et al. (2000)

marmosets could be measured up to 4 weeks after exposure using GC–NICI–MS. However, in the case of toxicokinetic studies of sulfur mustard, typical concentrations at 10–100 pg/mL in blood were found and could not be measured with a normal GC–MS configuration. The utilization of large-volume sample introduction by thermodesorption and two-dimensional chromatography (analog to the configuration for chiral nerve agent analysis) enabled the detection of sulfur mustard levels as small as 10 pg/mL (Oostdijk et al., 2007). In addition to the methods based on instrumental analysis, sulfur mustard exposure can also be detected using immunoassays. Antibodies that can recognize DNA adducts have been raised. In an immune slotblot assay, exposures to sulfur mustard could be verified in DNA obtained from blood or skin (Benschop et al., 1997; van der Schans et al., 2004). The number of methods to verify an exposure to lewisite is limited. Chlorovinylarsonous acid can be found as the main metabolite in urine, and this compound can also be found as an adduct to hemoglobin (Table 61.3). The analyte can be analyzed with GC–MS using normal configurations.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The development of sophisticated analytical instruments, mainly based on mass spectrometry, enabled several analyses for bioanalysis of CWAs. Toxicokinetic studies at relevant levels (as small as 10 pg/mL blood) can now be performed. Exposures to CWAs can be verified up to several weeks after exposure because of the persistence of the biomarkers and also because of sensitive and selective instrumentation. It is anticipated that future equipment will be even more sensitive, enabling exposures that occurred weeks after the event to be discovered. Better sensitivity is also desirable to meet the criteria that are in place for identification of a compound meeting the forensic standards. Normally, identification by two techniques is needed. If these techniques are not available, then special requirements for the MS analyses are demanded. A link with the doping control analysis can be made that accepts that three ions in an SIM or SRM are needed, provided that the retention time is within a certain time window (Black, 2008). In case of analyses with tandem MS detection, two transitions are also sufficient for forensic identification. When instrumentation becomes more sensitive, it is possible that these requirements can be met. The new mass spectrometers, based on time of flight or Orbitrap, offer high-resolution mass determination that will make the identification more accurate and therefore more reliable. Criteria for their added value in unambiguous identification of CWAs and related compounds are still undergoing development, however. The newest developed mass spectrometers will offer higher scan speeds allowing the possibility

to acquire many transitions at the same time without losing sensitivity. Using that configuration, it will be possible to perform generic analyses without previous knowledge of the identity of the nerve agent.

Another interesting development is comprehensive GC–MS. Comprehensive GC offers great selectivity and resolution and is ideal for complex samples such as biomedical samples. Its combination with time-of-flight mass spectrometry offers the possibility to analyze in full-scan mode, not target-directed, with the availability of a full mass spectrum that will meet the forensic standards. The utility has been demonstrated for the detection of CWAs in complicated matrices such as fuel (Reichenbach et al., 2003). The method has also been used for the analysis of regenerated sarin in inhibited plasma (van der Meer et al., 2010). Finally, it may be expected that the sample preparation will be more or less automated. Recently, a promising result was published by Carol-Visser et al. (2008), who described the digestion and analysis of sulfur mustard adducts in albumin and the sarin adduct to BuChE.

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CHAPTER

62

Biosensors for the Detection of OP Nerve Agents

Jun-ichi Anzai

INTRODUCTION

Organophosphorus (OP) pesticides and nerve agents have been detected based on various principles, including spectroscopy, chromatography, microgravimetry, and electrical and electrochemical techniques. Among the devices used for detecting OP agents, electrochemical devices (such as modified electrodes) are relatively inexpensive, small, and easy to operate. One of the characteristic features of electrochemical devices is that the function and performance of electrodes can be arbitrarily regulated by modifying the surface with functional molecules, proteins, DNA, and other substances. Thus, one can develop electrochemical devices that can be used for detecting chemicals in sample solutions and in the gas phase. The modified electrodes whose surface is modified with proteins and other biological molecules are often called biosensors. For this reason, modified electrodes or biosensors have been widely used for detecting a variety of analytes, including OP agents. So far, the majority of biosensors have been developed not for environmental and toxicological purposes, but for biomedical or clinical use; this is because of the large market for the latter. A typical example of a biomedical biosensor is a biosensor used for monitoring glucose levels in the blood of diabetics. This chapter describes recent progress in the development of electrochemical biosensors and related devices for detecting OP nerve agents and pesticides.

BIOSENSORS

It is well established that electrochemical techniques are useful for determining ions and molecules in solution and gas phases. Oxidizable and reducible chemical species dissolved in solution can be detected by measuring oxidation or reduction current that is produced upon electrolysis of the species on the surface of electrodes. Usually, the output current of the system depends on the concentration of redox species in the solution. Thus, one can quantitatively determine the concentration of the analyte. Another merit of electrochemical techniques is easy identification of an analyte by recording its redox potential because the redox species can be characterized by its own potential. In other words, an appropriate electric potential has to be applied to the electrode to electrochemically oxidize or reduce specific ions or molecules for the purpose of obtaining output signals from the electrochemical reaction. For example, hydrogen peroxide (H_2O_2) is known to be oxidized according to reaction (1) (Scheme 62.1) on the surface of metal or carbon electrode set at 0.4–0.6 V. This current value often depends on the type of electrode material and on the solution conditions, while no oxidation current can be observed at electrode potentials below this value because H_2O_2 cannot be oxidized at lower electrode potentials. Thus, one can identify the redox species based on the redox potential applied to the electrode and quantitatively determine the concentration from the magnitude of the output current.

However, this is true only for the sample solutions which contain a single component, H_2O_2 in this case. The electrochemical response is more complicated if sample solutions contain two or more redox species because both species would be oxidized or reduced, and thus they contribute concurrently to the output current. This is often the case for the electrochemical determination of drugs and metabolites or other biological components in blood because many kinds of redox-active species are intrinsically contaminated in blood. Ascorbic acid (vitamin C) and uric acid (UA) are redox species found in blood, and these compounds often disturb electrochemical measurements of blood because the redox potential

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$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad O_2 \qquad (1)$$

SCHEME 62.1 Electro-oxidation of H_2O_2.

of vitamin C and UA overlaps with those of many kinds of drugs and biological components (Anzai et al., 1998; Hoshi et al., 2001). Another drawback of electrochemical measurements is that target analytes are often electrochemically inert, so no electric signal can be obtained with an electrode. In other words, the redox potential of many target molecules is too high to be oxidized or reduced in the reasonable potential range. The operation of electrochemical systems in high electrode potentials is impractical because the oxidation of H_2O and reduction of dissolved O_2 often disturb the measurements. For these reasons, analytes and samples to which electrochemical protocol can be successfully applied by using unmodified electrodes are rather limited.

Electrochemical biosensors are devices that are fabricated by combining proteins or other biological molecules and electrodes (Ramanavicius et al., 2006; Xu et al., 2006; Yun et al., 2007). Usually, functional proteins are immobilized on the surface of metal or carbon electrodes to effect selective determination of analytes in sample solution. Figure 62.1 schematically shows the concept and structure of electrochemical biosensors, where the surface of the electrode is modified with biomaterial that can selectively bind target analyte in a sample solution contaminated with many interfering substances. It is also possible to replace electrodes with other transducers, such as thermistors and optical devices, to prepare calorimetric and optical sensors. Among the biosensors developed so far, enzyme-based biosensors have been most extensively studied due to their high sensitivity and wide applicability in biomedical and environmental analyses. Enzyme-catalyzed reactions occur on the surface of electrode and the reaction products are oxidized or reduced electrochemically, resulting in generation of electric current as an output signal. Thus, chemical signals (i.e., the type of analyte and its concentration) can selectively be converted into electric signals through the enzyme/electrode interface. For example, the principle of operation of glucose biosensors is based on the glucose oxidase (GOx)–catalyzed oxidation reaction of glucose (reaction (2)) and electrochemical oxidation of reaction product of the enzymatic reaction (H_2O_2) (reaction (3)) (Scheme 62.2). It is clear that 2 mol of electron can be produced from 1 mol of glucose, and the concentration of glucose in the sample solution is related to the recorded electric current. It should be noted here that oxidation or reduction potential of glucose is too high to be oxidized or reduced directly on unmodified electrodes. Consequently, electrochemical determination of glucose is not possible without using GOx-modified

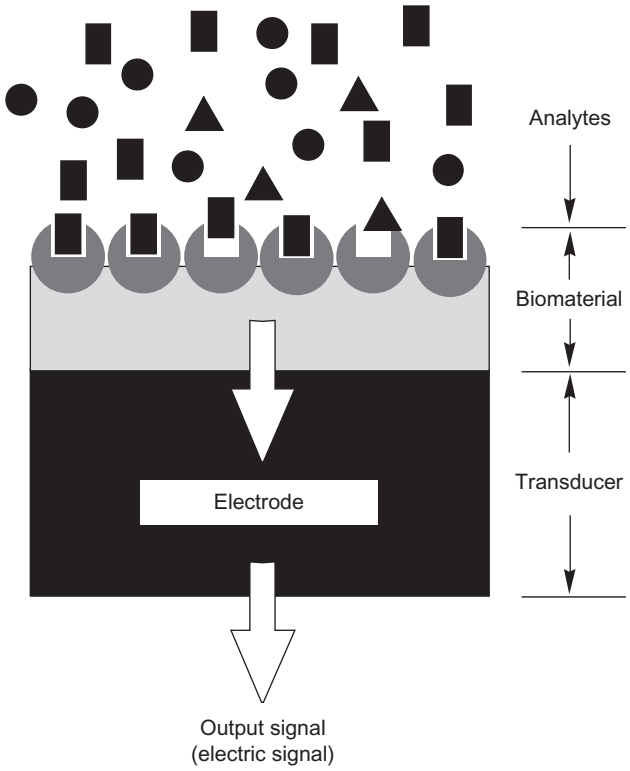


FIGURE 62.1 A schematic illustration of the structure of biosensors.

<Enzyme reaction>

$$\text{Glucose} + O_2 \longrightarrow \text{Gluconolactone} + H_2O_2 \qquad (2)$$

<Electrochemical reaction>

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \qquad (3)$$

SCHEME 62.2 Enzyme and electrochemical reactions in a glucose biosensor.

electrodes (or GOx biosensors). It is clear from reactions (2) and (3) that the role of enzymes in the function of biosensors is to catalytically produce redox-active species that can be electrolyzed by the electrode.

One of the merits of an enzyme biosensor is its versatility. The selectivity of biosensors directly relates to the type of enzyme used for constructing biosensors. Up to now, many kinds of enzyme sensors have been reported, other than glucose biosensors, using a variety of enzymes. A miniaturization of the sensor body is another possible advantage of enzyme sensors. In fact, miniature sensors sized in microns have been prepared

for the purpose of detecting target analyte in a single cell. The miniaturization of the sensor body would make it possible to construct a sensor array on a single tip. However, one should keep in mind that the output current decreases as the size of the electrode decreases, resulting in a suppressed signal-to-noise ratio. For routine purposes, therefore, biosensors of pencil size are usually used for easy handling. A small disk of metal or carbon material (3–5 mm in diameter) is often mounted in an insulating plastic rod (about 10 mm in diameter and 50–100 mm in length) to prepare disk electrodes.

Figure 62.2 illustrates an experimental setup for biosensor measurements. Electrochemical measurements using biosensors require three electrodes, which are called the *working electrode (enzyme-modified electrode)*, *reference electrode*, and *counter electrode.* In some cases, the three electrodes can be assembled into a single-body electrode. It is also possible to eliminate the reference electrode and use the counter electrode as a pseudo-reference electrode for specific reasons, such as miniaturization. The experimental setup shown in Figure 62.2 is a batch system, in which the electrodes are immersed in the sample solution to obtain an output signal. For constructing flow systems, the electrodes are set at a suitable place in the fluidic device, which is often used to measure a large number of samples.

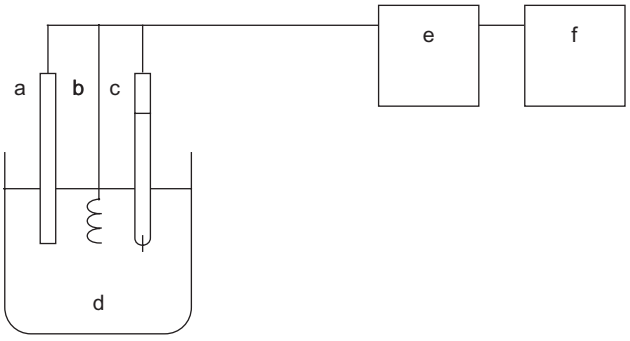


FIGURE 62.2 An experimental setup for the operation of electrochemical biosensors. (a) Biosensor, (b) counter electrode, (c) reference electrode, (d) sample solution, (e) potentiostat, and (f) recorder.

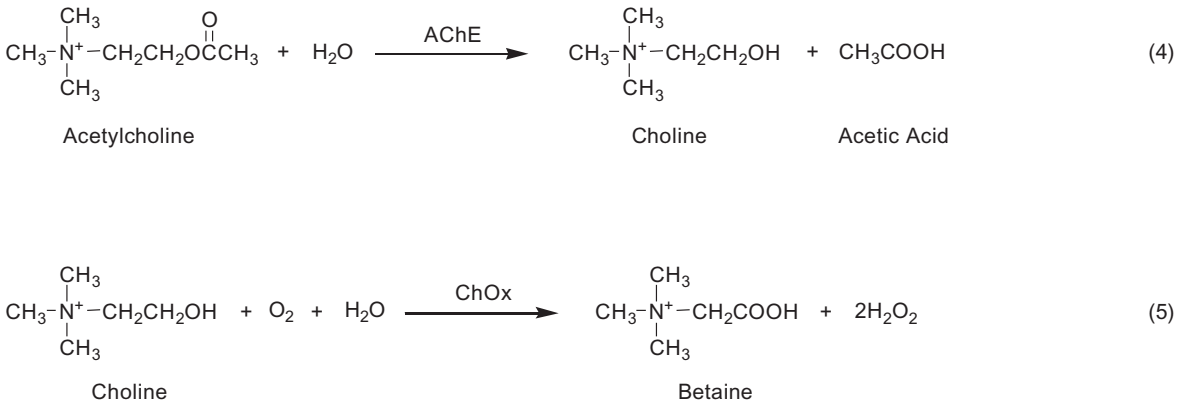
In the next section, recent studies on the determination of OP nerve agents and pesticides based on electrochemical biosensors will be discussed. The electrochemical biosensors used for detecting OP compounds can be divided into three types, depending on the type of enzymes used for constructing biosensors: (i) choline esterase (ChE)–ChOx bienzyme-modified biosensors, (ii) ChE-modified biosensors, and (iii) organophosphorus hydrolase (OPH)–modified biosensors. Also, OP biosensors based on transducers other than electrochemical devices are discussed in this chapter.

ELECTROCHEMICAL BIOSENSORS FOR DETECTING OP COMPOUNDS

ChE–ChOx Bienzyme-Modified OP Biosensors

The primary objective of ChE–ChOx bienzyme biosensors was to determine neurotransmitter acetylcholine (ACh) in biological samples (Chen et al., 1998). The redox potential of ACh is too high to be determined directly using an electrochemical reaction. For this purpose, acetylcholinesterase (AChE) was employed as ChE. AChE and ChOx were immobilized on the surface of the electrode to attain electrochemical analysis according to reactions (4) and (5) (Scheme 62.3).

The enzymatic reactions of AChE–ChOx bienzyme biosensor consist of AChE-catalyzed hydrolysis of ACh into choline and acetic acid (reaction (4)) and oxidation reaction of choline catalyzed by ChOx (reaction (5)). The latter reaction produces H_2O_2, which can be electrochemically oxidized on the electrode to generate output signal depending on the concentration of AChE. It is also possible to use an oxygen electrode in place of a metal or carbon electrode because ChOx consumes dissolved O_2 during the oxidation of choline. Therefore,



SCHEME 62.3 Enzyme reactions in AChE–ChOx bienzyme sensors.

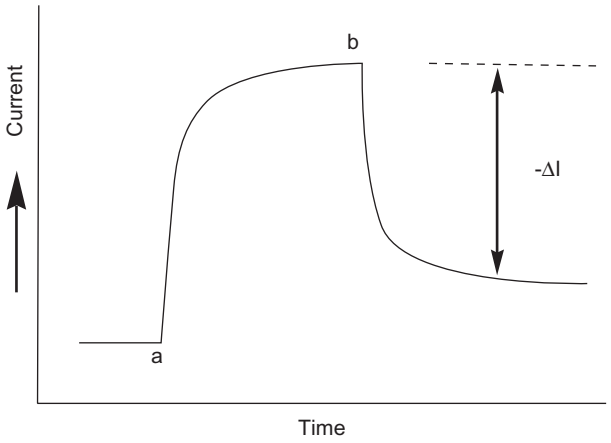


FIGURE 62.3 A typical response of inhibition-mode AChE–ChOx bienzyme OP sensors. (a) Addition of AChE and (b) addition of OP sample. – ΔI corresponds to the output signal.

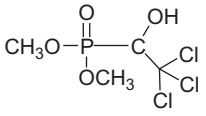


FIGURE 62.4 The chemical structure of trichlorfon.

the O_2 consumption can be detected using a commercially available O_2 electrode. The reason why AChE–ChOx bienzyme biosensors can be used for detecting OP compounds is that the catalytic activity of AChE is inhibited by OP compounds. OP compounds are known to irreversibly bind to AChE to disturb its catalytic activity. It is thus clear that reaction (4) is suppressed, in part depending on the concentration of OP, and the rate of choline production becomes lower in the presence of OP compounds, resulting in reduced response in the output current of the sensor. In fact, the change in output current of the sensor upon addition of an OP-containing sample is detected in the presence of a known concentration of ACh, as illustrated in Figure 62.3. The ΔI value depends on the concentration of OP compounds in the sample solution. Butyrylcholinesterase (BChE) is sometimes used in place of AChE, in which butyrylcholine (BCh) is used as a substrate.

According to this protocol, we have determined trichlorfon (Figure 62.4) in water samples using AChE–ChOx biosensors, which were constructed by a layer-by-layer deposition of the enzymes on the surface of a platinum disk electrode (Shi et al., 2005). The response of the sensor to trichlorfon was evaluated by a successive addition of trichlorfon into the solution of 2 mM of ACh. The output current of the sensor decreased as the concentration of trichlorfon increased over the concentration range of 1×10^{-8} to 2×10^{-5} g/mL. The lower detection limit of the sensor was found to be about 1×10^{-9} g/mL (1 ppb). A key point for constructing high-performance OP sensors in bienzyme systems may be to suitably control the ratio of the catalytic activity of AChE and ChOx in the sensor. In the present case, the catalytic activity of ChOx should be higher than that of AChE; the overall rate of the reactions is determined by the rate of the AChE-catalyzed reaction because OP compounds disturb this step. Therefore, excess amounts of ChOx usually are mixed with AChE, and the ChOx–AChE mixture is immobilized on the surface of the electrode to make the AChE-catalyzed reaction a rate-limiting step. In this situation, the overall reaction rate can be determined by the rate of the ChE-catalyzed reaction because the subsequent electrochemical oxidation of H_2O_2 is sufficiently fast. On the other hand, in our sensors, ChE and ChOx were immobilized separately, layer by layer, for optimization of the amounts of enzymes.

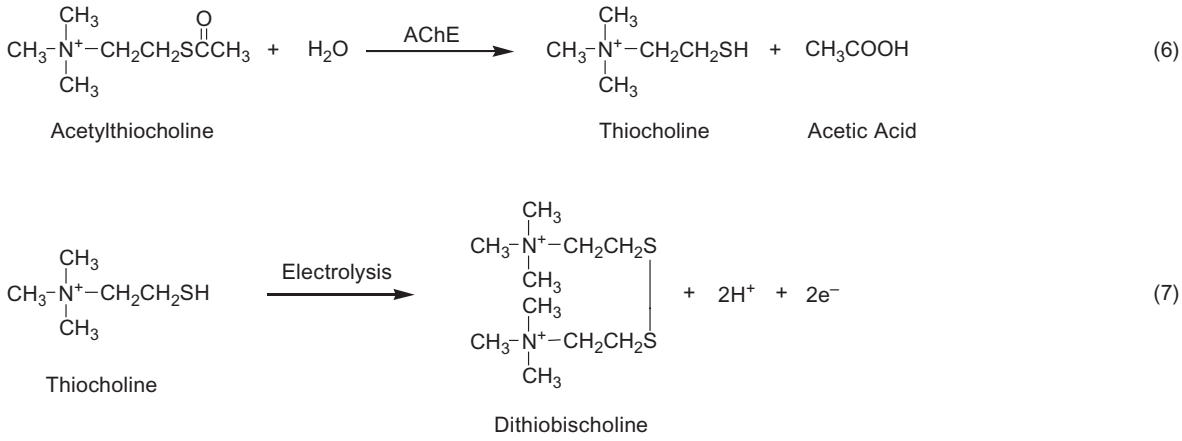
TABLE 62.1 AChE-ChOx Bienzyme Biosensors for Detecting OP Compounds

Sensor	OP Compound (Lower Detection Limit)	Reference
Dendrimer-coated	Dichlorvos (5 pM)	Snedarkova et al. (2003)
Gold electrode	Carbofuran (50 pM)	
Screen-printed	Methyl parathion (50 nM)	Lin et al. (2004)
Carbon electrode		
Gold electrode	Trichlorfon (4 pM)	Shi et al. (2005)

ChE–ChOx bienzyme-modified biosensors have been widely used, mainly for detecting OP pesticides, which are summarized in Table 62.1. AChE and BChE are often used as a ChE enzyme. This type of ChE–ChOx sensor can be a convenient way to detect OP compounds, and detection time for qualitative analysis is reasonably fast. For quantitative determination of OP compounds, however, operation of the sensor is somewhat time-consuming because multiple steps (a measurement of the response to AChE and inhibition) are required.

AChE-Modified OP Biosensors

As described in the previous section, the use of a bienzyme system sometimes induces complexity in the design of biosensors originating from different catalytic activity of the enzymes. In the ChE–ChOx systems, ChOx is used to oxidize choline that is generated through ChE-catalyzed reactions because the oxidation potential of choline is too high to be electrochemically directly oxidized. It is reasonable to assume that if the reaction products can be oxidized on the electrode directly, one can remove the ChOx enzyme from the sensor design, and a ChE-modified electrode may be



SCHEME 62.4 Enzyme and electrochemical reactions in acetylthiocholine-based sensors.

TABLE 62.2 AChE Biosensors Using Acetylthiocholine

Sensor	OP Compound (Lower Detection Limit)	Reference
Screen-printed	Paraoxon (19 nM)	Andreescu et al. (2002)
Carbon electrode	Chlorpyrifos ethyl oxon (1 nM)	
Screen-printed	Carbaryl (55 nM)	Carlo et al. (2004)
Carbon electrode	Methyl parathion (100 nM)	
CNT electrode	Paraoxon (0.4 pM)	Liu and Lin (2006)
Screen-printed	Sarin gas (0.1 mg/m^3)	Arduini et al. (2007)
Carbon electrode[a]	Sarin in solution (12 ppb)	
(Prussian blue)	VX in solution (14 ppb)	

[a]*BChE was used in place of AChE.*

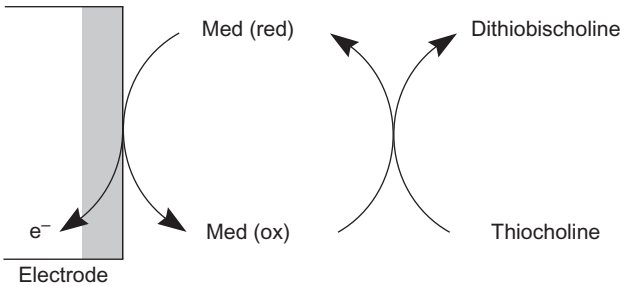


FIGURE 62.5 An electrochemical oxidation of thiocholine through electron transfer mediator. Med (red) and Med (ox) represent electrode transfer mediator in reduced and oxidized forms.

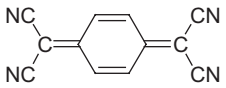


FIGURE 62.6 The chemical structure of TCNQ.

used for detecting OP compounds. This concept was tested using an AChE-modified electrode as sensor in the presence of acetylthiocholine in place of AChE as the substrate of AChE (Scheme 62.4). It was found that acetylthiocholine decomposes into thiocholine and acetic acid (reaction (6)), and the resulting thiocholine can be electrochemically oxidized to its dimer (dithiobischoline) on the surface of electrode (reaction (7)). Therefore, the output current of the sensor in the solution of a constant concentration of acetylthiocholine would depend on OP compounds in the solution. Thus, one can determine the OP concentration from the decrease in the output current, as shown in Figure 62.3.

Table 62.2 collects some properties of AChE-modified biosensors based on acetylthiocholine. As shown in reaction (7), thiocholine can be directly oxidized to dithiocholine. However, the oxidation potential of thiocholine is relatively high (approximately + 0.7 V), and thus a high background current and possible interference from oxidizable contaminants cannot be excluded. To circumvent this, electron transfer mediators (Med) are often used to oxidize thiocholine at milder potentials, as depicted in Figure 62.5, where thiocholine is oxidized into dithiobischoline by an oxidized form of the mediator, Med (ox); and the resulting reduced form of the mediator, Med (red), is oxidized at the electrode to generate the current output. In this scheme, 1 mol of thiocholine generates 1 mol of electron. For example, Carlo and coworkers modified the surface of a screen-printed carbon electrode with a thin layer of perfluorinated ion-exchanger film containing 7,7,8,8-tetracyanoquinodimethane (TCNQ) (Figure 62.6) as Med. It was found that the reduced form of TCNQ is oxidized at + 0.4 V, as opposed to the pseudo reference electrode (Ag wire; Carlo et al., 2004). The TCNQ-based biosensors were applied to a recovery test using egg, bovine meat, milk,

and honey as food matrices. No false negative or false positive samples were detected in the assay. These results indicate that TCNQ-based biosensors may be useful as a prescreening device in the analysis of food safety.

Another recent topic in the detection of OP compounds based on ChE-modified biosensors is to use carbon nanotubes (CNTs) as an electrode modifier. CNTs are known to exhibit excellent electrochemical properties originating from high catalytic activity of edge-plane-like graphite sites at the ends, as well as a large effective surface area. Lin et al. (2004) have reported that stable CNT-modified glassy carbon (GC) electrodes can be prepared by coating the surface of GC electrodes with a suspension of CNT, and they can be used for sensitive determination of thiocholine (Liu et al., 2005). Voltammetric studies have demonstrated that thiocholine is oxidized at + 0.15–0.20 V on CNT-modified GC electrodes, as compared to its higher oxidation potential of unmodified GC (+0.7 V), carbon paste (+ 0.6 V), and gold electrodes (+0.9 V). A lower detection limit of 5×10^{-6} M thiocholine was obtained under the optimal batch conditions, while the detection limit was further reduced to 3×10^{-7} M by the electrochemical detection in flow injection analysis.

The same authors further improved the CNT-modified biosensors for detection of OP compounds based on a layer-by-layer immobilization of CNTs, polymer, and AChE on the surface of GC electrodes (Liu and Lin, 2006). AChE was sandwiched with poly(diallyldimethylammonium chloride) layers on the surface of CNTs. The OP biosensor developed in this way was used to detect paraoxon (Figure 62.7) at levels as low as 4×10^{-13} M with a 6 min response time in flow injection analysis. The high stability of the sensors was another merit of this biosensor; no deterioration in the response was observed after 1 week of continuous use. Only a 15% decrease in the activity of the enzyme was observed after 3 weeks, although the authors recommended recalibration after 2 weeks. The high stability of the biosensors was ascribed to the assembling of enzymes in the sandwichlike layer structure, providing a favorable microenvironment to maintain the catalytic activity of the enzyme.

The effects of organic solvents on the performance of ChE-modified sensors were studied because OP compounds are sometimes extracted from samples using organic solvents. For this purpose, 4-aminophenyl acetate (Figure 62.8) was used as the substrate of the AChE enzyme in place of acetylthiocholine because of its high solubility in organic solvents and the redox-active nature of 4-aminophenol produced through enzymatic reaction (Scheme 62.5). Marty and coworkers prepared an AChE-modified carbon electrode by coating AChE-containing polymer film on the electrode (Andreescu et al., 2002). 4-Aminophenol was found to be oxidized on the electrode at + 0.1 V, while no redox response was observed in the range of 0–0.4 V for 4-aminophenyl acetate. An AChE enzyme modified on the electrode retained about 80% catalytic activity even in the aqueous solution containing 1–5% acetonitrile or ethanol, although more than 20% organic solvent induced nearly complete deactivation of the enzyme. This suggested that the sensor could be used in the presence of a small amount of water-miscible organic solvent in aqueous media. In fact, the AChE-modified sensor could be successfully used for determining the 10^{-9}–10^{-8} M level of OP compounds in the aqueous solutions containing 1–5% acetonitrile and ethanol.

Model OP compounds are usually used for studying the detection of nerve agents with biosensors because of the difficulty of working with nerve agents. Recently, ChE-modified disposable electrodes have been used for detecting sarin and *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX; Figure 62.9) in solution and in air. Arduini and coworkers have tested ChE inhibition in sarin and VX standard solution and

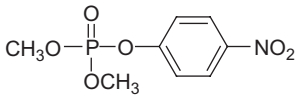


FIGURE 62.7 The chemical structure of paraoxon.

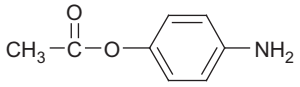


FIGURE 62.8 The chemical structure of 4-aminophenyl acetate.

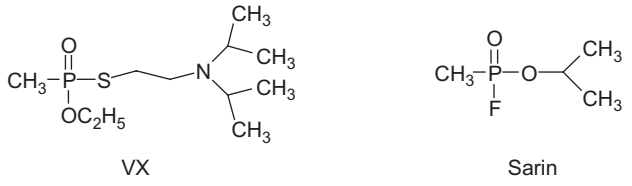
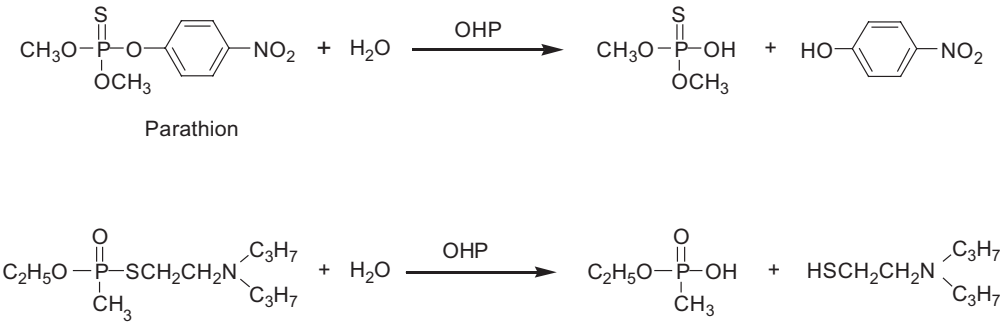


FIGURE 62.9 The chemical structures of VX and sarin.

SCHEME 62.5 Hydrolysis reaction of 4-aminophenyl acetate by AChE.



SCHEME 62.6 OPH-catalyzed hydrolysis of parathion and VX.

found lower detection limits of 12 and 14 ppb, respectively (Arduini et al., 2007). For detecting sarin gas in air, the surface of the sensor (which had been covered with a small amount of 0.05 M phosphate buffer) was exposed to a sarin stream for 30 s, and then ChE inhibition of the sensor was evaluated in solution. The sensor was found to be effective to detect sarin gas at a concentration of 0.1 mg/m^3. Thus, low concentrations of sarin gas can be detected in a few minutes using ChE-modified electrochemical biosensors.

OPH-Modified OP Biosensors

The detection of OP compounds by biosensors modified with ChE–ChOx or ChE enzymes relies on an inhibition of catalytic activity of ChE enzymes by OPs. Therefore, it is a drawback of the sensors that the magnitude of the output signal correlates inversely with the concentration of OP compounds in the sample. The protocol in measurements is somewhat complicated because the substrate of ChE has to be added in the sample solution before measurements; as a result, multiple steps are needed for OP detection. In addition, inhibition of ChE by OP compounds is usually irreversible, so reactivation of ChE activity is required with repeated use of the sensors, although this problem can be circumvented by using disposable sensor tips.

In contrast to the inhibition mode of detection in these biosensors, a direct detection of OP compounds has been proposed by means of OPH enzymes, which hydrolyze OP compounds as shown in Scheme 62.6. OPH enzymes have a rather wide spectrum of substrate selectivity and hydrolyze many kinds of OP compounds, including OP pesticides, VX, and sarin. It is obvious from the reactions shown in Scheme 62.6 that the reaction products such as 4-nitrophenol and 2-(diisopropylamino)ethanethiol can be electrochemically oxidized on the electrode to generate electric current as an output signal. Thus, OPH-modified electrodes directly detect OP compounds in a single step, as compared to the two steps required for biosensors constructed using ChE–ChOx and ChE enzymes.

FIGURE 62.10 The chemical structures of 2-(diethylamino)ethanethiol and 2-(dimethylamino)ethanethiol.

Mulchandani et al. (1999) prepared V-type nerve agent biosensors by immobilizing OPH on the surface of a CNT-modified carbon electrode (Joshi et al., 2006). They used a mutant OPH that exhibits a 20-fold greater catalytic activity toward demeton-*S* (a frequently used mimic of V-type nerve agents) than does wild-type OPH. The OP biosensors prepared in this way exhibited an electrochemical response to demeton-*S* at + 0.4 V, the higher and lower detection limits being 8.5×10^{-5} and 1×10^{-6} M, respectively, and with a sensitivity of 8×10^{-6} A/mM. A high response of the biosensors to the hydrolysis product of RVX (2-(diethylamino)ethanethiol; shown in Figure 62.10) and mimic of the hydrolysis product of VX (2-(dimethylamino)-ethanethiol; also shown in Figure 62.10) was also demonstrated, suggesting that they could be used for detecting both VX and RVX. An advantage of this sensor is its high selectivity to demeton-*S* over many possible interfering compounds belonging to OP and carbamate families, which excludes false positives in the field use of the biosensors. This biosensor was successfully applied to analyze lake water spiked with demeton-*S*. The same group developed a flow injection electrochemical detection system using an OPH-modified gold electrode. The response of the system depended linearly on the concentration of OP compounds over the range of 1×10^{-6} to 1×10^{-5} M, with a lower detection limit of 1×10^{-7} M. The authors suggested a possible use of the flow injection system for rapid screening of OP compounds (Wang et al., 1999).

Wang and coworkers prepared OPH-modified carbon electrodes as a submersible biosensor for the purpose of remote monitoring of OP compounds (Wang et al.,

1999). The biosensor mounted in a plastic tube was connected to a 50-foot shielding cable via environmentally sealed rubber connectors. It was found that, despite the 50-foot cable, the noise level of the sensor in the response to paraoxon and methyl parathion was very low. The authors suggested a possible use of the sensor for remote sensing of OP warfare agents in the field. The same group recently reported an interesting electrochemical/optical route for detection of OP nerve agents based on OPH-mediated biometallization (Arribas et al., 2005). They found that the formation of cupric-ferrocyanide (CuFeCN) particles was accelerated by nitrophenol produced through OPH hydrolysis of OP agents. Therefore, the rate of formation of CuFeCN nanoparticles (NPs) depended on the concentration of OP agents. The detection of CuFeCN particles can be carried out by either electrochemistry or optical measurement. CuFeCN is known to be redox-active, and CuFeCN deposited on the surface of a carbon electrode exhibits an oxidation peak at about + 0.7 V in its voltammogram, peak current being dependent on the concentration. Optical detection of OPs is also possible based on the color changes of the CuFeCN solutions. A light yellow color was observed in the absence of OP, while the solution became darker (orange-red) upon addition of OP due to the formation of a CuFeCN colloid. It is a merit of this system that no seed is required for CuFeCN biometallization, in contrast to seed-induced growth of gold NPs (as discussed previously).

Potentiometric OP biosensors were also prepared by coupling OPH and a glass pH electrode. The sensors were constructed by immobilizing OPH on the surface of a pH-sensitive layer of a commercially available pH electrode by crosslinking with bovine serum albumin (BSA) to form a thin film. This biosensor detects pH changes (or changes in electrode potential) originating from hydrolysis reaction of OP compounds catalyzed by OPH in the OPH-BSA film (Mulchandani et al., 1999). The best sensitivity of the sensor was obtained in the operation in a pH 8.5, 1 mM buffer, in which the sensor could detect OPs, including paraoxon, ethyl parathion, and methyl parathion, at levels as low as 2×10^{-6} M. In a similar mechanism of signal transduction, one can use pH-sensitive field effect transistors (FETs) to develop enzyme-modified FETs. Use of a FET provides superiority in miniaturization of the sensor body because a FET is usually produced through mass production on semiconductor materials (Simonian et al., 2004).

An electrochemical OP sensor by the nonenzymatic route was reported based on chemical modification of the surface of a gold electrode with ferrocene derivative (Fc). For this purpose, the gold electrode was modified with a dithioFc derivative to form an aminoFc-monolayer-modified electrode (Khan et al., 2007). The principle of operation of the aminoFc-modified electrode for OP sensing is that chloro- or cyano-substituted OP compounds covalently bind to aminoFc moieties, by which the redox potential of the surface-confined Fc can be altered. In fact, shifts of approximately 110 and 60 mV in the redox potential were observed, suggesting a possible use of the sensors for detecting OPs from the potential shifts.

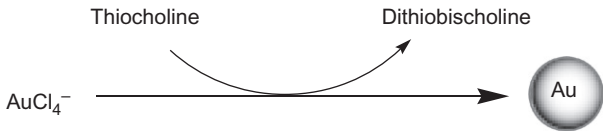


FIGURE 62.11 A thiocholine-induced growth of Au NPs.

MISCELLANEOUS BIOSENSORS FOR OP DETECTION

Different types of transducers other than electrodes have also been used to construct OP biosensors. Recent progress in the study of metal and semiconductor NPs prompted researchers to apply these materials to detect OP compounds. Willner and coworkers found that thiocholine-stimulated growth of gold NPs is inhibited by OP nerve agents depending on their concentration, enabling the NPs to be used for detecting OP compounds (Figure 62.11; Pavlov et al., 2005). A visible absorption of gold NP solution at about 550 nm originating from the plasmon absorption band of NPs increased as a result of NP growth in the presence of AChE and acetylthiocholine; this occurred because enzymatically produced thiocholine promotes the growth of NPs. In the presence of 1×10^{-8} to 1×10^{-6} M of OP compounds in the solution, however, the growth rate was significantly suppressed. NP seeds were confined on the surface of a glass plate for constructing an optical sensor system and the absorbance of the glass plate was monitored in the presence of OP agents. The NP-confined glass plate exhibited a maximum absorption at about 570 nm, and the intensity and wavelength clearly depended on the concentration of OP agent in the sample solution. The optical sensor can detect the 10^{-6} M level of OP compounds. Another merit of the NP-confined glass plate is the fact that the glass plate yields a blue-colored surface without OP agents, while the OP agents generate a weak, pale pink color, enabling OP compounds to be detected with the naked eye.

Polymer microbeads were also used for constructing an OP detection system using the fluorescence response of the microbeads. Carboxylate-functionalized polymer microbeads that had been covered with a polyvinylpyridine (PVP) layer were modified with fluorescamine (FLA). When the microbeads were exposed to the vapor

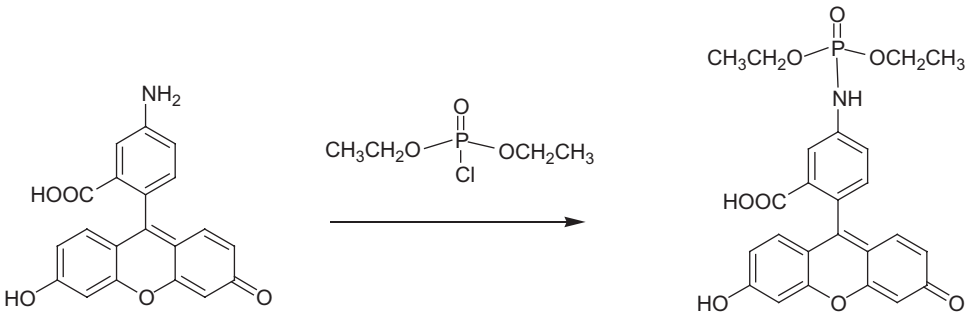


FIGURE 62.12 An enhanced fluorescence upon binding of an OP compound to FLA.

of diethyl chlorophosphate as a model OP, the fluorescence of the microbeads increased rapidly as a result of formation of a phosphoramide of FLA (Figure 62.12; Bencic-Nagale et al., 2006). A PVP layer on the surface of the microbeads served as an acceptor of hydrochloric acid (HCl) generated from phosphoramide formation. A potential use of the microbeads for constructing a sensor array was suggested for screening OP vapor.

Microgravimetry has often been used for studying mass changes on the surface using a quartz-crystal microbalance (QCM). A probe of QCM is a thin, vibrating quartz resonator sandwiched between metal electrodes. The QCM detects adsorption of substance on the surface of the quartz resonator as changes in resonance frequency of the resonator. Thus, one can detect a nanogram level of mass changes on the surface. It is a great merit of QCM that it can be operated in air for detecting adsorption of gases, as well as in solution for detecting dissolved analytes. For this reason, QCM is quite suitable for detecting nerve agents in air. Jiang and coworkers prepared a polymer film-coated quartz resonator for detecting dimethyl methylphosphonate (DMMP) vapor as a stimulant of nerve agents. The polyvinylidene fluoride (PVDF) film-coated quartz resonator was found to be sensitive to DMMP vapor at 5–60 ppm with a rapid and reversible response (Ying et al., 2007). A zeolite film was also utilized as a coating of QCM for detecting DMMP (Xie et al., 2005).

The progress in the development of microelectromechanical systems accelerated the use of microdevices for constructing biosensors. Microcantilevers (MCLs) have been chemically modified to use as sensitive probes for detecting OP compounds. It is known that adsorption of molecules on the surface of an MCL induces it to bend due to changes in surface tension, depending on the amounts of mass adsorbed, in both gas and solution phases. Usually, one side of the MCLs is modified with functional materials to effectively induce them to bend upon binding of analytes. Recently, the surface of V-shaped silicon MCLs, whose dimensions were 180 μm in length, 25 μm in width, and 1 μm in thickness, was modified with thin films containing the OPH enzyme for preparing OP biosensors (Karnati et al., 2007). The MCL-based sensors exhibited a bending response to paraoxon with a dynamic range of 10^{-7}–10^{-3} M. The conformational changes of OPH enzyme associated with the catalytic reaction are suggested to be a main factor inducing the MCL bending. It was also possible to construct MCL-based OP sensors using organic polymers and metal oxides as surface coating in place of the OPH enzyme. In these cases, adsorption OP compounds on the surface of MCLs induced bending, depending on the concentration of OPs, resulting in detection of DMMP as a model OP in air (Voiculescu et al., 2005; Zhao et al., 2006).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This chapter described recent developments of biosensors for detecting OP nerve agents. Different detection modes using various enzymes are applicable for constructing OP biosensors using electrochemical principles. Among them, the use of the OPH enzyme is superior to other protocols due to the fact that OPH-based biosensors directly detect OP compounds in a single step, as compared to the inhibition-mode biosensors based on ChE–ChOx and ChE enzymes which require a two-step operation. A merit of electrochemical biosensors in OP detection is that a portable device can easily be prepared for in-field analysis. The easy operation of biosensors compared to chromatographic or spectroscopic measurement is another advantage. Therefore, biosensors have high potential for fast on-site screening of OP nerve agents.

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CHAPTER

63

Neuropathy Target Esterase as a Biomarker and Biosensor of Delayed Neuropathic Agents

Rudy J. Richardson, R. Mark Worden, Sanjeeva J. Wijeyesakere, Nichole D. Hein, John K. Fink and Galina F. Makhaeva

INTRODUCTION

Delayed neuropathic (DN) agents represent a new functional class of potential organophosphorus (OP) threats that have heretofore not been used in warfare or terrorist acts. Nevertheless, it is vital to develop effective defenses against them for three reasons. First, these materials can readily be produced by rather straightforward modifications of the structures of conventional nerve agents. Second, warning signs and symptoms of toxicity may be lacking until 1–4 weeks after exposure, when the permanent sensory deficits and paralysis associated with OP compound–induced delayed neurotoxicity (OPIDN) develop. Third, there are no established means of preventing or treating OPIDN. This chapter begins with a review of current understanding about the chemistry and mechanism of action of DN agents. It then describes how existing knowledge can be applied to generate biomarkers and biosensors for discriminating between conventional and DN agents. It ends by indicating future work needed to address the potential threat of DN agents.

OP COMPOUNDS

Conventional Nerve Agents Versus DN Agents

Conventional nerve agents, such as sarin and soman, are OP compounds of pentavalent phosphorus that are well known as threats in the context of warfare and terrorism (Sidell and Borak, 1992). These substances produce acute cholinergic toxicity and death via inhibition of acetylcholinesterase (AChE) in the central and peripheral nervous systems (Marrs et al., 1996).

In contrast to conventional nerve agents, DN agents produce permanent neurological dysfunction in the form of OPIDN rather than cholinergic toxicity and death (Richardson et al., 2013; Richardson and Makhaeva, 2014). Like conventional nerve agents, DN agents are OP compounds of pentavalent phosphorus that are readily synthesized, but they are designed to inactivate neuropathy target esterase (neurotoxic esterase (NTE)) with preference to AChE (Wu and Casida, 1995; Makhaeva et al., 2012, 2013). Because of this selectivity, DN agents may elicit little or no warning signs of acute cholinergic toxicity, so that victims of DN agents might not know they have been exposed until OPIDN develops 1–4 weeks later.

Cholinergic toxicity from OP compounds can be elicited solely by inhibition of AChE (Richardson, 2010). However, the AChE–OP conjugate can undergo an additional reaction ("aging") entailing loss of an OP ligand to yield a negatively charged phosphoryl adduct on the active site serine. Although the aging reaction has practical implications for therapy, it does not change the qualitative nature of the toxicity (Figure 63.1).

In contrast to the situation with AChE inhibitors and cholinergic toxicity, inhibition of NTE on its own is insufficient to produce OPIDN. Inhibition is the obligatory first step, but this must be followed by aging of the NTE–OP conjugate to trigger axonal degeneration

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(Figure 63.2) (Moser et al., 2012; Richardson et al., 2013; Richardson and Makhaeva, 2014).

Thus, DN agents are distinct from conventional nerve agents in several respects. Furthermore, especially in view of their ease of synthesis, absence of initial signs or symptoms of exposure, and lack of prophylactic or therapeutic measures, it is conceivable that rogue nations or terrorist groups might consider DN agents attractive as weapons of permanent incapacitation against military and/or civilian populations. Therefore, part of an effective chemical defense strategy is to develop methods for detecting DN agents and distinguishing them from conventional nerve agents. This can be performed by exploiting the sensitivity and selectivity afforded by appropriate biomarkers and biosensors (Makhaeva et al., 2003; Malygin et al., 2003; Kohli et al., 2007a, 2010, 2014; Sigolaeva et al., 2010, 2013; Srivastava et al., 2010).

OP Compounds of Pentavalent Versus Trivalent Phosphorus

In this chapter, DN agents are defined as OP compounds of pentavalent phosphorus that produce OPIDN. Examples shown in Figure 63.3 are

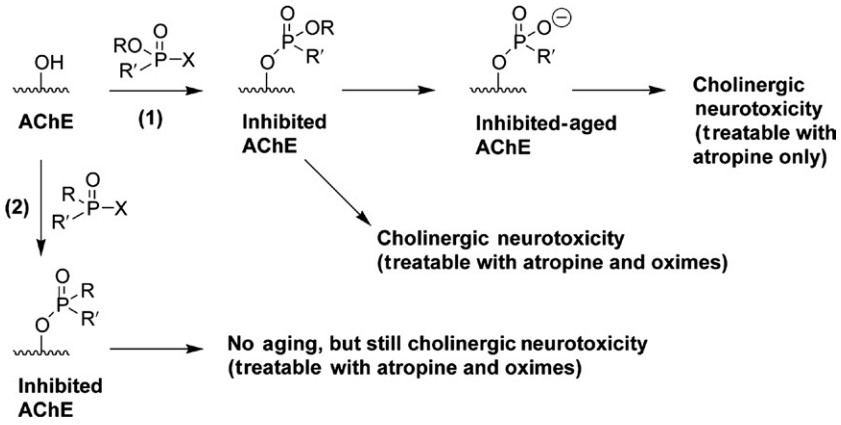

FIGURE 63.1 Reaction of AChE with an organophosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited and aged enzyme. Inhibition alone produces cholinergic toxicity that is treatable with both atropine to counteract excess acetylcholine at muscarinic acetylcholine receptors and oximes to reactivate AChE. Aging does not change the type of toxicity, but it renders the enzyme intractable to reactivation, so that treatment with oximes becomes ineffective. Reaction of AChE with an organophosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme and produces cholinergic toxicity, but because of the stability of the C—P bonds linking the R- and R′ groups to phosphorus, the phosphinyl moiety on AChE does not undergo aging, so that both atropine and oximes can be used in therapy. R, R′ = substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X = primary leaving group (e.g., fluoride). *Source: Reproduced with permission from Richardson and Makhaeva (2014).*

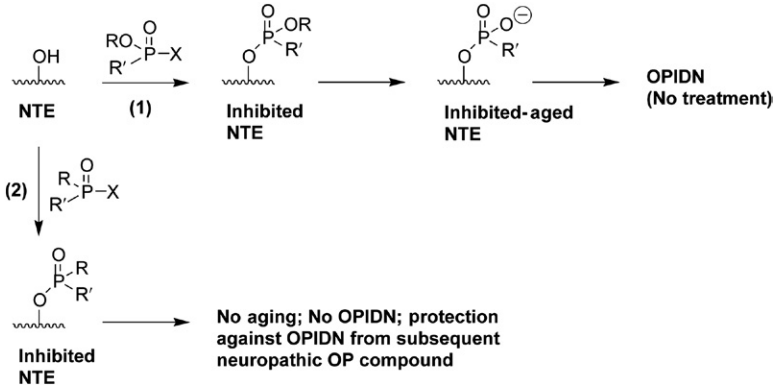

FIGURE 63.2 Reaction of NTE with an organophosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited and aged enzyme. Inhibition alone does not produce OPIDN; both inhibition and aging are required, and there is no treatment for the neuropathy. Reaction of NTE with an organophosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme, but because of the stability of the C—P bonds linking the R- and R′-groups to phosphorus the phosphinyl moiety on NTE does not undergo aging, so that OPIDN does not occur. Inhibition of NTE with a nonaging inhibitor is not biologically inert—it protects against subsequent exposures to neuropathic (ageable) NTE inhibitors. R, R′ = substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X = primary leaving group (e.g., fluoride). *Source: Reproduced with permission from Richardson and Makhaeva (2014).*

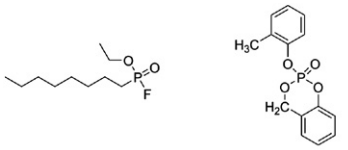

FIGURE 63.3 Examples of DN agents containing pentavalent phosphorus: EOPF and 2-(2-methylphenoxy)-4*H*-l,3,2-benzodioxaphosphorin-2-oxide (2-(2-methylphenoxy)-BDPO or CBDP), the active metabolite of the archetypal DN agent, TOCP (Wu and Casida, 1995).

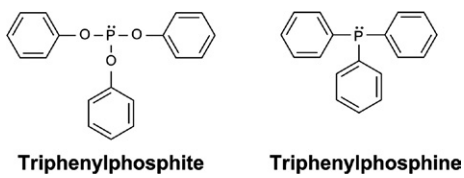

FIGURE 63.4 Examples of neuropathic OP compounds containing trivalent phosphorus: triphenylphosphite and triphenylphosphine (Eto, 1974).

ethyl *n*-octylphosphonofluoridate (EOPF) and 2-(2-methylphenoxy)-4*H*-l,3,2-benzodioxaphosphorin-2-oxide, abbreviated as (2-(2-methylphenoxy)-BDPO) or CBDP, the active metabolite of the archetypal DN agent tri-*o*-cresyl phosphate (TOCP) (Wu and Casida, 1995).

However, there are also OP compounds of trivalent phosphorus that produce neurological injury. Examples shown in Figure 63.4 are triphenylphosphite and triphenylphosphine. Comparatively little is known about the neuropathic trivalent OP compounds, but the clinical course and spatial–temporal pattern of lesions that they produce vary from OPIDN (Lehning et al., 1996), and the mechanisms of action of these compounds appear to differ from that of the DN agents. For example, triphenylphosphite produces a syndrome of delayed axonopathy and other neurodegenerative changes that could represent a variant of OPIDN, possibly via inhibition/aging of NTE (Padilla et al., 1987; Tanaka et al., 1990; Abou-Donia, 1992; Fioroni et al., 1995; Carlson and Ehrich, 2004). However, although triphenylphosphite inhibits NTE *in vitro* and *in vivo*, the nature of the adduct formed with NTE has not been elucidated, and the aging reaction of triphenylphosphite-inhibited NTE has apparently not been studied. Triphenylphosphine is another trivalent OP compound that produces delayed axonopathy, but its pathogenesis and mechanism appear to differ from those of triphenylphosphite or neuropathic OP compounds of pentavalent phosphorus. Among other differences, triphenylphosphine does not inhibit NTE *in vitro* or *in vivo* (Davis et al., 1999; Padilla et al., 1987). The remainder of this chapter deals exclusively with OP compounds of pentavalent phosphorus.

OPIDN

OPIDN, also known as OP compound–induced delayed polyneuropathy, can be produced within 1–4 weeks of a single exposure to a DN agent. With compounds that are highly selective for NTE, the first week of this period can be clinically quiescent (Davis and Richardson, 1980; Lotti, 1992; Lotti and Moretto, 2005; Richardson et al., 2013; Richardson and Makhaeva, 2014). Pathogenesis involves progressive distal degeneration of sensory and motor axons in peripheral nerves and spinal cord tracts. Accordingly, symptoms begin with paresthesias in the distal extremities, followed by sensory loss, ataxia, and flaccid paralysis. Once the disease is initiated, it advances inexorably. In keeping with its unknown mechanism of neurodegeneration, there is no cure or treatment other than general supportive therapy. Over a period of months to years, some regeneration of peripheral nerve axons may occur to reinnervate muscle. Nevertheless, the concomitant persistence of injury to descending inhibitory pathways in the spinal cord prevents complete functional recovery and permits only a shift from flaccid to spastic paralysis (Richardson et al., 2013; Richardson and Makhaeva, 2014).

Because of the insidious onset and permanent debilitating effects of OPIDN, it is essential to be able to predict the potential of a given OP compound to produce this disease as opposed to causing acute cholinergic toxicity. Furthermore, it is important to develop specific and stable biomarkers and biosensors of exposure to DN agents and devise countermeasures against them (Makhaeva et al., 2003; Malygin et al., 2003; Kohli et al., 2007a; Sigolaeva et al., 2010, 2013). To accomplish these goals, it will be necessary to acquire a level of mechanistic understanding of interactions between DN compounds and target macromolecules akin to what has been achieved for conventional (anti-AChE) nerve agents. Although there is much left to be learned about the pathogenesis and mechanism of OPIDN, sufficient knowledge exists about the apparent initiating events, such as inhibition and aging of NTE, to provide a foundation for the development of biomarkers and biosensors of DN agents.

NTE

Definition of NTE and its Potential Normal or Pathogenic Roles

NTE (UniProtKB/Swiss-Prot Q81Y17; PLPL6_HUMAN) has been categorized as a lysophospholipase (EC 3.1.1.5) because of its ability to hydrolyze phosphatidylcholine to glycerophosphocholine (UniProt, 2014). It is also known as patatin-like phospholipase

domain–containing protein 6, whose gene name is *PNPLA6*, because the catalytic domain of NTE encompasses a region with sequence homology to patatin, a phospholipase found in potatoes and other plants. Thus, it appears that NTE can function biochemically as a phospholipase, but its precise physiological role remains to be firmly established (Wijeyesakere and Richardson, 2010; Richardson et al., 2013).

The human *PNPLA6* gene encoding NTE has 35 exons (33 of them protein-coding) and 24 transcripts (Ensembl, 2014). Thus, it is not surprising that the UniProt entry for human NTE lists five protein isoforms attributed to alternative splicing (UniProt, 2014). Although UniProt has adopted isoform-1 as the canonical sequence, this chapter defines human NTE as isoform-2, which is the sequence initially reported by Lush et al. (1998) and consists of 1,327 amino acids and a molecular weight without posttranslational modifications of 146 kDa. The protein has a transmembrane domain near the N-terminus (residues 9–31) and three putative cyclic nucleotide binding domains (residues 163–262, 480–573, and 597–689), in addition to the patatin domain (residues 933–1,099), which contains the active site (Wijeyesakere et al., 2007; Wijeyesakere and Richardson, 2010).

The three-dimensional structure of NTE has not been experimentally determined, but a homology model of the patatin domain indicates that the active site serine (Ser^{966}) is located on a "nucleophilic elbow" characteristic of serine hydrolases (Wijeyesakere et al., 2007). Moreover, the model indicates that the catalytic site of NTE consists of a novel Ser-Asp catalytic dyad, as in patatin and mammalian cytosolic phospholipase A2 ($cPLA_2$), rather than the classical catalytic triad (Ser-Asp/Glu-His), as found in many serine hydrolases including AChE. Recently, mutations have been identified in NTE that are associated with motor neuron disease (NTE-MND) (Rainier et al., 2008, 2011). Constructs containing NTE-MND mutations within the catalytic domain have altered inhibition and aging kinetics (Hein et al., 2010a). In addition, cultured fibroblasts from human subjects with NTE-MND exhibit decreased NTE catalytic activity (Hein et al., 2010b). However, although fibroblasts from human subjects heterozygous for an insertion mutation expected to truncate the NTE protein had markedly reduced NTE activity (40–43% of control), these subjects were asymptomatic, indicating that decreased NTE activity alone is insufficient to produce disease.

NTE was first identified as the presumptive target of neuropathic OP compounds in the initiation of OPIDN (Johnson, 1970). Its activity in cells and tissues is operationally defined as the enzymatic hydrolysis of the nonphysiological substrate, phenyl valerate, which is resistant to inhibition by diethyl 4-nitrophenyl phosphate (paraoxon) and sensitive to inhibition by *N,N′*-diisopropylphosphorodiamidic fluoride (mipafox) under specified conditions of preincubation with inhibitors and subsequent incubation with substrate (Johnson, 1977; Kayyali et al., 1991; Makhaeva et al., 2007).

Because the full-length protein is difficult to isolate or produce, human recombinant NTE esterase domain (NEST) has been used as an NTE surrogate for studies *in vitro* that require pure protein rather than a crude preparation containing NTE (Kropp et al., 2004). NEST comprises residues 727–1,216 of NTE; it has been shown to be the shortest segment of NTE that retains esterase activity. Moreover, the catalytic properties of NEST, including its response to OP inhibitors, closely resemble those of full-length NTE (Atkins and Glynn, 2000; Atkins et al., 2002; van Tienhoven et al., 2002; Kropp et al., 2004). The catalytic activity of NEST has been shown to alter the fluidity of bilayer lipid membranes (Greiner et al., 2010), and NEST has been incorporated into a biosensor for the detection of DN agents (Kohli et al., 2007a, 2014).

Role of NTE in OPIDN

Early studies indicated that the initial molecular events in OPIDN are the concerted inhibition and aging of a threshold level (>70%) of NTE in the central and peripheral nervous systems (Johnson, 1974, 1982; Lotti, 1992; Glynn, 1999). As with other serine esterases, inhibition of NTE is thought to occur by nucleophilic attack of the active site serine (Ser^{966}) at the phosphorus of the OP compound, with displacement of a primary leaving group. Aging of the phosphylated enzyme presumably involves loss of a second ligand, leaving a negatively charged phosphyl moiety covalently attached to the active site. Note that "phosphyl" is used as a generic term for the various types of OP compounds that might be used as inhibitors of serine esterases, such as phosphates, phosphonates, phosphinates, and phosphoramidates.

The apparent necessity for aging of NTE as a trigger for OPIDN came about from analyses of structure–activity relationships. These studies revealed that NTE inhibitors fall into two functional categories, type A and type B (Johnson, 1975; Davis and Richardson, 1980; Davis et al., 1985) (Figure 63.5).

Type A NTE inhibitors produce OPIDN and include certain phosphates, phosphonates, and phosphoramidates. When type A compounds inactivate NTE, it rapidly becomes intractable to reactivation by powerful nucleophiles, such as oximes or fluoride. This inability to be reactivated has been interpreted as evidence of aging. Because loss of reactivatability takes place within minutes of inhibition by type A inhibitors and OPIDN takes 1–4 weeks to develop, aging of NTE is not the rate-limiting step in the disease process (Johnson, 1982).

Type B NTE inhibitors do not produce OPIDN and include certain phosphinates, carbamates, and

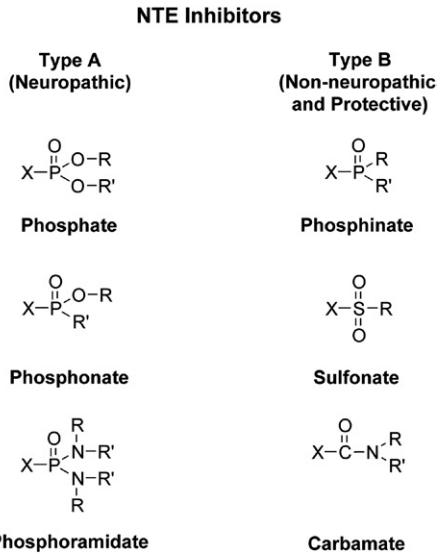

FIGURE 63.5 Subclasses of NTE inhibitors. Type A NTE inhibitors include phosphates, phosphonates, and phosphoramidates; these are neuropathic and capable of aging. Type B NTE inhibitors include phosphinates, sulfonates, and carbamates; these are nonneuropathic and not capable of aging. However, inhibition of NTE with a type B inhibitor will protect against OPIDN from subsequently administered type A inhibitors. *Source: Reproduced with permission from Richardson and Makhaeva (2014).*

sulfonates. NTE conjugates with type B compounds are considered not to undergo aging; those formed from carbamates and phosphinates can be reactivated. Moreover, carbamylated, phosphinylated, or sulfonylated NTE cannot undergo further reactions to produce a stable charged species. Despite the fact that inhibition of NTE by type B compounds does not result in OPIDN, this reaction is far from being biologically inert. Pretreatment of animals with type B inhibitors protects them against OPIDN from subsequently administered type A inhibitors, presumably by blocking the formation of aged NTE (Richardson, 1984; Davis et al., 1985; Johnson, 1990) (Figure 63.2). Protection against type A inhibitors by type B inhibitors is effective as long as inhibition of NTE by type B compounds is more than 30%, thus preventing inhibition and aging of the more than 70% of NTE required for induction of OPIDN.

It would appear that the ostensible requirement for aging of NTE in the development of OPIDN is not merely to ensure irreversible inhibition of the enzyme. Prolonged suprathreshold inhibition (>70%) of neural NTE *in vivo* by repeated dosing of nonaging inhibitors does not cause neuropathy (Johnson, 1970, 1974, 1982). Moreover, repeated low-level dosing with aging inhibitors to produce a prolonged steady-state inhibition that is below threshold (<70%) does not result in OPIDN until a higher single dose is superimposed that raises the inhibition level above threshold. In the latter scenario, there is still a delay of 10–14 days from the high point of inhibition (and presumed concomitant aging) until signs of neuropathy appear (Johnson and Lotti, 1980; Lotti and Johnson, 1980). These results suggest that simple loss of catalytic activity of NTE is not the damaging event in OPIDN. Thus, the notion has arisen that the "biochemical lesion" that triggers OPIDN is the formation of a chemically modified (aged) protein that has gained a toxic function in the neuron (Richardson, 1984; Glynn, 2000; Richardson et al., 2013).

The pathogenic role of aged NTE is not known, but it is tempting to speculate that it may be involved in an axonal self-destruct program that has been proposed to be an important mechanism in neurodegenerative disorders, such as motor neuron disease (Raff et al., 2002). Such a program might be triggered by chemically modified NTE acting through a toxic gain of function mechanism. However, experiments with conventional NTE knockouts (Winrow et al., 2003; Moser et al., 2004; Mühlig-Versen et al., 2005) and conditional NTE knockouts (Akassoglou et al., 2004), along with demonstrations of a likely role of NTE in membrane lipid metabolism (van Tienhoven et al., 2002; Quistad et al., 2003; Zaccheo et al., 2004), indicate that lethality or neuropathology can result from a loss of function of NTE (Glynn, 2006). Nevertheless, while the physiological and pathogenic roles of NTE are being deciphered, the fact that an excellent correlation exists between inhibition/aging of NTE and induction of OPIDN is sufficient to enable the pragmatic use this information for the development of biomarkers and biosensors for DN agents (Makhaeva et al., 2003, 2007).

KINETICS OF OP INHIBITOR–SERINE HYDROLASE INTERACTIONS

Introduction

The inhibitory and postinhibitory steps in the interaction of an OP compound with a serine hydrolase (E-OH) such as NTE or AChE are illustrated in Figure 63.6. The mathematical relationships describing the kinetics of irreversible inhibition of serine esterases by OP compounds and postinhibitory reactions of the enzyme–inhibitor adduct (conjugate) summarized here were elegantly set forth in the classic work by Aldridge and Reiner (1972), and synopses are available in other sources (Main, 1980; Clothier et al., 1981; Richardson, 1992). The equations featured here provide a basic foundation for determining the inhibitory potency of OP compounds against serine hydrolases, as well as the rates of reactivation or aging of the inhibited hydrolases. Such approaches can be used to assess the neuropathic potential of compounds and to distinguish conventional nerve agents from DN agents using

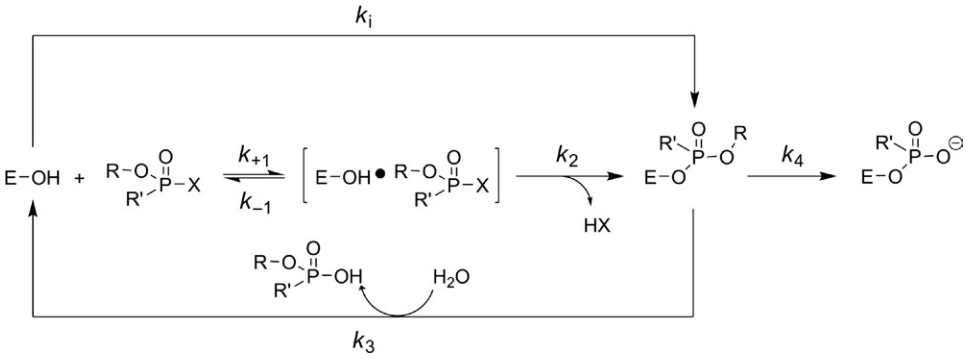

FIGURE 63.6 Reactions between a serine hydrolase and an organophosphonate inhibitor showing associated rate constants. The initial reversible reaction with forward (k_{+1}) and back (k_{-1}) rate constants forms a Michaelis-type complex. The complex can undergo phosphonylation (rate constant k_2) with expulsion of the primary leaving group, X. The overall progress of the reaction from enzyme to phosphonylated (inhibited) product is characterized by the bimolecular rate constant of inhibition, k_i. The phosphonylated enzyme can undergo spontaneous reactivation (hydrolysis in the presence of excess water) described by the pseudo-first-order rate constant, k_3, or undergo net loss of the R-group (aging) characterized by the first-order rate constant, k_4 (Richardson, 1992).

biomarkers and biosensors. For more complex scenarios beyond the scope of this chapter, other equations have been developed (Estevez and Vilanova, 2009).

Inhibition

The first step leading to E-OH inhibition is a reversible association of the OP compound (depicted in Figure 63.6 as a phosphonate) and enzyme to form a Michaelis-type complex (shown in square brackets in Figure 63.6). The associated rate constants for the forward (k_{+1}) and back (k_{-1}) reactions are shown on the double arrow (Figure 63.6). The active site serine hydroxyl of the hydrolase then engages in a nucleophilic attack on the phosphorus atom of the OP inhibitor, displacing the primary leaving group (X^-), resulting in a phosphonylated enzyme with net loss of HX. The primary leaving group, X^-, is typically a conjugate base of a strong acid, HX, such as HF. The phosphonylation rate constant is shown as k_2 (Richardson, 1992).

Considering only the first two steps in the reaction sequence, which is appropriate for most acylating inhibitors, the affinity of the inhibitor for the enzyme is given by the Michaelis-type equilibrium constant, K_a, as shown in Eq. (63.1).

$$K_a = (k_{-1} + k_2) / k_{+1} \tag{63.1}$$

The overall progress of the reaction from E-OH and inhibitor to phosphonylated enzyme is characterized by the bimolecular rate constant of inhibition, k_i. This important measure of inhibitory potency is determined by measuring the activity remaining as a function of time of preincubation with various concentrations of inhibitor. The substrate is added after the preincubation interval (Richardson, 1992). When the inhibitor concentration [OP] $\ll K_a$, k_i is given by Eq. (63.2).

$$k_i = k_2 / K_a \tag{63.2}$$

So-called ideal first-order kinetics of inhibition are obtained under the commonly observed conditions when the concentration of the Michaelis-type complex is small, k_2 is high, k_3 is much smaller than k_2, and [OP] >10[E-OH], where [E-OH] = concentration of native enzyme. In such cases we have Eq. (63.3).

$$\ln(v/v_0) = k_2[\mathrm{OP}]t/([\mathrm{OP}] + K_a) \tag{63.3}$$

In Eq. (63.3), v is the rate of enzymatic hydrolysis of its substrate at time = t, and v_0 is the rate at time zero. Substituting (% activity/100) for (v/v_0) and letting $k' = k_2[\mathrm{OP}]/([\mathrm{OP}] + K_a)$ yields Eq. (63.4).

$$\ln(\%\,\text{activity}/100) = -k't \tag{63.4}$$

Thus, primary plots of ln(% activity/100) versus t will be straight lines with slopes= $-k'$, as shown in Figure 63.7A. In addition, the experimentally determined dependence of %(activity remaining) on the preincubation time (t) and inhibitor concentration [OP] is given by Eq. (63.5).

$$\ln(\%\,\text{activity})/100 = -k_i[\mathrm{OP}]t \tag{63.5}$$

Setting Eqs. (63.4) and (63.5) equal to each other gives Eq. (63.6).

$$k' = k_i[\mathrm{OP}] \tag{63.6}$$

Therefore, a secondary plot of $-k'$ versus [OP] will yield a straight line with slope = k_i, as shown in Figure 63.7B. The k_i value thus obtained is an indication of the inhibitory potency of a given compound against a particular serine hydrolase. As stated, k_i is a measure of the

overall progress of the reaction between the inhibitor and enzyme to yield the organophosphylated product. However, it is important to realize, as shown in Eq. (63.2), that k_i is a composite of K_a, which is an indication of the affinity of the inhibitor for the enzyme, and k_2, which is an indication of the rate of phosphylation of the enzyme by the inhibitor. Inspection of Eqs. (63.2) and (63.5) shows that the units of k_i are $[OP]^{-1}t^{-1}$; for example, the k_i obtained for chlorpyrifos methyl oxon (CPMO) against hen brain microsomal NTE at pH 8.0 and 37°C (Figure 63.7) is $5.82 \times 10^4 M^{-1} min^{-1}$ (Richardson, 1992; Kropp and Richardson, 2003).

Under certain conditions, it is possible to determine the K_a and k_2 components of k_i separately. For example, if the secondary plot is not linear, then this is an indication of an appreciable concentration of a Michaelis-type complex. In this case, the K_a term must be included; by combining Eqs. (63.3) and (63.4) and rearranging, we have Eq. (63.7).

$$[OP]/k' = K_a/k_2 + [OP]/k_2 \qquad (63.7)$$

Thus, a Wilkinson plot of $[OP]/k'$ versus [OP] will yield a straight line with slope $1/k_2$, a y-intercept of K_a/k_2, and an x-intercept of K_a. Moreover, in Eq. (63.2), $k_i = k_2/K_a$; therefore, the reciprocal of the y-intercept gives k_i (Richardson, 1992).

An especially useful relationship is given by substituting a % activity of interest into Eq. (63.5) to yield the inhibitor concentration at a given time of preincubation with an enzyme that would yield the particular percent activity. For example, when $[OP]_{50} = I_{50}$ = the inhibitor concentration required to produce 50% inhibition of the enzyme at a given time, t, of incubation of enzyme and inhibitor at defined conditions of pH, temperature, and ionic strength before adding substrate, then we have Eq. (63.8).

$$[OP]_{50} = I_{50} = \ln 2/k_i t \approx 0.693/k_i t \qquad (63.8)$$

Note that in Eq. (63.8), k_i and I_{50} are reciprocally related and I_{50} is time-dependent. It is valid to calculate an I_{50} from a k_i value when ideal kinetics are observed. However, it is not valid to calculate a k_i from an experimentally determined fixed-time I_{50}, because the I_{50} alone contains no information about the kinetic behavior of the inhibition reaction. In addition, if inhibitory potency is assessed by measuring fixed-time I_{50} values, it is essential to report the time of preincubation along with the concentration, because the I_{50} decreases as the preincubation time increases (Aldridge and Reiner, 1972; Clothier et al., 1981; Richardson, 1992). For example, using Eq. (63.8), the 20-min I_{50} for CPMO against hen brain microsomal NTE at pH 8.0 and 37°C may be calculated from the k_i given as $0.693/(5.82 \times 10^4 M^{-1} min^{-1})(20 min) = 5.95 \times 10^{-7} M \approx 0.60 \mu M$ (Kropp and Richardson, 2003).

Reactivation

In Figure 63.6, the phosphonylated enzyme can undergo spontaneous reactivation via hydrolysis to yield free enzymes and a phosphonic acid, with an

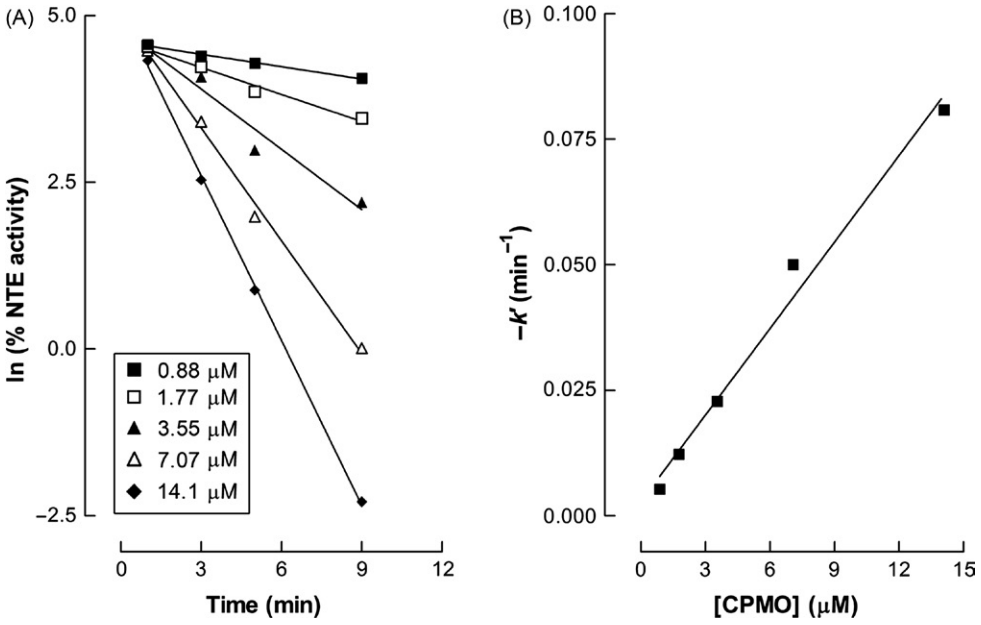

FIGURE 63.7 (A) Primary kinetic plots of ln(% NTE activity) versus time for various indicated concentrations of CPMO. (B) Slopes ($-k'$) of primary kinetic plots versus inhibitor concentration. Data are for hen brain microsomal NTE at pH 8.0 and 37°C. *Source: Reproduced with permission from Kropp and Richardson (2003).*

associated pseudo-first-order rate constant, k_3 (the reactivator, water, is present in great excess). The spontaneous rate constant of reactivation, k_3, can be obtained from Eq. (63.9).

$$\ln(100/\%\,\text{inhibition}) = k_3 t \tag{63.9}$$

The % reactivation in a reactivation experiment is determined by Eq. (63.10).

$$\%\,\text{inhibition} = [(A - A_t) / (A - A_0)] \times 100 \tag{63.10}$$

In Eq. (63.10), A is the activity of the control, A_t is the activity of the inhibited enzyme at time t, and A_0 is the activity of the inhibited enzyme at $t = 0$. In practice, reactivation is studied by preincubating the enzyme with a concentration of inhibitor that rapidly produces essentially complete inhibition. The inhibitor and enzyme are then rapidly separated or the preparation is diluted sufficiently to prevent further appreciable inhibition of free enzyme, and the return of enzyme activity is measured at timed intervals. The slope (k_3) of the least-squares best-fit line from a plot of ln(100/% inhibition) versus t is determined using linear regression from the initial linear portion of the curve. From Eq. (63.9), it can be seen that the units of k_3 are t^{-1} (e.g., min^{-1}), and that the half-life, $t_{1/2}$, the time required for 50% reactivation, can be determined from Eq. (63.11).

$$t_{1/2} = \ln 2/k_3 \approx 0.693/k_3 \tag{63.11}$$

The apparent half-life of reactivation can range from minutes to weeks, depending on the enzyme, inhibitor, pH, and temperature. Reactivation can appear to be slow or nonexistent if aging has occurred, because the aged form of the phosphylated enzyme is stable to hydrolysis and will not reactivate (Richardson, 1992; Jianmongkol et al., 1999).

Aging

As shown in Figure 63.6, the inhibited esterase can also undergo aging, such as via net loss of a labile R-group to yield the negatively charged phosphonyl adduct on the active site serine of the enzyme. Aging is characterized by a first-order rate constant, k_4, and the operational definition of this reaction is the time-dependent loss of the ability to reactivate the inhibited enzyme by powerful nucleophiles, such as fluoride ion or oximes. The method of determining aging is similar to that for reactivation. Enzyme is preincubated with a concentration of inhibitor sufficient to produce essentially complete inhibition in a relatively short time. Aliquots are then treated with a reactivator such as fluoride or an oxime, and the amount of activity relative to a nonreactivated sample is measured. The first-order rate constant of aging, k_4, can be determined from Eq. (63.12).

$$\ln[100/(\%\,\text{reactivation})] = k_4 t \tag{63.12}$$

In an aging experiment, the % reactivation is determined by Eq. (63.13).

$$\%\,\text{reactivation} = [(AR_1 - AR_2)/(AR_0 - AI_0)] \times 100 \tag{63.13}$$

In Eq. (63.13), AR_1 = activity of reactivated enzyme at t_{aging}, AR_0 = activity of reactivated enzyme at $t = 0$, AI_1 = activity of inhibited enzyme without reactivator at t_{aging}, and AI_0 = activity of inhibited enzyme without reactivator at $t = 0$. The slope (k_4) of the least-squares best-fit line from a plot of ln(100/% inhibition) versus t is determined using linear regression from the initial linear portion of the curve. From Eq. (63.12), it can be seen that the units of k_4 are t^{-1} (e.g., min^{-1}), and that the aging half-life, $t_{1/2}$, the time required for 50% aging, can be determined from Eq. (63.14).

$$t_{1/2} = \ln 2/k_4 \approx 0.693/k_4 \tag{63.14}$$

The apparent half-life of aging depends on the enzyme, inhibitor, pH, and temperature. However, NTE inhibited with most neuropathic agents exhibits aging half-lives on the order of several minutes, so that the delay of 1–4 weeks between exposure and appearance of signs of OPIDN is not due to the time required for aging of phosphylated NTE (Johnson, 1982; Richardson, 1992; Jianmongkol et al., 1999; Kropp and Richardson, 2007).

Relative Inhibitory Potency

The neuropathic potential of an OP compound depends on its ability to inhibit NTE (and presumably for the NTE–OP conjugate to undergo aging). However, an OP compound will not be able to inhibit NTE to a sufficient degree to produce OPIDN if its cholinergic toxicity is too high to permit survival of a neuropathic dose. Therefore, neuropathic potential of an OP compound is a relative concept that depends on the ability of the compound to inhibit NTE compared to its anti-AChE activity. Compounds can be screened *in vitro* for relative inhibitory potency (RIP) by measuring k_i or I_{50} values and calculating a ratio, as shown in Eq. (63.15).

$$\text{RIP} = k_i(\text{AChE})/k_i(\text{NTE}) \tag{63.15}$$

Because of the reciprocal relationship between the k_i and I_{50} described by Eq. (63.8), the RIP for fixed-time I_{50} values (determined at the same fixed-time, t) is given by Eq. (63.16).

$$\text{RIP} = I_{50}(\text{NTE})/I_{50}(\text{AChE}) \quad (63.16)$$

Thus, when the RIP is more than 1, the compound is a more potent inhibitor of AChE than NTE, and cholinergic toxicity would be expected to predominate over OPIDN. In fact, when the RIP is more than 1, the dose required to produce OPIDN tends to be greater than the median lethal dose (LD_{50}) for the compound (Richardson, 1992; Kropp and Richardson, 2003). Note that the RIP is simply a ratio of inhibitor potencies, and it could have been defined the other way around, with the k_i for NTE in the numerator. However, the convention used in the present chapter defines the RIP as shown in Eqs. (63.15) and (63.16).

For example, although CPMO, the active metabolite of the insecticide chlorpyrifos methyl, would seem to be a potent inhibitor of NTE, with $k_i = 5.82 \times 10^4\,M^{-1}\,min^{-1}$ and 20 min $I_{50} = 0.595\,\mu M$, it is a much more potent inhibitor of AChE, with $k_i = 1.09 \times 10^7\,M^{-1}\,min^{-1}$ and 20 min $I_{50} = 3.18 \times 10^{-9}\,M$ (3.18 nM), so that the RIP for this compound is 187. This value is $\gg 1$, indicating that OPIDN could not be produced unless the dose were $\gg LD_{50}$ and aggressive therapy for cholinergic toxicity were instituted (Kropp and Richardson, 2003). In contrast, the compound di-*n*-pentyl 2,2-dichlorovinyl phosphate (*n*-pentyl DCV) has an RIP of 0.03 and produces OPIDN at a dose of 2 mg/kg, which is much lower than the LD_{50} dose of 26 mg/kg (Richardson, 1992). OP inhibitors with extremely high selectivity for NTE have been synthesized (e.g., EOPF) (Figure 63.3) (Wu and Casida, 1995), which has 20-min I_{50} values for mouse brain NTE and AChE of 0.02 and 54 nM, respectively, yielding an RIP value of 3.7×10^{-4} (i.e., approximately 2,700-times more potent for NTE than AChE) (Wu and Casida, 1996). Note that EOPF is a chiral compound, and the reported I_{50} and RIP values are for the racemic mixture. Given that inhibitory potency can vary considerably between *R* and *S* forms of OP compounds (Wu and Casida, 1994), it would be of interest to perform such measurements on resolved isomers.

Because RIP values can encompass several orders of magnitude above and below 1, it is convenient to visualize such data by using log (RIP) values as shown in Figure 63.8. It can readily be seen that higher positive numbers correspond to greater cholinergic propensity, as typified by the insecticide active metabolite, CPMO. Likewise, larger negative numbers correspond to greater neuropathic potential, as exemplified by the DN agent, EOPF. It is also interesting to see the steady progression from cholinergic to neuropathic tendency exhibited by the homologous series of dialkyl 2,2-dichlorovinyl phosphates (dichlorvos (DCV)) compounds. In this and other series of alkyl OP inhibitors, increasing length of alkyl chains tends to produce decreasing potency against AChE and increasing potency against NTE until maximum selectivity is reached, after which potencies and selectivity decline (Richardson, 1992; Malygin et al., 2003). The highest potency for NTE appears to have been achieved with certain phosphonofluoridates, where the sum of alkyl and alkoxyl chain length is 12–16 atoms, including O and P; in this series, EOPF has the highest potency and selectivity for NTE (Wu and Casida, 1995, 1996).

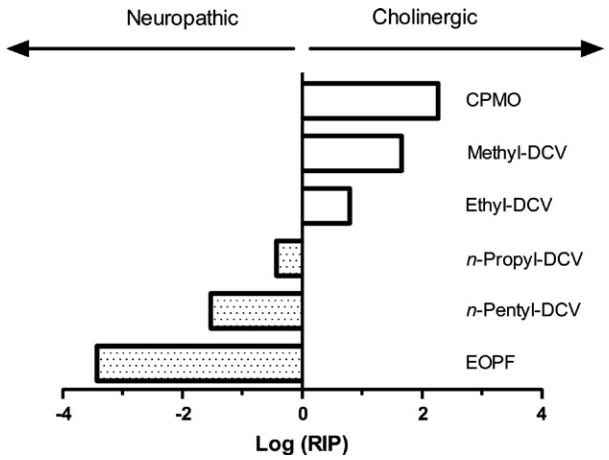

FIGURE 63.8 Spectrum of log values of RIP = k_i(AChE)/k_i(NTE), where k_i = bimolecular rate constant of inhibition. As log(RIP) becomes more positive, cholinergic potential increases; as log(RIP) becomes more negative, DN potential increases. DCV derivatives refer to symmetrical dialkyl–2,2–dichlorovinyl phosphate (dichlorvos) compounds. *Source: Data from Richardson (1992); Wu and Casida (1996); and Kropp and Richardson (2003).*

Determinations of RIP performed *in vitro* using preparations of AChE and NTE can only assess the neuropathic potential of OP compounds that are direct-acting inhibitors (i.e., compounds that do not require metabolic activation). Such preparations would also not detect effects of detoxification that might occur *in vivo*. To account for the possibility of metabolic activation/deactivation, the system can be augmented by preincubating the compound of interest with xenobiotic metabolic enzymes (or chemical activators, e.g., bromine) before mixing with AChE or NTE preparations (Barber et al., 1999). The concept of the RIP can also be extended to assaying AChE and NTE *ex vivo* and calculating activity ratios after dosing laboratory animals with the compound of interest. Because the adult hen is the species of choice for assessing the neuropathic potential of OP compounds, this species could also be used for *ex vivo* RIP determinations (Richardson, 1992; Richardson et al., 1993). However, despite the fact that rats and mice are not as suitable as hens for studying the clinical and histopathological effects of DN agents, rodent species have AChE and NTE in their nervous systems and can therefore serve as sources of these enzymes for *in vitro* or *ex vivo* determinations of neuropathic potential (Novak and

Padilla, 1986; Wu and Casida, 1996; Quistad et al., 2003; Makhaeva et al., 2014).

BIOMARKERS

Introduction

A widely quoted definition of "biomarker" is that it is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al., 2001). Although this definition was developed from the standpoint of pharmacology and therapeutics, it can easily be modified to apply to toxicology. In a toxicological setting, a biomarker would consist of an appropriate measure of a chemically induced pathological process (e.g., a response to a toxicant that could be considered injurious or diagnostic of potential injury). Ideally, a toxicological biomarker would represent a biological response that would be pathognomonic of a specific agent or at least a selective indicator of the effects of a class of agents. Thus, a biomarker of a DN agent would involve more than measuring the mere presence of the compound; instead, it would assess a distinctive and preferably early feature of the pathogenesis of OPIDN. Because the earliest known molecular events associated with OPIDN are the inhibition and aging of NTE, the appropriate biomarkers of DN agents would be measurements of these reactions, which could be performed via determination of inhibition and aging using enzymology, or through identification of the resulting OP–NTE conjugates using mass spectrometry.

Enzymological Measurements of NTE Inhibition and Aging

NTE activity in lymphocytes and platelets was discovered by Dudek and Richardson (1978, 1982), characterized in human populations (Bertoncin et al., 1985; Maroni and Bleecker, 1986), and used in animal (Richardson and Dudek, 1983; Schwab and Richardson, 1986; Richardson et al., 1993; Makhaeva et al., 2003) and human (Lotti et al., 1983, 1986; McConnell et al., 1999) studies to monitor exposures to potentially neuropathic OP compounds. NTE activity is not found in erythrocytes or in plasma or serum. These studies indicate that NTE in lymphocytes, platelets, or whole blood can be assayed and used as a biomarker of exposure to DN agents, particularly if the blood samples are taken within 24 h of acute exposures. Attempts to measure the aging of NTE in lymphocytes or platelets of exposed subjects have apparently not been performed. However, given that aging of NTE inhibited by DN agents typically occurs within a half-life of a few minutes (Clothier and Johnson, 1979; Johnson, 1982), aging would be complete by the time that lymphocyte samples could be taken and assayed for activity. Nevertheless, as stated, given the apparent requirement for aging as well as inhibition of NTE in OPIDN, and to help rule out false positives arising from inhibition by type B (nonaging) NTE inhibitors (Figure 63.5), aging studies should be undertaken in future work. As noted, mass spectrometry can be used to identify the phosphyl adduct on the inhibited enzyme, thus differentiating between inhibited and aged forms.

In keeping with the concept of the RIP discussed, inhibition of lymphocyte and/or platelet NTE and possibly erythrocyte LysoPC hydrolase should be used in conjunction with inhibition of erythrocyte AChE and plasma butyrylcholinesterase (BChE) to assess the likelihood that an exposure to OP compounds would produce cholinergic and/or DN effects. Erythrocyte AChE inhibition has long been used as a biomarker of exposure to conventional nerve agents or OP insecticides (Wilson and Henderson, 1992; Lotti, 1995). BChE can be sensitive to both conventional and DN agents, and its inhibition could thus serve as a general biomarker for OP agents (Kropp and Richardson, 2007; van der Schans et al., 2008; Jiang and Lockridge, 2013). For additional biomarkers of OPs, readers are referred to Gupta and Milatovic (2014).

Identification of NTE–OP Conjugates Using Mass Spectrometry

Mass spectrometry has proved to be an excellent tool for identifying the OP conjugates (adducts) of serine hydrolases such as AChE and BChE (Doorn et al., 2000, 2001b, 2003; Elhanany et al., 2001; Jennings et al., 2003; Kropp and Richardson, 2007; Li et al., 2007; van der Schans et al., 2008; Jiang et al., 2013). One approach is to reactivate inhibited esterases with fluoride and to detect the liberated OP fluoridate using gas chromatography–mass spectrometry; however, this technique cannot be used if the esterase conjugate has aged (Degenhardt et al., 2004; Solano et al., 2008). Because NTE inhibited with DN agents undergoes rapid aging (Johnson, 1982), it is important to be able to detect the aged adducts. Therefore, peptide mass mapping would be the current method of choice, whereby the active site peptide containing the phosphyl moiety on the active site serine is identified using mass spectrometry after digestion of the inactivated esterase with trypsin or other proteases (Doorn et al., 2003; Jiang et al., 2013). Although some DN agents have extremely high potency and selectivity for NTE, in general there will be some degree of inhibitory overlap with other serine hydrolases (see Figure 63.8). Accordingly, in addition to examining NTE, it would be useful to acquire mass spectrometry data for OP conjugates of other potential esterase targets, such as AChE

and BChE, with particular attention being given to the aged species.

The classical view of the aging reaction of OP-inhibited serine esterases, based on studies of soman-inhibited AChE, is that aging involves an S_N1 reaction at a ligand carbon and transient formation of a carbocation that dissociates from the enzyme (Michel et al., 1967; Shafferman et al., 1996). The traditional model has been confirmed using mass spectrometry for BChE inhibited by a variety of phosphates and phosphonates and allowed to age in the presence of [^{18}O]-H_2O (Li et al., 2007). Thus, it appears that cholinesterases inhibited by phosphates or phosphonates undergo aging via S_N1 cleavage of an O—C bond rather than S_N2 cleavage of a P—C bond.

However, it is important to realize that mechanisms of inhibition and aging can differ depending on the esterase, inhibitor, and stereochemistry of the inhibitor. For example, the aging of AChE inhibited by ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun) was shown to proceed via hydrolytic P—N bond scission with loss of dimethylamine (Elhanany et al., 2001). In addition, the mechanism of inhibition of AChE, BChE, and cholesterol esterase by the resolved stereoisomers of isomalathion has been shown to differ between the (1*R*) and (1*S*) forms, and the mechanism of aging in each case appears to proceed via S_N2 displacement of a secondary leaving group by water, involving cleavage of a P—S bond (Doorn et al., 2000, 2001a,b, 2003). A subsequent study of BChE inhibited with racemic isomalathion confirmed that the major pathway for aging involved scission of the P—S bond, but relatively small peaks in the mass spectrum indicated that O—C and S—C cleavage could also occur as minor pathways (Li et al., 2007).

For NTE inhibited by phosphates or phosphonates, the aging mechanism has been thought to be consistent with an S_N2 reaction at phosphorus (Johnson, 1982), although this hypothesis has not been rigorously tested. With respect to stereochemical influences on aging, limited studies indicate that both enantiomers of certain phosphonates inhibit NTE, but only one enantiomer undergoes aging (Johnson et al., 1985, 1986). As expected, the isomer that inhibits and ages NTE produces OPIDN. In contrast, the isomer that inhibits NTE without aging does not produce OPIDN. Furthermore, the nonaging isomer protects against neuropathy from subsequent administration of a neuropathic OP compound (Johnson and Read, 1987; Johnson et al., 1988). Some racemic phosphonates have been found to produce OPIDN at lower than expected threshold levels of inhibited/aged NTE (i.e., <70%), possibly because of potentiation or promotion of subclinical axonopathy by the nonaging enantiomer, presumably by the action of the nonaging enantiomer on one or more targets other than NTE that could be involved in repair or regeneration of neural lesions (Lotti, 1992).

Perhaps the most thoroughly investigated compound with respect to inhibition and aging of serine hydrolases is diisopropylphosphorofluoridate (DFP). Using a variety of techniques, this compound has been shown to inhibit AChE (Clothier and Johnson, 1979; Ordentlich et al., 1996, 1998; Millard et al., 1999a,b; Kropp and Richardson, 2006), BChE (Masson et al., 1997; Kropp and Richardson, 2007; Li et al., 2007), NTE (Clothier and Johnson, 1979; Williams and Johnson, 1981; Williams, 1983), and NEST (Kropp et al., 2004) by organophosphorylation of the active site serine followed by aging to yield a monoisopropylphosphoryl adduct, as shown in Figure 63.9. Esterase adducts have been directly demonstrated using mass spectrometry for AChE (Kropp and Richardson, 2006), BChE (Kropp and Richardson, 2007; Li et al., 2007), and NEST (Kropp et al., 2004), and, in the case of BChE, the mechanism of aging has been unequivocally demonstrated to involve scission of an O—C bond in an isopropyl group (Li et al., 2007). These results, taken together with those for soman-inhibited AChE (Michel et al., 1967) and BChE inactivated with a variety of phosphonates and phosphates (Li et al., 2007), indicate that it is reasonable to expect that aging of phosphonylated or phosphorylated serine esterases, including NTE and NEST, will proceed via O—C bond scission. This insight enables accurate prediction of the biomarker species to be sought in the mass spectra of serine esterases inhibited with phosphonates or phosphates.

Among the type A inhibitors of NTE (Figure 63.5), the phosphoramidates are perhaps the most enigmatic to be studied to date. For example, mipafox, the phosphoramidate analog of DFP, is capable of producing suprathreshold inhibition (>70%) of neural NTE *in vivo* and causing OPIDN (Davis and Richardson, 1980). Therefore, mipafox-inhibited NTE was expected to undergo aging (Davis et al., 1985). Early studies showed that NTE inhibited by mipafox was intractable to reactivation, indicating the possibility of aging as a mechanism for yielding a nonreactivatable enzyme (Johnson, 1982). In the case of mipafox-inhibited NTE, however, nonreactivation was thought to be too rapid to measure by standard kinetic approaches (Johnson, 1982). Consequently, aging

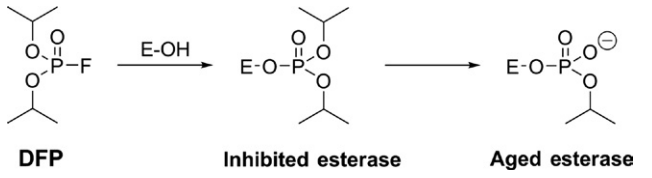

FIGURE 63.9 Inhibition and aging of serine esterases by DFP. The active site serine is organophosphorylated in the inhibition step. Aging results in net loss of an isopropyl group to yield the monoisopropylphosphoryl esterase. This mode of inhibition and aging has been established for AChE, BChE, and NTE catalytic domain (NEST) (Kropp and Richardson, 2007).

of mipafox-inhibited NTE had only been inferred and not directly demonstrated. Subsequently, Milatovic and Johnson (1993) showed that NTE inhibited by mipafox could be reactivated by prolonged treatment with KF at acidic pH. This surprising finding led to the conclusion that aging had not occurred after all. Instead, it was surmised that an unusual property of the bond between the phosphoramidate and the active site serine was responsible for the stability of the complex as well as the perturbation of NTE that leads to OPIDN.

It had been known for some time that esterases inhibited by mipafox are resistant to reactivation, but conventional aging could not be proved or disproved by classical techniques. It is also known that the phosphoramido hydrogen on *N*-alkylphosphoramidates is acidic and that its removal yields a resonance-stabilized anion (Eto, 1974). Such a mechanism would explain the relative stability of phosphoramidated esterases in a manner that does not require loss of an alkylamino group to yield a negative charge on the adduct (Richardson, 1995). Moreover, reprotonation of the phosphoramide anion would generate a neutral species subject to attack by a nucleophile and account for the reactivation of mipafox-inhibited NTE at low pH. Under the acidic conditions usually used for peptide mass spectrometry, the putative anion would be reprotonated. Thus, it can be hypothesized that the species attached to NTE after its inhibition by mipafox and allowing time for aging is the intact *N*,*N*′-diisopropylphosphorodiamido moiety. Such a result would indicate that the inhibited enzyme has not lost an isopropyl or isopropylamino group due to an aging process analogous to the net loss of an isopropyl group in the aging of DFP-inhibited NTE or NEST.

In contrast to the results for mipafox-inhibited NTE, early limited studies indicated that mipafox-inhibited BChE or AChE undergo irreversible aging, because the enzymes could be reactivated soon after inhibition, but not after 18 h (Milatovic and Johnson, 1993). The chemistry of phosphoramidates suggest that aging could involve hydrolytic loss of an alkylamino group. For example, acid-catalyzed P—N bond fission has been observed for certain *N*-alkyl phosphoramidates (Eto, 1974), and the aging of tabun-inhibited AChE has been shown to proceed via P—N bond scission with loss of dimethylamine (Elhanany et al., 2001).

Considerations of probable mechanisms of aging for mipafox-inhibited serine esterases suggest that mass spectrometry studies would support a deprotonation mechanism for NTE or NEST and hydrolytic P—N bond scission for AChE and BChE. As summarized in Figure 63.10, these expectations were borne out for human recombinant NEST, which was used as a surrogate for NTE (Kropp et al., 2004) and BChE, respectively (Kropp and Richardson, 2007). However, mipafox-inhibited AChE gave the surprising result, confirmed by immunoprecipitation and mass spectrometry, of loss of both isopropylamine groups to yield a simple phosphate adduct on the active site peptide (Kropp and Richardson, 2006). Thus, in the case of phosphoramidates, aging appears to be nonclassical for the three esterases investigated; moreover, the biomarker is distinctly different in each case.

Recently, mass spectrometry was successfully used to detect BChE adducts arising from reaction with 2-(2-methylphenoxy)-BDPO (CBDP), which has been implicated in "aerotoxic syndrome" (Carletti et al., 2011). Interestingly, the final aging product was a phosphoserine adduct, as found previously in the aging of mipafox-inhibited AChE (Kropp and Richardson, 2006).

BIOSENSORS

Nanostructured Electrochemical Biosensors to Measure Enzyme Activity

Recently developed nanofabrication methods using layer-by-layer (LBL) self-assembly provide exciting new opportunities to design multilayered, functional, biosensor interfaces to measure enzyme activity. Since the LBL technique was first introduced (Decher and Hong, 1991), significant work has been performed to fabricate polymer and organic thin films through alternating deposition of positively and negatively charged polyelectrolyte species on solid surfaces (Decher et al., 1992; Lvov et al., 1994; Decher, 1997; Schmidt et al., 1999; Ruths et al., 2000).

Polyelectrolyte layers typically have a thickness of approximately a nanometer, allowing ultrathin interfaces to be fabricated that have minimal mass-transfer resistance. LBL self-assembly using polyelectrolytes is thus well-suited for biosensor development studies, because, by minimizing mass-transfer resistances, the intrinsic enzyme kinetics can be measured. LBL assemblies of polyelectrolytes have been used to develop nanostructured biosensor interfaces that encapsulate enzymes (Sokolovskaya et al., 2005 ; Kohli et al., 2006, 2007a; Hassler et al., 2007) and functional nanoparticles (Kohli et al., 2005, 2007b).

Electrochemical biosensors that measure the activity of a protein have been widely used to determine analyte concentrations in both research and commercial applications (Prodromidis and Karayannis, 2002). Biosensors can detect protein activity either directly (Hassler and Worden, 2006) or indirectly through reaction coupling (Peteu et al., 1996, 1998). The indirect approach greatly expands the range of enzyme activities that can be characterized using electrochemical biosensors. For example, reaction coupling has been used to measure NTE activity outside of the electrode (e.g., in blood samples)

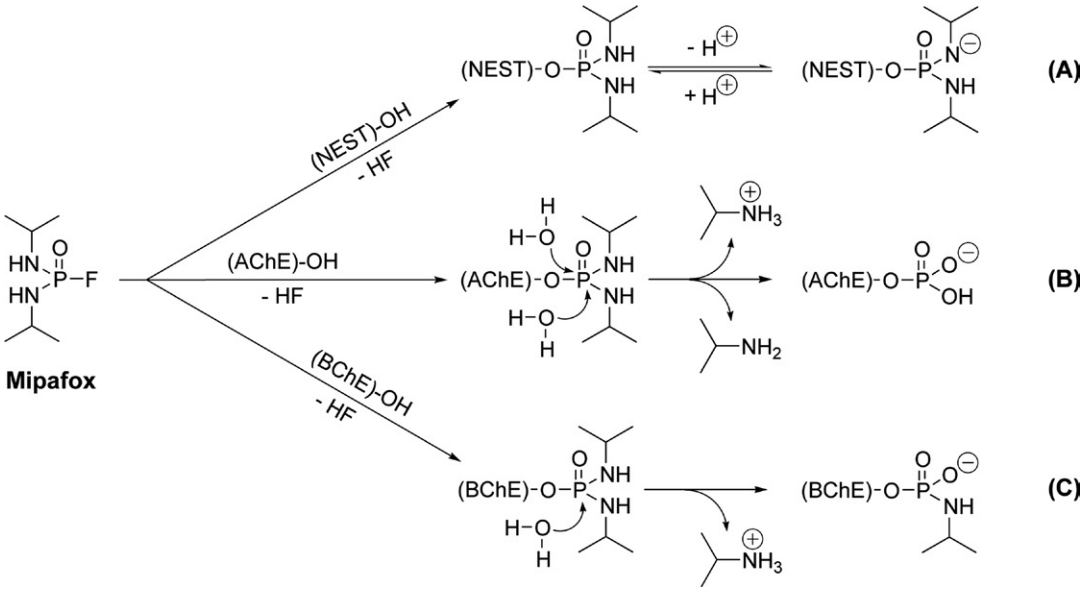

FIGURE 63.10 Inhibition and aging of serine esterases by *N*,*N*′-diisopropylphosphorodiamidic fluoride (mipafox). (A) NTE catalytic domain (NEST) showing aging by reversible deprotonation of a phosphoramido nitrogen. (B) AChE showing aging by displacement of both isopropylamine groups by water. (C) BChE showing aging by displacement of a single isopropylamine group by water. *Source: Reproduced with permission from Kropp and Richardson (2007).*

(Makhaeva et al., 2003, 2007; Sigolaeva et al., 2010, 2013) or AChE, BChE, and NEST activity incorporated into the electrode (Kohli et al., 2007a, 2014).

Electrochemical Biosensor Arrays for High-Throughput Analysis

Microsystems technologies, particularly thin-film deposition of microelectrodes and formation of microfluidic channels, have been widely applied to biological analysis systems (Bergveld, 1996; Urban, 2000), such as DNA-processing chips (Raisi et al., 2004) and other lab-on-chip implementations (Ziegler, 2000; McFadden, 2002; Kovacs, 2003). Most of these devices rely on optical transduction mechanisms (Rabbany et al., 1994; Vo-Dinh et al., 2003) that often cannot quantitatively measure several important protein activities, such as redox reactions.

In contrast, enzymatic redox reactions are readily measured using versatile electrochemical techniques. Traditionally, electrochemical measurements are performed at the "beaker scale," but newer technologies allow such systems to be miniaturized for improved performance and high-throughput analysis. Complete three-electrode electrochemical cells, including thin-film Ag/AgCl reference electrodes, have been integrated on silicon surfaces (Yun et al., 2004). Thin-film reference electrode stability has been improved using Nafion or polyurethane coatings (Nolan et al., 1997) and complex micromachined structures (Suzuki et al., 1999; Suzuki, 2000). A remaining challenge is to adapt these new technologies to the construction of miniaturized electrochemical arrays for high-throughput protein activity characterization. Such biosensor array platforms must be compatible with the self-assembled biosensor interfaces, thus establishing requirements for electrode materials, geometries, surface smoothness, and overall array architecture (Huang et al., 2013).

Assembly of Electrochemical Biosensor Interfaces for Serine Hydrolases

The natural reaction substrates and products of serine esterases are not electrochemically active. To transduce these esterase activities into an electrical signal, a two-enzyme reaction pathway can be assembled on a gold electrode. In the first reaction, the esterase of interest converts a phenyl ester (e.g., phenyl valerate) into phenol (Figure 63.11). In the second reaction, tyrosinase converts phenol first to catechol, and then to *o*-quinone (Figure 63.12), which can be electrochemically reduced back to catechol at an electrode, yielding a measurable current (Eq. (63.17)) (Kohli et al., 2007a, 2010).

$$o\text{-Quinone} + 2H^+ + 2e^- \rightarrow \text{Catechol} \quad (63.17)$$

Tyrosinase is a copper-containing oxidase (Coche-Guerente et al., 2001; Forzani et al., 2000) that possesses the two different activities illustrated in Figure 63.12. In the first step, referred to as the hydroxylase or cresolase activity, molecular oxygen is used to hydroxylate phenol to form catechol. In the second step, known as

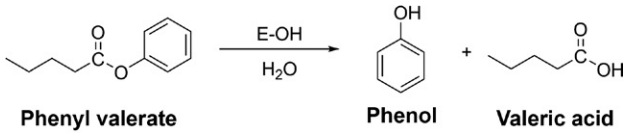

FIGURE 63.11 Reaction of a serine esterase with phenyl valerate to yield phenol and valeric acid (Kayyali et al., 1991).

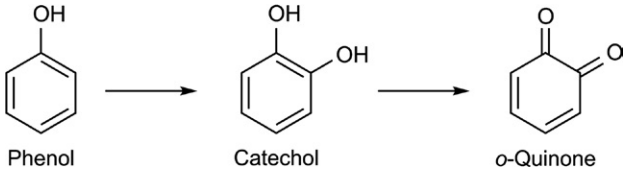

FIGURE 63.12 Reactions catalyzed by the two activities of tyrosinase. Phenol is first oxidized to catechol and then to *o*-quinone. *Source: Reproduced with permission from Kohli et al. (2007a).*

the catecholase activity, the enzyme oxidizes catechol to *o*-quinone, which is simultaneously oxidized by oxygen to its original form, with the production of water. The *o*-quinone is electrochemically active and can be reduced back to catechol, as illustrated in Eq. (63.17). Such electrodes have been used to determine esterase activities in blood samples (Sigolaeva et al., 2013).

By co-immobilizing tyrosinase with a serine esterase on a gold electrode, it is possible to establish a multistep reaction pathway that allows the activity of the esterase to be determined indirectly via measurement of *o*-quinone reduction at the electrode. The molecular architecture of a bi-enzyme sensor interface is shown schematically in Figure 63.13 (Kohli et al., 2010).

In practice, the fabrication of these bi-enzyme biosensors is relatively straightforward. Gold electrodes are scrupulously cleaned in "Piranha solution" ((concentrated) H_2SO_4: 30% (w/w) H_2O_2, 7:3 (v/v)) and then dipped in a 5 mM solution of thioctic acid in ethanol for 30 min. The electrodes are washed with ethanol, dried under nitrogen, and dipped in positively charged poly-L-lysine (PLL) solution for 45 min. The PLL solution is prepared by adding 12 mg of PLL in 50 mL of 20 mM phosphate buffer (pH 8.5). The electrodes are then rinsed with water and dipped in an aqueous solution of negatively charged tyrosinase (0.2 mg/mL) for 1 h. The last two steps are repeated 3.5 times to create 3.5 PLL-Tyr bilayers, with PLL being the topmost layer. The electrodes are washed with water and dipped in a 0.1 mg/mL solution of a serine esterase (e.g., AChE, BChE, or NEST) in 100 mM phosphate buffer, pH 7.0, for 1 h. The electrodes are then washed with water, dried under nitrogen, and dipped in phosphate buffer (0.1 M, pH 7.0) for testing. All steps are performed at room temperature (approximately 22°C) (Kohli et al., 2007a).

Electrochemical Measurements of Serine Esterase Activity

The electrochemical properties of serine esterase-containing biosensor interfaces are readily characterized. Each biosensor is immersed in a stirred buffer solution and maintained at a potential of − 100 mV (compared with an Ag/AgCl reference electrode). For example, NEST activity was measured indirectly via the output current of the electrode for a variety of phenyl valerate concentrations. As shown in Figure 63.14, the first step involves the diffusion of the ester (e.g., phenyl valerate) through a stagnant film from bulk solution to the enzyme layer. The NEST enzyme then hydrolyzes phenyl valerate to phenol, which is then oxidized to *o*-quinone by tyrosinase. Reduction of *o*-quinone at the surface of the electrode generates a current and regenerates catechol, which can then be reoxidized by tyrosinase. This internal recycling between catechol and *o*-quinone provides a mechanism for signal amplification, thereby enhancing biosensor sensitivity (Kohli et al., 2007a).

The response of the biosensor to aliquots of ester can be measured at a variety of pH values and applied potentials. For a typical NEST biosensor, the highest signal-to-background ratio is obtained at pH 7.0 and a working electrode potential of − 0.1 V relative to an Ag/AgCl reference electrode. At this potential, addition of phenyl valerate to a stirred aqueous solution triggers a rapid increase in the NEST biosensor signal, with a time constant of approximately 5 s. BChE can be substituted for NEST, with a similar current versus time response using phenyl valerate as substrate. For AChE, phenyl acetate is a better substrate than phenyl valerate. Current increases linearly with phenyl acetate concentration ($R^2 = 0.989$) in the range of 0.5–16 μM, reaching saturation at approximately 40 μM. Current versus time response and concentration calibration curves similar to those obtained for NEST and AChE can be obtained for BChE in the bi-enzyme electrode. Sensitivities of the electrodes obtained from the slopes of the initial linear portions of the concentration calibration curves were found to be 180, 25, and 87 $nA\mu M^{-1}cm^{-2}$ for AChE, BChE, and NEST, respectively, when AChE used phenyl acetate and when BChE and NEST used phenyl valerate as substrate. Control experiments were also performed in which each of the substrates was delivered to a gold electrode containing only PLL-Tyr bilayers. The current sensitivities obtained in these control experiments were negligibly small—always less than 0.5 $nA\mu M^{-1}cm^{-2}$—indicating that the signal is mediated by the esterase activity (Kohli et al., 2007a).

The ability of the biosensors to detect chemical agents was confirmed by adding a known serine esterase inhibitor, phenylmethylsulfonyl fluoride, to the solution. The electrode response decreased in a

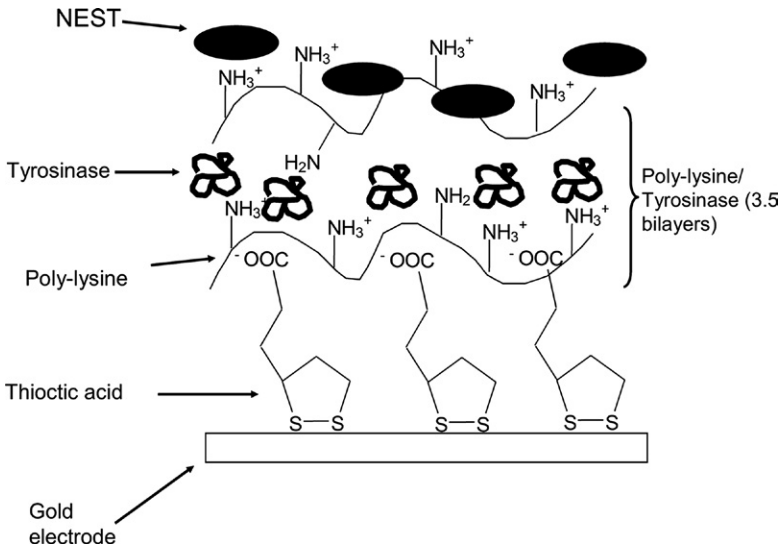

FIGURE 63.13 Molecular architecture of a bi-enzyme biosensor electrode containing tyrosinase and NTE catalytic domain (NEST). *Source: Reproduced with permission from Kohli et al. (2007a).*

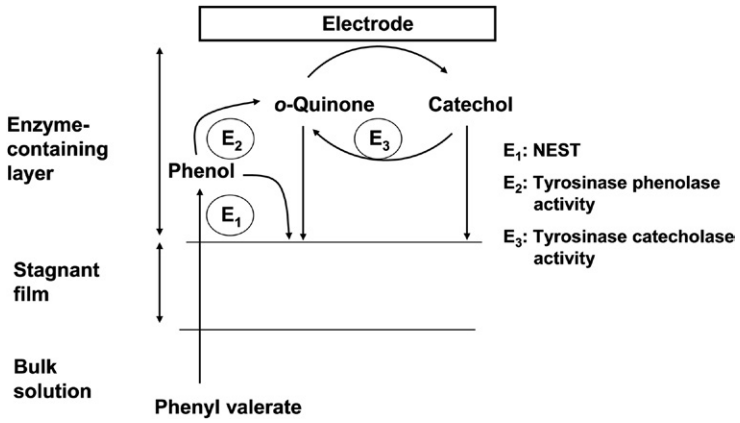

FIGURE 63.14 Schematic representation of the pathway that leads to the generation of current in the bi-enzyme biosensor containing tyrosinase and NTE catalytic domain (NEST). *Source: Reproduced with permission from Kohli et al. (2007a).*

concentration-dependent manner (Kohli et al., 2007a). Using a combination of biosensors containing different target enzymes (e.g., AChE and NEST), it would be possible to obtain real-time differential data that could be used to distinguish exposures to conventional versus DN agents.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Fortunately, DN agents have not yet been used in warfare or terrorism. However, their possible use must be considered seriously, because it is straightforward to design and synthesize these compounds based on established methods in OP chemistry. Thus, a conventional insecticide or nerve agent can be reengineered to produce a DN agent. The use of DN agents would be catastrophic, because there are no accepted means of prophylaxis or treatment, and the effects are devastating and permanent. NTE is the presumptive target for initiation of OPIDN. Accordingly, lymphocyte NTE has been used as a biomarker of exposure, and the NTE catalytic domain (NEST) has been incorporated into a biosensor for detection of DN agents. However, the precise physiological function of NTE and its role in the pathogenesis of OPIDN remain to be elucidated. Therefore, further mechanistic understanding is needed to improve on existing biomarkers and biosensors as well as to develop preventive and therapeutic measures.

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CHAPTER

64

Biomarkers of Exposure to Organophosphorus Poisons: A New Motif for Covalent Binding to Tyrosine in Proteins That Have No Active Site Serine

Oksana Lockridge, Lawrence M. Schopfer and Patrick Masson

INTRODUCTION

Schaffer et al. (1954) reported the astonishing finding that the irreversible inhibition of eel acetylcholinesterase (AChE) by the organophosphorus (OP) agent, diisopropylfluorophosphate (DFP), was the result of covalent binding to serine. In 1959, horse butyrylcholinesterase (BChE) that had been inactivated by treatment with DFP was found to have diisopropylphosphate-labeled serine in the sequence FGESAGAAS (Jansz et al., 1959). Despite this evidence, serine was not immediately accepted as the esteratic site of cholinesterases, because the pKa of serine was so high that significant reaction with OP agents was not expected (Bergmann, 1955). The crystal structures of AChE and BChE confirmed that OP agents make a covalent bond with serine. These structures also provided an explanation for the special reactivity of the active site serine (Sussman et al., 1991; Nachon et al., 2005). Nearby histidine and glutamic acid form a catalytic triad with serine that lowers the pKa of the serine, consistent with the expectation that the hydroxy-anion of serine is necessary to make a nucleophilic attack on the phosphorus. The oxyanion hole activates the OP for attack by the serine.

The covalent bond between OP and the active site serine of intact cholinesterase is stable, but not irreversible. Hydrolysis can occur with a half-life of between 10 and 35,000 min, depending on the enzyme, OP, temperature, pH, and buffer composition. The adduct becomes irreversibly bound to the enzyme after one of the alkyl groups on the OP is lost in a step called *aging* (Benschop and Keijer, 1966; Michel et al., 1967). The dealkylated OP makes a stable salt bridge with the protonated histidine of the catalytic triad, so that histidine is no longer available for the dephosphorylation step that would otherwise have restored the enzyme to an uninhibited state. Hundreds of scientists have contributed to this understanding of the mechanism of OP inhibition of AChE and BChE activity. Their studies are the foundation for the use of AChE and BChE as biomarkers of OP exposure.

OP-inhibited AChE and BChE are the established biomarkers of OP exposure (Gupta and Milatovic, 2012, 2014). The special features that make them good biomarkers are the following: (i) they react rapidly with OP at low concentrations; (ii) symptoms of acute toxicity always correlate with inhibition of AChE and BChE; (iii) AChE is present in human red blood cells, while BChE is present in human plasma, making it possible to test for OP exposure by measuring enzyme activity in a blood sample; (iv) the OP adduct (nonaged and aged) for common pesticides and nerve agents is relatively stable, making it possible to detect exposure days after the actual event; and (v) the mechanism of irreversible inhibition of AChE and BChE activity by OP is understood.

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USE OF AChE AND BChE BIOMARKERS IN THE CLINIC

Most hospitals and forensic laboratories are capable of performing cholinesterase activity assays. Two examples of the usefulness of AChE and BChE activity assays are given next.

Tokyo Subway Attack with Sarin

AChE and BChE were useful biomarkers for identifying the poison that intoxicated more than 5000 people in the Tokyo subway. In March 1995, members of the Aum Shinrikyo sect dispersed the nerve agent sarin in trains on the Tokyo subway. Japanese health workers identified the poison as a cholinesterase inhibitor within 2h of seeing the first patient (Nozaki and Aikawa, 1995). A cholinesterase inhibitor was suspected because victims had physiological signs of cholinergic intoxication, including pinpoint pupils. Laboratory assays showed that red blood cell AChE and plasma BChE activities were inhibited, confirming that the poison was a cholinesterase inhibitor. There are many cholinesterase inhibitors, including OP pesticides and carbamates (CMs). In the case of some OPs, AChE inhibiting compounds are precursor molecules that need bioactivation before they are toxic to humans. The rapid onset of toxic symptoms meant that the agent used in the subway was already activated or a direct AChE inhibitor was involved that needed no activation. The fact that people were becoming intoxicated by breathing the air meant that the poison was volatile. These characteristics suggested that the poison was a nerve agent, very likely sarin. The Forensic Science Laboratory used gas chromatography–mass spectrometry (GC–MS) to identify sarin in crime scene samples (Seto, 2001). Identification of the poison as sarin was crucial to the police in their search for the perpetrators. Years later, new MS methods were developed and used to retroactively identify sarin bound to red blood cell AChE (Nagao et al., 1997) and to plasma BChE from the Tokyo subway victims (Polhuijs et al., 1997; Fidder et al., 2002).

Suicide Attempts

Exposures to OP pesticides are declining in the United States following the banning of chlorpyrifos and diazinon for residential use in 2000 (Sudakin and Power, 2007). In 2004, the number of OP exposure cases reported to the American Association of Poison Control Centers (AAPCC) was 7,181, with 4 fatalaties (Bronstein et al., 2007). In 2012, the number of cases was 3,179, with 1 fatality (Mowry et al., 2013). The subjects intentionally ingested OP pesticides for the purpose of committing suicide. In contrast, the ingestion of OP pesticides in suicide attempts is a frequent occurrence in rural communities of Sri Lanka, India, and China, where an estimated 200,000 persons die annually from OP pesticide poisoning (Eddleston and Dawson, 2012). Diagnosis is made on the basis of cholinergic signs of toxicity, smell of pesticides or solvents, and reduced BChE and AChE activity in blood. Patients who survive the suicide attempt are monitored in the hospital for several days; their plasma BChE is assayed daily because recovery of BChE activity is a marker of OP elimination from the body.

METHODS TO DETECT OP ADDUCTS ON AChE AND BChE

Cholinesterase Activity Assay

Exposure to a toxic dose of OP results in inhibition of AChE and BChE activities. The most common method to measure OP exposure is to assay AChE and BChE activities in blood using a spectrophotometric method (Ellman et al., 1961; Worek et al., 1999; Wilson et al., 2005). The drawbacks of activity assays are that they do not identify the OP agent in question. They show that the poison is a cholinesterase inhibitor, but they do not distinguish between nerve agents, OP pesticides, CM pesticides, and tightly bound, noncovalent inhibitors like tacrine and other anti-Alzheimer drugs. In addition, low-dose exposure, which inhibits less than 20% of the cholinesterase, cannot be determined by measuring AChE and BChE activity because individual variability in activity levels is higher than the percentage of inhibition.

Fluoride Reactivation Followed by GC–MS

A new method for identifying exposure to nerve agents was introduced by Polhuijs et al. (1997). The method is based on the finding that incubation of sarin-inhibited BChE with 2M potassium fluoride at pH 4 results in the release of sarin. Sarin is then extracted and analyzed by GC–MS. Polhuijs et al. (1997) applied their new method to positively identify sarin in serum samples from Japanese victims of the Tokyo subway attack. This method has the advantage that it positively identifies nerve agents and other OPs (van der Schans et al., 2004). The method has been validated by Adams et al. (2004), who found that potassium fluoride released sarin and soman from human BChE, as well as from covalent attachment to human albumin.

Identification of OP-BChE Adducts by Electrospray–Ionization Tandem MS

In clinical diagnosis of OP exposure, the tissue most readily available for study is blood. OP adducts of BChE are better candidates for study than OP adducts on

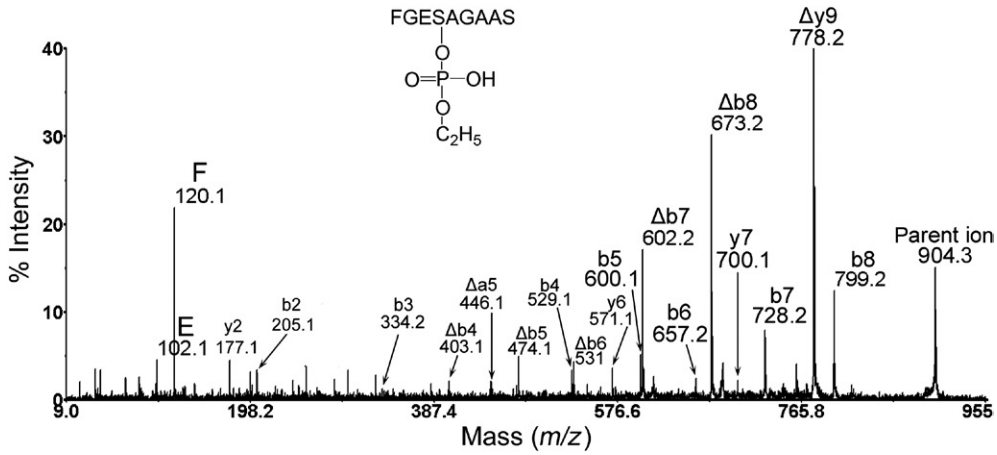

FIGURE 64.1 MS/MS spectrum of the active site peptide of human AChE, covalently bound to tabun and dealkylated by aging or by acid. The identical modified peptide is derived from human BChE treated with tabun, chlorpyrifos oxon, paraoxon, or diazoxon. The Δ symbol indicates ions that have lost the OP and a molecule of water, converting serine to dehydroalanine. The peak height for 778.2 m/z was 2.5-fold higher than indicated; it was shortened to show the less intense ions.

AChE for the following reasons. Human blood contains 5 mg of BChE and 0.5 mg of AChE per liter. The BChE is in plasma, whereas the AChE is bound to the membranes of red and white blood cells. Most OPs, with the exception of chemical warfare nerve agents, react more rapidly with BChE than with AChE.

Fidder et al. (2002) introduced an electrospray–ionization tandem MS method for diagnosing OP exposure by measuring the mass of the OP-labeled active site peptide of human BChE. His starting material was 0.5 mL of human plasma from a victim of the Tokyo subway attack. The mass of the active site peptide was higher by 120 atomic mass units than the mass of the unlabeled active site peptide. This added mass was exactly what was expected from sarin. The peptide's MS/MS fragmentation spectrum yielded the sequence of the peptide and verified that the OP label was on serine 198, the active site serine. Two important technical details described by Fidder et al. (2002) are the advantage of digesting with pepsin rather than trypsin, and the fact that during collision-induced dissociation, the OP-labeled serine decomposes to dehydroalanine, with loss of the OP plus a molecule of water.

Pepsin digestion of BChE is preferred because pepsin yields a 9-residue active site peptide $FGES_{198}AGAAS$, whereas trypsin digestion yields a 29-residue peptide $SVTLFGES_{198}AGAASVSLHLLSPGSHSLFTR$. Short peptides give a more intense signal in the mass spectrometer than long peptides. Figure 64.1 shows the MS/MS spectrum of the active site peptide FGESAGAAS, modified on serine 198 by monoethoxyphosphate. The 904.3 m/z parent ion is particularly interesting because it represents the deamidated (aged) tabun adduct, as well as the dealkylated (aged) chlorpyrifos oxon adduct, and

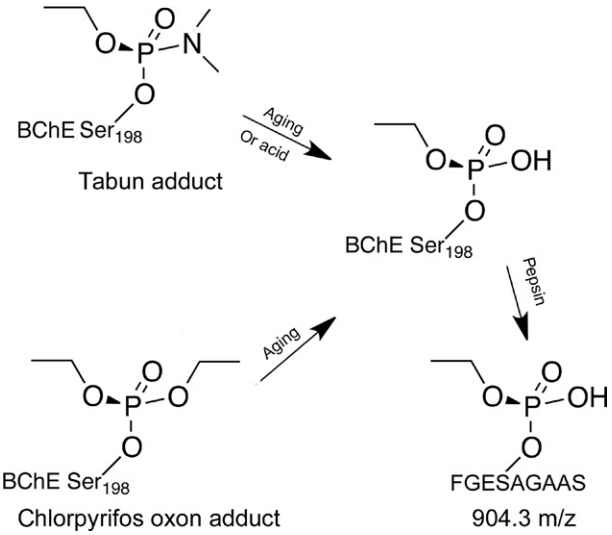

FIGURE 64.2 Aging or exposure to acid during pepsin digestion results in deamidation of the initial tabun adduct. Aging dealkylates the initial chlorpyrifos oxon adduct. Pepsin digestion yields the identical 904.3 m/z FGESAGAAS peptide covalently modified by monoethoxyphospate from human AChE, human BChE, and horse BChE inhibited by tabun, chlorpyrifos oxon, diethyl paraoxon, diazoxon, or other diethyxophosphate pesticides.

adducts with other common activated pesticides, including diethyl paraoxon and diazoxon (Figure 64.2). Thus, exposure to the nerve agent tabun yields an adduct that is indistinguishable from exposure to common pesticides. Another notable feature of the peptide in Figure 64.1 is that pepsin digestion of human AChE (Swiss protein # P22303), human BChE (Swiss protein # P06276), and horse BChE (NCBI # gi 7381418) yields the same FGESAGAAS active site peptide.

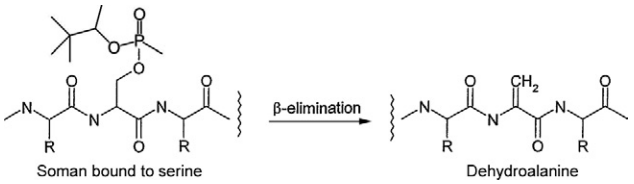

FIGURE 64.3 OP-serine loses the OP (soman in this case) plus a molecule of water when the peptide is fragmented in the mass spectrometer. This beta-elimination reaction converts OP-serine to dehydroalanine.

The acid condition for pepsin digestion causes the phosphorus–nitrogen bond to break in adducts with tabun and iso-OMPA (tetraisopropyl pyrophosphoramide). This can be used to distinguish between the reactions with Sp and Rp stereoisomers of tabun (Jiang et al., 2013a).

Mass spectrometers convert a majority of the OP-labeled serine to dehydroalanine during acquisition of MS/MS spectra. For example, all ions labeled with the Δ symbol in Figure 64.1 have dehydroalanine in place of OP-serine. Investigators who are not aware of this gas phase chemistry have a difficult time interpreting their MS/MS spectra. The conversion of OP-serine to dehydroalanine is called *beta-elimination.* The beta-elimination reaction is well documented for phosphoserine peptides (Tholey et al., 1999). Figure 64.3 shows a schematic diagram of the beta-elimination reaction for soman-labeled serine in human BChE. The ease with which the OP-serine bond breaks during collision-induced dissociation is indicated by the fact that the most intense ion in Figure 64.1 is the 778.2 m/z ion. The peptide at 778.2 m/z is the parent ion that has lost the OP and 1 molecule of water. However, serine is not restored. A molecule of water is abstracted along with the OP, converting the active site serine to dehydroalanine.

Fidder et al. (2002) showed that MS could detect adducts on human BChE with sarin, soman, dimethyl paraoxon, diethyl paraoxon, and pyridostigmine when purified BChE was treated with these poisons. Studies from other laboratories confirmed that OP-BChE adducts could be identified by MS (Tsuge and Seto, 2006; Sun and Lynn, 2007; Li et al., 2008b).

Single-Step Purification of BChE from Human Plasma

A technical difficulty with MS of OP adducts on BChE, in a clinical setting, is the need to enrich plasma samples for BChE to make it possible to detect the labeled peptide. Previously, the BChE protein was enriched from plasma by affinity chromatography on procainamide Sepharose (Fidder et al., 2002; Li et al., 2009b; Liyasova et al., 2011; van der Schans et al., 2013). In this case, the level of purity was inadequate when procainamide affinity chromatography was the only enrichment step. We had to further enrich by using gel electrophoresis or ion exchange chromatography, or by selectively extracting the OP-labeled active site peptide on titanium oxide microcolumns (Jiang et al., 2013c). A big advance was the introduction of a single-step purification method by binding to an immobilized monoclonal (Sporty et al., 2010). The commercially available monoclonal 3E8 (HAH 002-01-02 from Thermo Scientific Pierce) selectively extracts BChE from human plasma when the monoclonal is immobilized on Dynabeads Protein G (Sporty et al., 2010; Carter et al., 2013) or on Dynabeads epoxy (Marsillach et al., 2011). Two other monoclonals, mAb2 (created in the laboratory of Jacques Grassi) and B2 18-5 (created in the laboratory of Steven Brimijoin), also immunopurify BChE from plasma in a single step (Schopfer et al., 2014). BChE is released from the antibody with acetic acid. Figure 64.4 is a Coomassie-stained gel showing the effectiveness of immunopurification of BChE from plasma in a single step. A band at 85 kDa for the BChE monomer is visible. The major contaminating proteins are immunoglobulins. Immunopurified BChE released with acid is suitable for MS analysis of adducts, but it is not suitable for applications that require active native BChE enzymes because acid denatures the BChE protein.

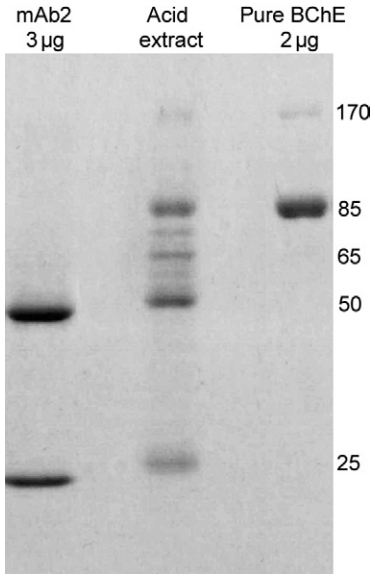

FIGURE 64.4 SDS gel stained with Coomassie Blue shows BChE purified by binding to immobilized monoclonal mAb2. The monoclonal was crosslinked to CNBr-Sepharose. About 98% of the BChE in 0.5 mL of plasma bound to 0.04 mL of beads. The BChE was released from beads with 0.4 M acetic acid. The band at 85 kDa is the BChE monomer, while the band at 170 kDa is the BChE dimer. Immunoglobulin heavy (50 kDa) and light (25 kDa) chains are major contaminants.

WHY ARE NEW BIOMARKERS NEEDED?

Not All OPs Inhibit AChE

Tri-o-cresyl-phosphate (TOCP), the contaminant in a homemade liquor called "Ginger Jake," which is responsible for delayed neuropathy and paralysis of the legs, is bioactivated in a form that inhibits neuropathy target esterase (NTE) and BChE, but not AChE at nonlethal doses (Casida and Quistad, 2004; Glynn, 2006). Large structures with a 12–20 carbon alkyl chain on the phosphorus atom inhibit fatty acid amide hydrolase but not AChE (Casida and Quistad, 2004). These examples show that OPs that do not affect AChE can react with other proteins, sometimes generating toxic symptoms. These examples also suggest that additional, unknown OP targets may exist that are sensitive to OP that do not affect cholinesterases. In order to detect exposure to such OPs, biomarkers other than cholinesterases are needed.

OP doses too Low to Inhibit AChE Cause Toxicity

Workers in India ($n = 59$) engaged in the manufacture of quinalphos had normal red cell AChE activity but complained of generalized weakness and fatigue. They had significantly low scores for memory, learning ability, and vigilance compared to controls. Plantar reflexes were abnormal in 50% of the workers. The average age of the workers was 30 ± 6 years, and the average duration of exposure to quinalphos was 5.7 years. There was no history of acute poisoning. The plant was situated in large tin sheds without adequate ventilation (Srivastava et al., 2000). The most logical explanation for their symptoms was low-dose exposure to OP.

Family tobacco farmers in Brazil ($n = 37$) used chlorpyrifos and acephate for 3 months a year, for 5.4 h a day. The average duration of exposure was 18 years. Their plasma cholinesterase activity was within the normal range and was not different between on- and off-exposure periods. Clinically significant extrapyramidal symptoms were present in 12 subjects during the pesticide application season, though this number was reduced to 9 after 3 months without exposure. Generalized anxiety disorder was diagnosed in 13 subjects, and major depression in 8 subjects. After 3 months without OP exposure, the number of subjects with psychiatric disorders declined to about half (Salvi et al., 2003). The most logical explanation for their symptoms was low-dose exposure to OP.

More examples of chronic low dose, subclinical exposure to OPs in humans, leading to chronic neurotoxicity, are cited in a number of studies (e.g., Abou-Donia, 2003; Kamel and Hoppin, 2004).

Animal studies have been directed at understanding the mechanism of low-dose OP toxicity. The studies agree that doses too low to inhibit AChE activity nevertheless have adverse effects on the animals, including disruption of adenylyl cyclase signaling (Song et al., 1997), hyperphosphorylation of calcium/cyclic adenosine monophosphate (cAMP) response element binding protein (Schuh et al., 2002), airway hyperactivity (Lein and Fryer, 2005), changes in expression levels of fibroblast growth factor in the brain (Slotkin et al., 2007), changes in serotonin receptors (Slotkin et al., 2008), inhibition of acylpeptide hydrolase (Richards et al., 2000), and disruption in lipid metabolism (Medina-Cleghorn et al., 2013).

Rats chronically treated with low doses of chlorpyrifos or DFP have long-term cognitive deficits in the absence of clinical signs of exposure (Jett et al., 2001; Terry et al., 2007, 2014). The mechanism for impairment of cognitive function may involve disruption of the microtubule transport of vesicles, organelles, and other cellular components that are synthesized in the neuronal cell body and moved down long axons to presynaptic sites (Gearhart et al., 2007).

A possible, noncholinesterase-based explanation for the neurotoxic symptoms observed in humans from the previous examples is that workers were also exposed to a combination of chemicals, including heavy metals, solvents, herbicides, and fumigants. The symptoms may actually have been caused by these chemicals and not by the OP (Kamel and Hoppin, 2004). However, the animal data cannot be explained this way because the animals were treated only with OP agents. In conclusion, OP targets that are not AChE or BChE are involved in chronic neurotoxicity. These unknown targets bind OP at doses too low to inhibit AChE.

Only Some People Have Symptoms

Not every person who has been chronically exposed to OP exhibits symptoms. For example, of 612 sheep dippers (workers who dip sheep into a chemical bath to kill insects in the wool) exposed to diazinon twice a year for 3 days at a time for 40 years, only 19% reported symptoms. In a control group of ceramics workers who had not been exposed to OPs, 5% reported similar symptoms (Pilkington et al., 2001).

The susceptibility of a minority of the population may be explained by variations in genes affecting OP metabolism. The best-studied example is paraoxonase, an enzyme that inactivates OPs (La Du et al., 2001; Furlong, 2007; Costa et al., 2013). Paraoxonase polymorphism in humans is proposed to explain why some people are resistant to OP toxicity, while others are susceptible. Another enzyme that may be involved in resistance to OP toxicity is BChE. BChE scavenges OPs, eliminating the poison before it reaches sites where it could cause harm (Doctor et al., 1991). Humans have a wide range of BChE activities, with some people having none whatsoever due to genetic variation (Manoharan et al., 2007). It is possible,

but unproven, that people with BChE deficiency are more susceptible to OP toxicity. Polymorphisms in liver cytochrome P450 enzymes may also contribute to individual susceptibility to OP toxicity. Specific cytochrome P450 enzymes bioactivate the parent organophosphorothioates to the highly toxic oxon forms. Other cytochrome P450 enzymes detoxify OPs by dearylation (Hodgson and Rose, 2007). The concentration of each isozyme varies among individuals. Thus, the relative rates for activation and detoxification and the circulating levels of the toxic form of the OP will vary between individuals.

Another possibility for variable susceptibility in the population could be genetic variation in the yet-unknown OP targets that are responsible for the symptoms. Some forms of these targets may be more sensitive to OPs than are others.

Toxic Symptoms Depend on the OP

High doses of OPs cause similar toxic effects, independent of the identity of the OPs. However, low-dose effects are not identical for all OPs (Moser, 1995). For example, a low dose of fenthion decreased motor activity in rats by 86% but did not alter the tail-pinch response, whereas a low dose of parathion did not affect motor activity but did decrease the tail-pinch response. In another example, rats given doses of different OPs that inhibited AChE to similar levels had more severe toxicity when the OP was parathion than when it was chlorpyrifos (Pope, 1999). Toxicological studies such as these have led to the conclusion that sites in addition to cholinesterase are targets of OPs. New biomarkers for these effects of OPs are needed.

In summary, AChE and BChE are not the only proteins modified by OP exposure in humans. Neurotoxicity from low doses of OPs may be explained by OP modification of heretofore unidentified proteins. Toxic symptoms from low-dose exposure to a particular OP are not identical to toxic symptoms from another OP, suggesting that the set of proteins modified by a particular OP does not overlap completely with the set of proteins modified by a different OP. Identification of new biomarkers of OP exposure could lead to new assays for OP exposure, and could lead to an understanding of the causes of low-dose toxicity.

NEW BIOMARKERS

Beta-Glucuronidase in Rat Plasma

A carboxylesterase in rat liver microsomes called *egasyn* is tightly linked to beta-glucuronidase (BG) by a complex. When egasyn binds OP, it releases BG into the blood. A single oral dose of chlorpyrifos (10 mg/kg) increased the level of BG activity in rat blood 100-fold within 2 h (Fujikawa et al., 2005). The BG activity decreased to control levels by 24 h. Thus, increased levels of BG in plasma may serve as a biomarker for OP exposure. However, increase in plasma BG activity has not been validated in other animal species or in humans. For further details on BG, refer to Satoh et al. (2010).

Acylpeptide Hydrolase in Rat Brain

Acylpeptide hydrolase is a member of the serine hydrolase family. It deacetylates the acetylated N-terminus of polypeptides. Rat brain acylpeptide hydrolase was inhibited 93% at a dose of dichlorvos (4 mg/kg, i.p.) which inhibited AChE only 47%. The *in vitro* sensitivity of acylpeptide hydrolase to chlorpyrifosmethyl oxon, dichlorvos, and DFP (IC_{50}) was 6–10 times greater than that of AChE (Richards et al., 2000). Acylpeptide hydrolase is also found in human erythrocytes, where it could potentially serve as a biomarker for low-dose exposure to OP in humans (Quistad et al., 2005), though human cases of OP exposure have not yet been tested for OP-modified acylpeptide hydrolase.

Albumin in Mouse and Guinea Pig Plasma

Mice treated with a nontoxic dose of a biotin-tagged OP called fluorophosphate (FP)–biotin had FP-biotinylated albumin in blood and muscle (Peeples et al., 2005). *In vitro* experiments identified the site in human albumin for covalent attachment of a variety of OPs as tyrosine 411 (Li et al., 2007).

Guinea pigs treated with the nerve agents soman, sarin, cyclosarin, or tabun had nerve agent-labeled albumin in their blood (Williams et al., 2007). The OPs were bound to tyrosine. The tabun-tyrosine and soman-tyrosine adducts were detected in blood 7 days postexposure, indicating that the adducts are stable. The adducts did not undergo aging and were not released from tyrosine by treatment of the guinea pigs with oxime, which is a common treatment for OP exposure that induces release of OP from AChE. MS identified OP-albumin adducts in rats treated with 1/5 LD_{50} of paraoxon (Gladilovich et al., 2010).

These examples show that OPs can bind covalently to albumin under physiological conditions, and that the resultant adducts are relatively stable. OP-albumin adducts could therefore be useful as biomarkers of OP exposure. In addition, unlike cholinesterases, the soman-albumin conjugate does not age (Li et al., 2008a), making it possible to discriminate between sarin and soman exposure.

Albumin in Human Plasma is a Biomarker of OP Exposure

The animal studies described previously provided the rationale for testing whether humans poisoned by OP have detectable OP-albumin adducts. To date, three

studies provide MS evidence that plasma from humans who ingested dichlorvos (Li et al., 2010b) or chlorpyrifos (Li et al., 2013b; van der Schans et al., 2013) contains OP-albumin adducts. Blood drawn as late as 49 days after exposure had detectable levels of OP adducts on tyrosine (van der Schans et al., 2013). Though the chlorpyrifos-poisoned patients had been treated with oximes to reverse OP binding to AChE, their albumin had detectable levels of OP adducts. This suggests that oximes do not reverse OP binding to albumin.

M2 Muscarinic Receptors in Heart and Lung

The ^{3}H-chlorpyrifos oxon binds covalently to rat heart M2 muscarinic receptors (Bomser and Casida, 2001). The site of attachment has not been identified. When guinea pigs were treated with chlorpyrifos, diazinon, or parathion at doses too low to inhibit AChE activity, the M2 muscarinic receptors lost their ability to inhibit acetylcholine release from parasympathetic nerves, causing bronchoconstriction (Lein and Fryer, 2005).

COVALENT BINDING OF OP TO TYROSINE

In a 1963 Pedler lecture, Sanger reported that ^{3}H-DFP makes a covalent bond with tyrosine in the sequence Arg*Tyr*ThrLys from human and rabbit albumin (Sanger, 1963). MS of human albumin treated with soman, chlorpyrifos oxon, FP-biotin, dichlorvos, and DFP confirmed OP modification on Tyr 411 in peptide LVry*tkKVPQVSTPTL (Li et al., 2007, 2008a), where the lowercase letters show Sanger's sequence and the asterisk indicates the labeled tyrosine.

At the time that we confirmed Sanger's observations, we thought that albumin was a special case, though we were aware that papain and bromelain had also been reported to bind ^{3}H-DFP on tyrosine (Murachi et al., 1965; Chaiken and Smith, 1969).

We soon learned differently. In a general search for proteins that bind OP, we treated live mice and mouse tissues with FP-biotin, a modified OP designed for identifying unknown targets of OP reaction. The FP-biotinylated proteins were isolated on immobilized avidin, washed with 0.1% sodium dodecyl sulfate (SDS), and separated on an SDS gel. Coomassie Blue-stained bands were excised and digested with trypsin and the proteins in the bands were identified by MS. Surprisingly, the majority of proteins that were identified were not serine esterases or proteases, which are the classical targets for OPs, nor did the identified proteins contain the consensus sequence GXSXG, which is characteristic of an active site serine. Typically, we found high-abundance proteins such as albumin and tubulin.

Though biotin–avidin complexation has been used widely to isolate specific targets, it is notorious for generating false-positive identifications. To rule out the possibility that our results were an artifact, we set out to find the OP-labeled peptide. We reasoned that convincing proof for OP labeling required identification of the labeled peptide and the labeled amino acid.

During years of MS analysis, we had consistently identified OP-labeled tubulin in mouse brain. Therefore, we studied pure bovine tubulin (Cytoskeleton, Inc., Denver, CO) by treating it with soman, sarin, FP-biotin, DFP, chlorpyrifos oxon, and dichlorvos. We isolated the OP-labeled tryptic peptides and analyzed them by fragmentation in the QTRAP 2000 and QTRAP 4000 mass spectrometers. We identified five OP-labeled peptides in tubulin (Grigoryan et al., 2008). In every peptide, the OP was covalently attached to tyrosine.

Similar MS experiments with pure human and mouse transferrin (Li et al., 2009a), and with human kinesin showed that the OP label was consistently on tyrosine. Studies with human plasma identified OP labeling on tyrosine in apolipoprotein and alpha-2-glycoprotein. Aggressive treatment of human albumin with FP-biotin and chlorpyrifos oxon led to the identification of seven OP-labeled tyrosines (Ding et al., 2008). Finally, we found that small synthetic peptides made a covalent bond with DFP, chlorpyrifos oxon, dichlorvos, and soman (Li et al., 2009a). For example, incubation of peptide RYGRK (ArgTyrGlyArgLys) with soman yielded the pinacolylphosphonate-modified peptide (Li et al., 2013a). MS analysis conclusively proved that the OP was attached to tyrosine.

MOTIF FOR OP BINDING TO TYROSINE

Comparison of the sequences of OP-labeled peptides shows no consensus sequence around the tyrosine to which the OP binds. What the peptides do have in common is the presence of a positively charged arginine, lysine, or histidine within five amino acids of the labeled tyrosine, most being within three amino acids. We suggest that these positively charged residues could interact with the phenolic hydroxyl of tyrosine to lower the pKa. Such ion-pair interactions have been shown to lower the pKa for the negatively charged partner, with a comparable rise of the pKa of the positively charged partner by as much as 4 pKa units (Johnson et al., 1981). Tyrosines with a lower pKa value would be better nucleophiles and thus better able to attack OPs.

In conclusion, most proteins that we have examined in detail using sensitive MS techniques have shown the capacity to become labeled by OP on tyrosine. However, only certain tyrosines in a protein are labeled (i.e., those on the surface and near a positively charged residue that

could potentially activate the phenolic hydroxyl group). The finding that even small synthetic peptides can be labeled by OP on tyrosine has led to the hypothesis that OP labeling on tyrosine is a general phenomenon. We propose that OP labeling on tyrosine is a new motif for OP binding to proteins.

CHARACTERISTICS OF OP BINDING TO TYROSINE

On-Rate

Little is known about the rate of OP binding to tyrosine because the recognition of this OP binding motif is new. Soman binding to Tyr 411 in human albumin has been measured and has been found to be slow, with a bimolecular rate constant of $15 \pm 3\,M^{-1}min^{-1}$ (Li et al., 2008a). We expect that other proteins will be identified whose rate of OP binding to tyrosine will be faster.

Off-Rate

The OP adduct on tyrosine 411 of human albumin is stable. The half-life for decay of the soman–Tyr 411 adduct is 20 days at pH 7.4, 22°C (Li et al., 2008a). The chlorpyrifos oxon–Tyr 411 adduct is even more stable. After 7 months at 22°C in pH 7.4 buffer, 80% of the Tyr 411 was still labeled with diethoxyphosphate, which is the adduct formed by chlorpyrifos oxon. However, at pH 8.3 and 22°C, 50% had lost the OP label in 3.6 months. OP-albumin adducts stored at −80°C are stable indefinitely at pH 8.3, 7.4, and 1.5. One advantage of a stable OP-tyrosine adduct is that it will survive in an animal long enough to allow the generation of antibodies. Another advantage of a stable OP-tyrosine adduct is that detection of OP can be made on samples long after exposure.

No Aging

OPs bound to tyrosine do not age. Aging of OP adducts on AChE and BChE is defined as the loss of an alkoxy group from the phosphorus atom. No masses representing aged OP-tyrosine adducts have been found.

Aging can confound the identification of the original OP. For example, aged sarin and soman adducts on AChE are indistinguishable because they yield the same methylphosphonate derivative. The absence of aging for OP-tyrosine adducts results in a species that is suitable for discriminating between sarin and soman adducts because the unaged adducts have different masses. Absence of aging for OP-tyrosine adducts has also been reported for albumin adducts in guinea pigs (Williams et al., 2007).

METHODS FOR DETECTING OP BINDING TO TYROSINE

MS for OP Adducts on Unknown Proteins

If the identity of the OP-labeled protein is unknown, a tagged OP (e.g., a biotinylated OP) can be used to identify the protein (Schopfer et al., 2005). After the identity of the OP-labeled protein is known, identification of the OP-labeled peptide depends on separating it from contaminating peptides. We have found that the OP-labeled peptide is frequently not found by MS unless it has been extensively purified. In some cases, it is possible to identify the labeled peptide simply by liquid chromatography–tandem MS (LC-MS/MS), where an enzymatic digest of the isolated protein is subjected to liquid chromatography (LC) on a C18 nanocolumn, and the effluent from the column is electrosprayed directly into the mass spectrometer. For other cases, offline high-pressure liquid chromatography (HPLC) purification of the enzymatic digest is necessary to obtain a purified fraction of peptides that can be introduced into the mass spectrometer.

Preliminary identification of the labeled peptides in HPLC fractions is made by mass, using matrix-assisted laser desorption/ionization (MALDI) MS (MALDI TOF–TOF 4800 from Applied Biosystems). The MS-Digest algorithm, available from the Protein Prospector website at the University of California, San Francisco (prospector.ucsf.edu/prospector/mshome.htm), is a useful tool for predicting the masses of peptides from a proteolytic digest both with and without the added mass from a particular OP. Another indispensable tool is the Fragment Ion Calculator (available from http://db.systemsbiology.net:8080/proteomicsToolkit/), which can be used to calculate the masses of ions generated during MS/MS fragmentation of a peptide. Our laboratory acquired MS/MS spectra with the MALDI TOF–TOF 4800, the QTRAP 4000, and Triple TOF 5600 mass spectrometers (AB-Sciex). Examples of MS/MS spectra for human albumin peptides labeled on tyrosine with chlorpyrifos oxon are shown in Figure 64.5.

Fluoride Treatment to Release OP from Albumin

The fluoride reactivation method has been applied to a human case of self-poisoning by chlorpyrifos (van der Schans et al., 2013). Blood drawn from the patient 49 days after she ingested chlorpyrifos yielded two products, both released from albumin with potassium fluoride. The released products were identified by LC-MS using multiple reaction monitoring (MRM). The products were diethoxyfluorophosphate (representing an albumin adduct with chlorpyrifos oxon) and

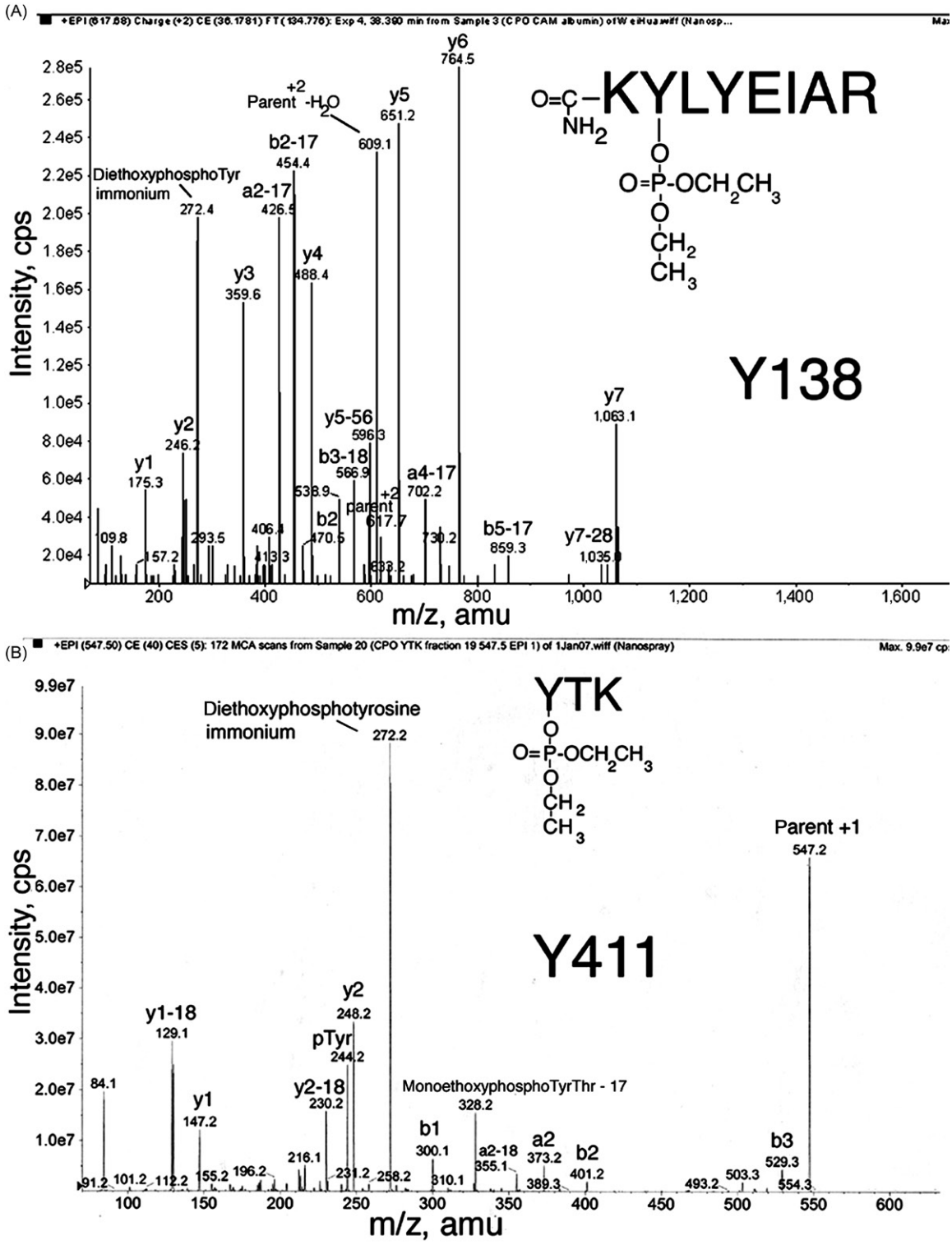

FIGURE 64.5 MS/MS spectra of human albumin peptides labeled on tyrosine with chlorpyrifos oxon. (A) The doubly charged parent ion (617.7 amu) for KYLYEIAR includes a mass of 136 amu from chlorpyrifos oxon and a mass of 43 amu from CM. Carbamylation was a by-product of denaturation in 8M urea. The presence of diethoxyphosphate on Tyr 138 is supported by the masses y7, a2—amine (17 amu), b2—amine (17 amu), b3—water (18 amu), a4—amine (17 amu), and b5—amine (17 amu), all of which include the 136 amu added mass for diethoxyphosphate. The ion at 272.4 amu is consistent with the mass of the diethoxyphosphotyrosine immonium ion; its presence supports chlorpyrifos oxon labeling of tyrosine. (B) The singly charged parent ion (547.2 amu) for YTK includes an added mass of 136 amu from chlorpyrifos oxon. The presence of diethoxyphosphate on Tyr 411 is supported by the masses of the b1 ion, the y2 ion, and the parent ion, all of which include the 136-amu mass of diethoxyphosphate. Furthermore, the characteristic ions at 272.2 amu for the diethoxyphosphotyrosine immonium ion, at 328.2 amu for the monoethoxyphosphoTyrThr ion minus amine (17 amu), and at 244.2 amu for phosphotyrosine support labeling on tyrosine. The loss of one or both alkyl groups from the diethoxyphospho adduct during collision-induced dissociation in the mass spectrometer is a common observation. *Source: Reprinted with permission from Ding et al. (2008). Copyright 2008 American Chemical Society.*

diethoxyfluorothiophosphate (representing an albumin adduct with the thiol form of chlorpyrifos). It is remarkable that the albumin adducts were still detectable 49 days after exposure, when the patient had recovered from the toxic effects.

MRM LC-MS/MS for OP Adducts When the Protein and the Modified Amino Acid Are Known

The most sensitive LC-MS/MS method for detection of OP exposure is a method called *multiple reaction monitoring* or *selective ion monitoring*. This method requires knowledge of the mass of the parent ion and masses of two or more product ions. The selection of product ions is determined experimentally with known samples because not all product ions give a good signal. The method is sensitive because the mass spectrometer selects only those ions that fit the given parent and product ions. An MS/MS spectrum is automatically acquired for samples that have the specified parent and product ions. The MS/MS spectrum allows the investigator to determine whether the ion is the OP peptide or whether it has the specified masses by coincidence. A list of the parent ion and product masses for nerve agent tyrosine adducts is published (Williams et al., 2007).

The MRM method has been used to detect nerve agent–tyrosine adducts in the blood of guinea pigs (Williams et al., 2007), carbofuran-BChE adducts in the blood of human subjects (Li et al., 2009b), OP-BChE adducts in the blood of humans poisoned by dichlorvos, chlorpyrifos, and aldicarb (Li et al., 2010a), OP-albumin adducts in the blood of humans poisoned by dichlorvos (Li et al., 2010b), nerve agent–tyrosine and nerve agent–BChE adducts in the blood of marmosets (Read et al., 2010), nerve agent–tyrosine in rat plasma (Bao et al., 2012), and OP-tyrosine adducts in the plasma of human subjects poisoned by chlorpyrifos (van der Schans et al., 2013).

The MRM method is sensitive and quantitative, but it has the disadvantage that unexpected adducts are not found. The investigator will find only known adducts. A new poison is likely to make adducts that are undetectable in an MRM method.

Pronase Digestion to Yield Single Amino Acids Modified by OPs

Digestion of OP-treated human plasma proteins with pronase yields free amino acids modified by OP (Black et al., 1999). Human plasma treated with sarin or soman contains OP-serine (from BChE) and substantially greater amounts of OP-tyrosine (from albumin and possibly other proteins as well; Black et al., 1999). This method can detect low levels of exposure because large volumes of plasma can be digested. The Porton Down laboratory has synthesized isotope-labeled internal standards (Williams et al., 2007). MRM methods have been published for the detection of nerve agent–tyrosine adducts (Williams et al., 2007; Bao et al., 2012).

Less than 1% of the albumin in plasma is modified by OP. A cleanup step is required that separates and concentrates the OP-tyrosine adduct, such as solid phase extraction (Williams et al., 2007; Read et al., 2010) or offline HPLC (Jiang et al., 2012), before the sample is analyzed by LC-MS/MS. However, the use of MRM in an LC-MS/MS protocol increases the sensitivity and specificity of detection, making it possible to identify OP-tyrosine in a pronase digest directly, without a cleanup step (van der Schans et al., 2013).

Enrichment of OP-Albumin Pepsin Peptides on PHOS-Select Iron Affinity Beads

Though several tyrosine residues on albumin can be modified by treatment with OP, the most reactive is tyrosine 411. Peptides VRY_{411}TKKVPQVST, and $LVRY_{411}$TKKVPQVST selectively bind to Fe(3+) beads at pH 11. Human plasma digested with pepsin and diluted with buffer to raise the pH to 11 is applied to a microcolumn of beads. The peptides are eluted with pH 2.6 buffer and identified by MS. The protocol enriches both the unmodified and OP-modified peptides, regardless of the identity of the OP, thus allowing quantitation of the OP-peptide relative to the unmodified peptide (Jiang et al., 2013b).

Antibody

Sensors that use an antibody to detect nerve agent–adducts on albumin are being developed but are not yet commercially available (Vandine et al., 2013).

OPS MAKE A COVALENT BOND WITH SERINE, THREONINE, TYROSINE, LYSINE, AND HISTIDINE

The first OP adducts were identified on the active site serine of AChE and BChE using a radioisotope-labeled OP. For 50 years, it was taken as dogma that an enzyme was defined as a serine hydrolase if it made a covalent bond with an OP. When a denatured protein sample made a covalent bond with an OP, the result was discarded as an outlier. Today, it is known that proteins need not be in their native structure to bind OPs. Small peptides make a covalent bond with OPs. The new tools of MS have demonstrated that proteins are modified

by OPs not only on serine, but also on threonine, tyrosine, lysine, and histidine (Grigoryan et al., 2008, 2009; Liyasova et al., 2012; Verstappen et al., 2012). To date, these additional binding sites have been demonstrated by *in vitro* reactions with high concentrations of soman, chlorpyrifos oxon, FP-biotin, dichlorvos, and cresyl saligenin phosphate, but it seems only a matter of time before new protein targets will be discovered that react with low doses of OP.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A new motif for OP binding to tyrosine has been identified. Almost all proteins appear to be capable of binding OP covalently on tyrosine. Whether or not OP will bind to tyrosine *in vivo* depends on the concentration of the protein, the concentration of the OP, and the ionization status of the tyrosine hydroxyl group. The latter factor appears to depend on the presence of nearby positively charged residues.

Albumin is the first protein outside the serine hydrolase family to be recognized as a target of OP binding in humans. The concentration of albumin is so high (600 μM in human plasma) that albumin binds OP despite its slow rate of reaction. The reaction of albumin's most sensitive tyrosine is aided by the presence of three positively charged residues within a five-residue stretch surrounding that tyrosine. It is expected that in the future, additional proteins will be identified as targets of OP binding.

Antibodies will be used to diagnose OP exposure in a biosensor assay with blood, saliva, sweat, or urine. New biomarkers of OP exposure will be identified using MS and antibodies. The identification of new biomarkers for low-dose OP exposure is expected to lead to an understanding of how neurotoxicity is caused by OP doses that are too low to inhibit AChE. For example, it is possible that disruption of microtubule polymerization by OP-adduct formation may explain cognitive impairment from OP exposure.

Identification of new biomarkers of OP exposure may also lead to an understanding of why some people are intoxicated by low doses of OP that have no effect on the majority of the population. The new motif for OP binding to tyrosine may lead to new antidotes for OP poisoning; for example, peptides containing several tyrosines and several arginines may be effective OP scavengers.

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CHAPTER

65

Monitoring of Blood Cholinesterase Activity in Workers Exposed to Nerve Agents

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INTRODUCTION

Determination of the activity of blood cholinesterases (ChEs; erythrocytes, red blood cells [RBCs], and acetylcholinesterases [AChEs], EC 3.1.1.7; and plasma butyrylcholinesterase [BChE] EC 3.1.1.8) is currently the most important procedure for confirmation of the diagnosis of poisoning with organophosphorus compounds (OPs), for monitoring of the recovery of intoxicated persons, and for forensic study purposes (Karalliedde et al., 2003; Bajgar, 2004, 2005, 2013; Eddleston et al., 2005; Pope et al., 2005; Radic and Taylor, 2006; Tavaselvam and Flora, 2014). It is necessary to investigate the whole picture of intoxication, including not only biochemical examinations but also clinical signs, thus allowing more precise assessment of further prognosis. The evidence supporting AChE as the primary site of action for both OP pesticides and nerve agents has been summarized by many authors (Bajgar, 1985, 1991, 2013; Taylor, 1985; Marrs, 1993; Marrs et al., 1996; Lotti, 2000). Their findings include the following observations: symptoms of OP poisoning are similar to those of the AChE inhibitor physostigmine; the *in vivo* LD_{50} value for a variety of OPs correlates well with the inhibition efficacy to AChE determined *in vitro*; and ChE reactivators (e.g., oximes), anticholinergics (e.g., atropine, benactyzine), and reversible AChE inhibitors (e.g., pyridostigmine) can attenuate their toxicity. However, there are ample documented data showing that AChE inhibition is not the only important biochemical change during intoxication; other important changes include changes in other enzymes, neurotransmitters, immune system changes, anaphylactoid reactions, and changes in behavior. Prophylactic/therapeutic drugs might also have multiple sites of action that are similar to those observed during intoxication (Bardin et al., 1987a,b; Bajgar, 1991, 1992; Cowan et al., 1996, Kassa, 1998). Nevertheless, the first reaction of OPs is their interaction with ChEs in the blood stream (Bajgar, 1985, 1991, 2013; Benschop and de Jong, 2001) and then in the target tissues—the peripheral and central nervous system (Green, 1958, 1983; Bajgar, 1985, 1991; Gupta et al., 1985, 1987a,b, 1991; Bardin et al., 1987a,b; Marrs, 1993; Marrs et al., 1996). The delayed neurotoxic effect is induced by inhibition of the neurotoxic esterase and is not caused by inhibition of ChEs; however, only some OPs are neurotoxic in that sense (Abou-Donia and Lapadula, 1990; Johnson, 1990, 1992; Lotti, 1992; Johnson and Glynn, 1995; Slotkin et al., 2008).

The mechanism of AChE inhibition for all the OPs and nerve agents is practically the same—phosphylation (phosphorylation or phosphonylation) of the serine hydroxyl group in the esteratic site of AChE.

Monitoring the ChE changes during the intoxication is, at present, the best marker of the severity of OP or nerve agent poisoning, as well as a basis for further antidotal therapy.

Both the toxicodynamics and toxicokinetics of OPs can be explained by biochemical characteristics of their interaction with ChEs and other hydrolases (e.g., human plasma paraoxonase [PON1]). Inhibition of ChEs in the blood is the first target for OPs according to the principle of "first come, first served" (Benschop and de Jong, 2001).

AChE and BChE differ in their enzymatic properties and physiological function (Massoulié et al., 1993; Darvesh et al., 2003). However, there are other types of

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ChEs such as benzoylcholinesterase and propionylcholinesterase. AChE splits the neurotransmitter acetylcholine (ACh) at the cholinergic synapses. A similar reaction is also observed in erythrocytes (RBC AChE), but its function is not yet known in detail, as is also the case with the function of BChE activity in plasma, although there is evidence that BChE plays an important role in cholinergic neurotransmission and could be involved in other nervous system functions, in neurological diseases, and in nonspecific detoxification processes (Darvesh et al., 2003).

DETERMINATION OF ChEs

Determination of ChE activity is based on a number of principles. In general, biological material containing the enzyme is added to the buffered or unbuffered mixture and the enzymatic reaction is initiated by adding the appropriate substrate. Different parts of the reaction mixture are determined (continuously or discontinuously), such as unhydrolyzed substrate or reaction products, either directly or indirectly (Witter, 1963; Augustinsson, 1971; Holmstedt, 1971). The conditions must be chosen very carefully because of different factors influencing the enzyme activity (Reiner and Simeon-Rudolf, 2000, 2006).

According to the procedure and laboratory instrumentation, the most common methods of ChE determination are as follows: electrometric (Michel, 1949); titration (Nenner, 1970); manometric (Witter, 1963); colorimetric detection of the unhydrolyzed substrate (Hestrin, 1949); measurement by the change of pH using an indicator (Winter, 1960); spectrophotometric (Siders et al., 1968; Voss and Sachsse, 1970; Worek et al., 1999a, 2012); fluorimetric (Sasaki 1964; Kusu et al., 1990); radiometric (e.g., Israel and Lesbats, 1987); calorimetric (Konickova and Wadso, 1971); polarographic (Fiserova-Bergerova, 1969); enzymatic (Abernethy et al., 1986; Israel and Lesbats, 1987); and others such as near-infrared spectroscopy (Domjan et al., 1998). These methods are also suitable for the detection of ChE inhibitors using biosensors (Brimijoin and Rakonczay, 1986; De Jong and Benschop, 1988; Cremisini et al., 1995; Walker et al., 2007) or immunochemical assays for detection of chemical warfare agents (Lenz et al., 1997a,b).

A very sensitive and commonly used method for ChE determination was described by Ellman et al. (1961). This method uses thiocholine esters as substrates (acetyl- and butyrylthiocholine or others). After enzymatic hydrolysis, the relevant acid and thiocholine are released and thiocholine by its SH-group is detected using 5,5′dithiobis-2 nitrobenzoic acid (DTNB) forming 5-mercapto-2-nitrobenzoate (3-thio-6-nitrobenzoate, TNB) with an absorbance maximum of approximately 412 nm (Figure 65.1).

Reaction is monitored spectrophotometrically using a spectrophotometer or microplate reader at a convenient wavelength, usually 405, 412, or 436 nm. ChE activity can be calculated in diluted buffered solutions using a TNB extinction coefficient of 14,150 $M^{-1}\cdot cm^{-1}$ (at 412 nm) (Collier, 1973).

Sometimes this method is used with specific inhibitors (tetraisopropyl pyrophosphoramide or profenamine for BChE inhibition and BW284C51 for AChE inhibition) for selective determination of AChE or BChE activity in whole blood samples and there are many modifications described in the literature. Ellman's method is sufficiently specific and sensitive, and it is routinely used for different purposes in many laboratories around the world. Expression of the activity varies greatly, usually as μmols of substrate hydrolyzed per minute (time) per milliliter of biological material examined (e.g., plasma, serum, blood hemolysate) or per milligram of weight tissue or tissue protein. From these values, the expression of the activity in units (U) can be derived (quantity of enzyme catalyzing μmol of substrate per minute at standard conditions). In the clinical laboratory, the enzyme activity can also be expressed as 1 mol of substrate hydrolyzed per liter or kilogram (cat/L; kg) of material per second, which is the hydrolysis of 1 mol of substrate hydrolyzed per second per liter or kilogram ($mol\,s^{-1}\,L^{-1}$ or kg^{-1}). There are many publications dealing with the review and modifications of ChE determination. One of the last methodical works describing improvements in Ellman's method (Ellman et al., 1961), including a description of the methods, is an article published by Worek et al. (1999a).

FACTORS INFLUENCING ACTIVITY OF ChEs

The influencing of BChE activity by gamma irradiation, stress, gravidity, certain neurological and psychiatric disorders, hormones, and medical drugs has been demonstrated (Brown et al., 1981; Bajgar, 1985, 1998). The elevation of BChE activity is not so frequent. An increase in children with nephritic syndrome has been observed; an increased ratio of BChE:LDL cholesterol indicates an increase in the risk of cardiovascular diseases (Kutty, 1980; Navratil and Bajgar, 1987). The involvement of BChE with fat (cholesterol) metabolism has been suggested by Van Lith et al. (1991) and Van Lith and Beynen (1993). The relationship between BChE activity and experimentally induced diabetes mellitus in rats was also mentioned (Annapurna et al., 1991).

In clinical biochemistry, BChE determination in the plasma or serum is more frequently used than that of AChE in RBCs. Except for intoxication with OPs or carbamates (CMs), a BChE decrease indicates either reduced

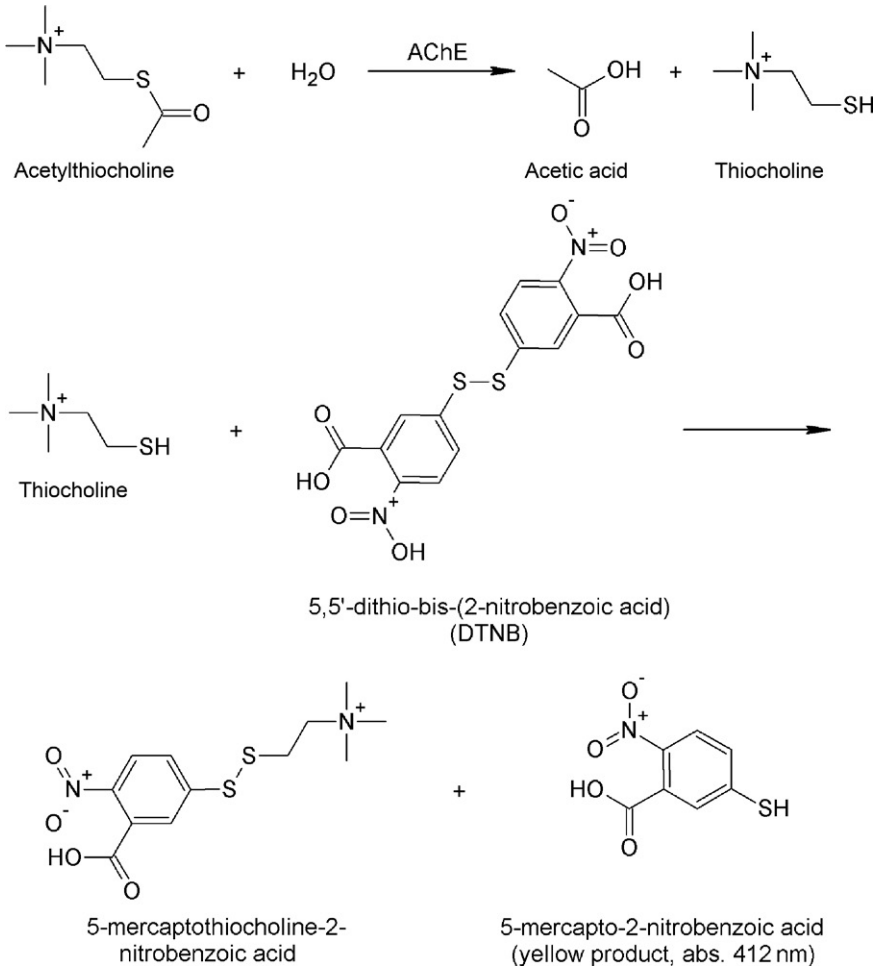

FIGURE 65.1 Acetylthiocholine is hydrolyzed by AChE to acetic acid and thiocholine, which forms yellow 5-mercapto-2-nitrobenzoic acid after reaction with Ellman reagent.

enzyme synthesis or a decrease in the number of production of cells in the liver (Masopust, 1983). A special case of diminished BChE activity is hereditary and is affected by the presence of atypical variants of BChE (Whittaker, 1980; Brown et al., 1981; Goodall, 2006).

There are many other factors influencing BChE activity, and the diagnostic importance of diminished BChE activity is important for the following states (except hereditary decrease of the activity): poisoning with nerve agents and OP/CM pesticides; congenital deficiency; liver damage; acute infection; chronic malnutrition; metastasis (especially liver); myocardial infarction; dermatomyositis; intoxication with carbon disulfide or mercury; and obstructive jaundice (Bajgar, 1991; Bardin et al., 1987a,b; Kutty, 1980; Molphy and Ratthus, 1964; Wyckoff et al., 1968).

Determination of AChE activity is not so widely used in clinical laboratories. A decrease in RBC AChE activity in pernicious anemia has been demonstrated; diminished erythrocyte AChE activity is typical for paroxysmal nocturnal hemoglobinemia and blood type ABO incompatibility (Rakonczay, 1988). AChE activity in the erythrocyte membrane can be considered an indicator of erythrocyte membrane integrity. Increased AChE activity during rectal biopsy is of great diagnostic significance in Hirschsprung disease, especially in the presence of its atypical molecular form (Bajgar and Hak, 1979; Rakonczay, 1988). There are other publications demonstrating increased AChE activity in the amniotic fluid during pathologic development of the neural tube (Bonham and Attack, 1983). AChE activity in erythrocytes and cerebrospinal fluid is also diminished in some endogenous depressions and other psychiatric disorders; however, the results presented are not quite clear at present (Skau, 1986; Bajgar, 1985; Rakonczay, 1988; Bohnen et al., 2007; Martucciello, 2008).

DIAGNOSIS OF OP POISONING

The determination of ChEs in the blood is the basic method for diagnosis and therapy monitoring for OP poisoning, although some doubts exist; some prefer the clinical signs of poisoning as a leading tool for OP poisoning diagnosis and monitoring (Bardin et al., 1987a,b). The determination of AChE and/or BChE activity in whole blood is possible. The decrease in these activities is a good marker but the diagnostic validity is limited to the statement that some factors causing a decrease in blood ChEs are present. In connection with the anamnestic data (exposure to OP or CM pesticide), this is important information. The determination of the RBC AChE or plasma BChE is more informative. There are some discussions dealing with AChE and BChE activity, but which is more important for the diagnosis? In general, AChE activity in RBCs can be considered to be more important for diagnosis of intoxication with nerve agents than plasma BChE activity. However, there are some discussions dealing with the validity of the BChE determination. This enzyme was described as not a very good marker of OP poisoning and its determination was proposed for exclusion from the recommended biochemical procedures (Molphy and Ratthus, 1964; Wyckoff et al., 1968; Bardin et al., 1987a,b). The temporal profile of BChE was studied in a cohort of 25 OP-poisoned patients to examine their relationship to the development of intermediate syndrome. The study suggests that BChE reflects the clinical course of poisoning and that intermediate syndrome may be associated with a persistent BChE inhibition (Khan et al., 2001). Israeli authors also described a direct correlation between the degree of BChE inhibition levels and the severity of intoxication with OP pesticides (Weissmann-Brenner et al., 2002). According to Aygun et al. (2002), in the acute phase of OP poisoning, low levels of AChE support the diagnosis of OP poisoning but do not show a significant relationship to the severity of poisoning. The preference of AChE determination has been demonstrated by Worek et al. (1999b)—BChE activity determination for diagnosis and therapeutic monitoring provides no reliable information about AChE status. This is in agreement with our experimental results (Bajgar, 1998). BChE activity in plasma is in some cases a good marker for diagnosis of OP pesticide poisoning. It is necessary to exclude a diminishing of BChE activity caused by the aforementioned reasons. In all cases, the simple ChE determination provides us with information about the decrease of enzyme activity without specification of the factor causing this phenomenon. A more detailed specification is possible using special methods not available in all clinical laboratories.

For the purposes of occupational medicine, the determination of ChEs in the blood of workers intoxicated with OPs is obligatory. A decrease in activity to less than 70% of normal values is an indicator that the worker should not come into contact with OPs. However, the normal values varied within the laboratories depending on the method of determination.

For practical purposes (individual and interindividual variation), determination of individual normal activity is recommended (this approach is better than that of calculating the decrease from an average value), as well as separate determination of ChEs, RBC AChE, and plasma BChE. The activity determined in human whole blood corresponds to approximately 10% of BChE and 90% of AChE. This is different in rats, in which this ratio is 29% of BChE and 71% of AChE (Bajgar, 1972). Erythrocyte AChE activity seems to be more useful for these purposes than BChE activity in plasma.

There are other biological materials available for special purposes—amniotic fluid, cerebrospinal fluid, and bioptic materials. From these samples, tissue obtained by rectal biopsy is used most frequently (diagnosis of Hirschsprung disease). Elevated AChE activity in the rectal tissue/homogenate (detected histochemically/biochemically) is one of the significant diagnostic markers indicating a need for surgical treatment of Hirschsprung disease and a criterion for diagnosis and management of obstipation (Kobayashi et al., 2002). The presence of an unusual AChE band after electrophoretic separation supports the diagnosis (Bajgar and Hak, 1979; Rakonczay, 1988). The same (either AChE elevation or the presence of a new electrophoretic AChE form) in the amniotic fluid can be applied for the diagnosis of malformation of the neural tube development during pregnancy (Bonham and Attack, 1983). AChE activity in cerebrospinal fluid is also changed in some pathological states; however, the diagnostic validity is not very high and can be considered as a complementary examination (Koponen and Riekkinen, 1991).

In OP poisoning, it is necessary to check vital functions (cardiac, ventilation) and other clinical signs and, according to the symptoms, to apply different biochemical examinations and treatment. Diagnostic criteria are mostly based on anamnesis and the state of ventilation (Bardin et al., 1987a,b; Bardin and Van Eeden, 1990). Serum electrolytes (especially potassium), Blood urea nitrogen (BUN), and creatinine are indicated to assess the degree of volume depletion by secretory losses. Arterial blood gas, blood pH, glucose, lactate, and pyruvate allow us to assess the degree of hypoxia/hypercapnia/acidosis in the presence of respiratory distress. The neurological examination involves procedures such as recording of muscle action potential of abductor digiti minimi after stimulation of nerve ulnaris. A quantitative correlation is shown between RBC AChE activity and paraoxon concentration in plasma. In these cases, muscle function was severely disturbed when the RBC AChE activity was inhibited by more than 90% (Thiermann et al., 2002).

Direct determinations of the toxic agent (nerve agent or OP pesticide) in the circulatory system are also possible. However, the parent compound will circulate intact for a short period of time and detection will not be possible for more than approximately a few hours after exposure. Metabolites circulate for a longer time and are mostly excreted in urine. A metabolite of sarin (*O*-isopropyl methylphosphonic acid) could be traced in urine and plasma from victims after the Tokyo subway sarin terrorist attack (Noort et al., 1998, 2002). For some OP pesticides (parathion, paraoxon), detection of *p*-nitrophenol in urine is an indicator of exposure (Bajgar, 1985). However, the retrospectivity of these methods is limited. Detection using an immunoassay of nerve agents was developed. The antibodies against soman may have the appropriate specificity and affinity for immunodiagnosis of soman exposure (Lenz et al., 1997a,b; Miller and Lenz, 2001).

Methods for the determination of blood ChE inhibition (AChE and BChE) do not allow the identification of the OPs and do not provide reliable evidence for exposure at inhibition levels less than 10–20%. Moreover, they are less suitable for retrospective detection of exposure due to *de novo* synthesis of enzymes. For this purpose, a method based on reactivation of phosphylated ChE and carboxylesterase (CarbE) by fluoride ions was developed. Treatment of the inhibited enzyme with fluoride ions can inverse the inhibition reaction, yielding a restored enzyme and a phosphofluoridate that is subsequently isolated and quantified by gas chromatography and phosphorus-specific or mass spectrometric detection (De Jong and Van Dijk, 1984; Polhuijs et al., 1997a,b). Human as well as monkey plasma does not contain CarbE, but its BChE concentration is relatively high (70–80 nM) (Myers, 1952; De Bisschop et al., 1987), much higher than the concentration of AChE in blood (approximately 3 nM) (Heath, 1961). The plasma of laboratory animals, such as rats and guinea pigs, contains considerable concentrations of CarbE in addition to ChEs. This method allows partial identification of the OP, whereas the lifetime of the phosphylated esterase (and consequently the retrospectiveness of the method) is only limited by spontaneous reactivation, *in vivo* sequestration, and aging. The rate of aging depends on the structure of the phosphyl moiety bound to the enzyme and on the type of esterase. Phosphylated CarbEs generally do not age. Based on this method for retrospective detection of exposure to OP, the exposure of victims of the Tokyo incident to a nerve agent, probably sarin, could be established from analysis of their blood samples (Polhuis et al., 1997a,b; Fidder et al., 2002). Fluoride-induced reactivation of OP-inhibited AChE is a reliable and retrospective method to establish OP exposure. It is limited to compounds that regenerate with fluoride ions. A novel and general procedure for diagnosis of exposure to OPs, which surpasses the limitations of the fluoride reactivation method, is described (Van der Schans et al., 2002). It is based on the rapid isolation of BChE from plasma by affinity chromatography, digestion with pepsin, and followed by liquid chromatography with the mass spectrometric analysis of phosphylated nonapeptides. The method can be applied for the detection of exposures to various OP pesticides and nerve agents including soman. This approach is very valuable and represents a new field for the improvement of diagnosis with OPs. A comprehensive review of the methods for retrospective detection of exposure to toxic scheduled chemicals has been published by Noort et al. (2001, 2002).

As mentioned previously, a decrease in ChE activity is the factor indicating (after the exclusion of other factors) an exposure to OP pesticides, nerve agents, or other ChE inhibitors. This simple determination does not allow us to make certain decisions dealing with the antidotal therapy (especially the repeated administration of reactivators) and has low prognostic validity. Therefore, a new test of the reactivation of inhibited enzyme has been described (Bajgar, 1991). The principle of the reactivation test is double determination of the enzyme activity, the first without and the second with the presence of a reactivator in the sample. The choice of reactivator is dependent on the availability of the oxime; however, in principle it is necessary to have in these parallel samples the same concentrations of reagents. The concentration of the reactivator (usually trimedoxime, but other oximes such as obidoxime, pralidoxime, or HI-6 are also possible) must be no higher than the oxime concentration that causes the hydrolysis of the substrate (acetyl- or butyrylthiocholine); in other words, the oxime concentration must be lower than 10^{-3} M (Patočka et al., 1973).

OP poisoning is very complex and there are many biochemical changes to be registered. Although the assessment of sensitivity and specificity was rather subjective, it is clear that there are two or three parameters that are most sensitive: ChE determination (depending on the type of OP, either AChE or BChE); the possible determination of OP metabolites in the blood; and determination of the phosphonyl moiety on the target enzyme (if possible). In the case of developed intoxication when convulsions occurred, tension of the blood gases is also a good marker; however, these changes are not very specific. The same approach can be applied to lactate. This is not surprising because of the existence of convulsions, including hyperactivity of the diaphragm (and thus disturbed ventilation, low oxygen saturation, and an increase in acid metabolites). It should also be mentioned that the validity of these parameters is changed during intoxication. The changes in transaminases, lactate dehydrogenase (LDH), and gamma glutamyl transferase (γ-GT), indicating liver damage, can be caused by solvents used in commercially available OP insecticides. Low validity in the number of RBC or leukocytes is also indicated. As for CS and

Thrombin-antithrombin complexes (TAT) stress markers, it is clear that OP intoxication represents a stress situation. In this context, an increase in Alanine aminotransferase (ALT) can also be considered as a stress marker and not indicative of liver damage.

Determination of inhibition for different AChE or BChE molecular forms is interesting and useful for improvement of the diagnosis of OP poisoning. It was demonstrated that these forms are inhibited in different manners—some of the forms are resistant (low-molecular-weight forms) and some of them are very sensitive (high-molecular-weight forms). When the total AChE activity is determined, the value obtained is a "mean" of the activities of these forms. The changes in the cyclic nucleotides are interesting but not valid for blood. They were determined during animal experiments with toxic OPs and are of more interest in connection with the nervous system. Esterases and AP, generally hydrolases, are sensitive but the inhibition potency of different OPs is very variable; for nerve agents, these hydrolases are not valid and, for some OP insecticides like malathion, they are sometimes more sensitive than ChEs. In conclusion, diagnosis of OP poisoning represents a serious problem. The development of the new specific methods mentioned (fluoride reactivation, tandem MS analysis of enzymatic digests of BChE) is of high importance for more precise diagnosis of OP nerve agent poisoning. The extensive review of Noort et al. (2002) dealing with biomonitoring of exposure to chemical warfare agents (not only nerve agents) is strongly recommended. From a practical point of view in the clinical laboratory, it is necessary to monitor basic physiological functions, ChEs, and other biochemical parameters, not only for diagnostic purposes but also for the regulation of treatment.

MONITORING OF BLOOD ChE ACTIVITY IN WORKERS WITH NERVE AGENTS

For the purposes of occupational medicine, the determination of ChEs in the blood of workers intoxicated with OP is obligatory. However, the normal values varied within the laboratories depending on the method of determination. Systematic monitoring of workers with nerve agents was performed at our department from 1962 to 1963; however, the results presented contain determinations during 40 years (1964–2004). Because of the large number of results, the results are limited to RBC AChE only, although plasma BChE activity was also determined.

Methods for Determination

Modified Hestrin's method (Hestrin, 1949) was used initially. This method is based on colorimetric detection of unhydrolyzed substrate, ACh. Acetylcholine reacts with hydroxylamine quantitatively in alkaline solution after reaction with $FeCl_3$. The activity was determined in RBC hemolysate with distilled water (1:20). The method was modified and activity was expressed as μmols of hydrolyzed acetylcholine per minute and per milliliter.

In 1964, a method described by Winter (1960) using an autoanalyzer (Technicon, USA) system was evaluated. The method is based on determination of acetic acid released from the substrate hydrolyzed (ACh) and detected with phenol red. AChE activity in RBC hemolysate with distilled water (1:10) was determined (pH 7.6, phosphate buffer 37°C). The activity was expressed as μmol of acetic acid released per 10 min.

From 1972 to 1975, Ellman's method (Ellman et al., 1961), modified for the autoanalyzer, was used. The activity was expressed as ncat/L. The same method was used for this determination using kinetic methods and instruments Vitatron (Eefde, Holland) (1976–1990) and Uvicon 952 (Kontron Instruments, Switzerland) from 1991 to 2004.

Correlation Among Methods

To achieve comparable results (unification of the activity, i.e., one scale), all the activities were recalculated to ncat/L. Recalculation of the activities was performed simply using the formula: U/L = 16.6667 ncat/L. However, in the case of different substrates (esters of choline and thiocholine), correction was made according to correlation analysis ($y = 2.0188x + 2.2458$ and $r_{xy} = 0.9998$). Moreover, this correlation was demonstrated previously including not only Ellman's and Winter's methods (Ellman et al., 1961; Winter, 1960) but also the potentiometric method.

Subjects

International inspectors included 28 males (two determinations, 2000 and 2002), seven were selected (more determinations), and age was unknown. There were 21 medical students (10 female, 11 male) aged 19–20 years. Workers in the department included 18 males (from these, 9 were selected for long-term study) and 23 females (from these, 13 were selected for long-term study). The last determination was performed at age 60 years (female) and 65 years (male), respectively. Ages at the beginning of the study were 24–40 years for males and 19–48 years for females. All determinations were performed during the years 1964–2004.

Data Analysis and Findings

Because of the large number of examinations, the results were demonstrated only for the RBC AChE. The

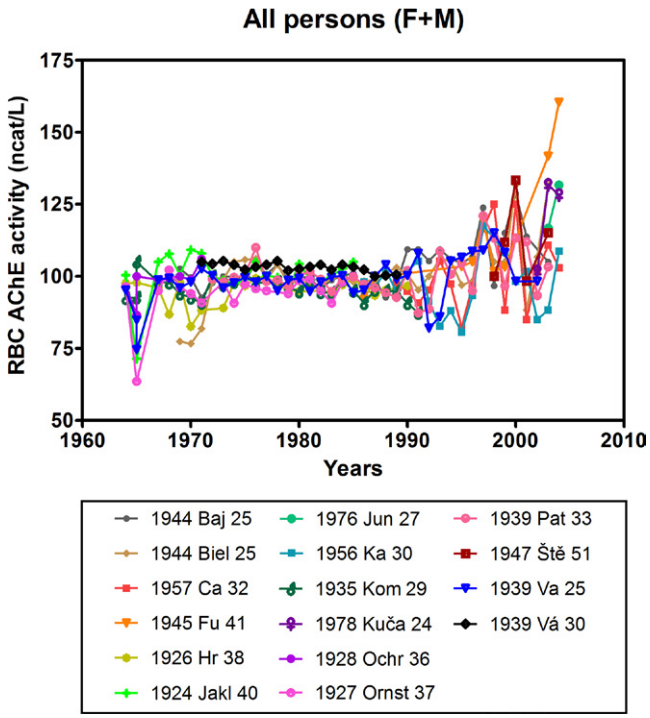

FIGURE 65.2 AChE activities of all monitored persons.

TABLE 65.1 Selected Results of RBC AChE Activity Determination

Dept. Toxicol.	All	M	F
(18 + 23)	101.2 ± 7.2	102.2 ± 6.9	100.2 ± 8.4
Selected (9 + 13)	98.0 ± 8.2	99.7 ± 6.3	93.9 ± 7.8
Inspectors (0 + 28)	–	110.7 ± 9.4	–
(2000 + 2002)	–	112.4 ± 14.7	–
Students (11 + 10)	110.2 ± 9.5	115.1 ± 9.4	105.3 ± 9.5
Susp. intox (3 + 4)	90.9 ± 4.8*	94.3 ± 6.9*	86.4 ± 2.3*

**Stands for significant differences among activities at $p \leq 0.05$.*

first step was a simple survey of AChE activities divided between females and males. The next step was analysis of individual results and trends. Some attempts to detect a decrease of AChE activity in the case of potential exposure to nerve agents were made.

The results of Hestrin's method (Hestrin, 1949) are documented as an average activity of the RBC AChE. Normal AChE activity was determined to be 104.9 ± 8.5 ncat/L and the coefficient of variation was 27.1%. The method was relatively difficult and is shown for information only. The results of AChE activities for all persons are shown in Figure 65.2. Selected results of RBC AChE activity determination are shown in Table 65.1.

The correlation of activities in hemolysate and whole blood is shown in Figure 65.3.

In general, there were certain tendencies to decrease ChE activity (especially RBC AChE activity) during

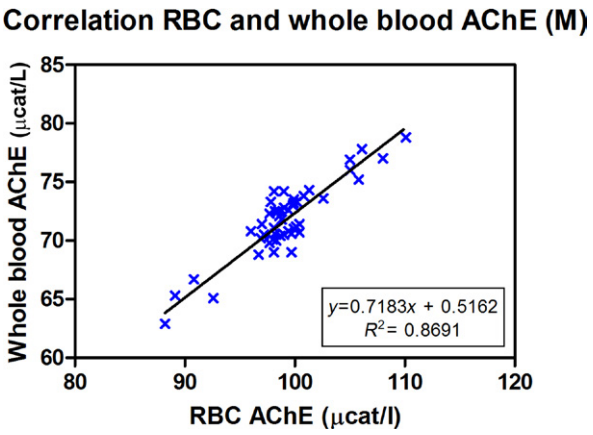

FIGURE 65.3 Correlation between RBC and whole blood AChE.

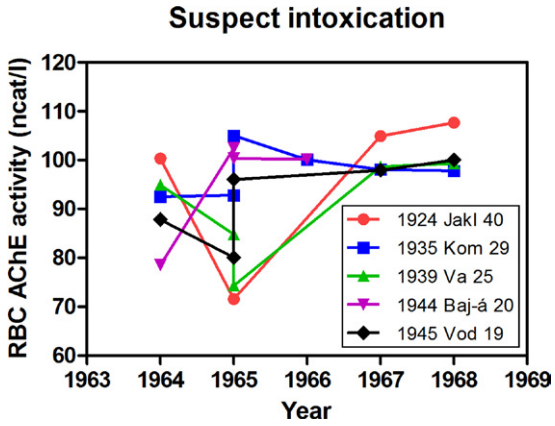

FIGURE 65.4 Suspect nerve agent intoxication.

work at the department. Some rare examples of suspect intoxications were observed in the ChE monitoring as well as an RBC AChE decrease in workers during their work with high concentrations of nerve agents (e.g., inhalation exposure experiments) (Figure 65.4).

In a practical way, the best appreciation of ChE was performed using individual norms for RBC AChE. It seems to be the most sensitive parameter for monitoring ChE changes in exposed workers, followed by sensitivity for whole blood hemolysate. Plasma BChE activity is not so specific and is a sensitive parameter. A decrease of 30% of individual norms seems to be critical for further consideration of intoxication. In conclusion, ChE determination in blood (especially RBC AChE) is a good parameter for monitoring and laboratory examination of workers with highly toxic nerve agents, reflecting possible exposure to these agents.

Variation of RBC AChE is relatively high. Therefore, individual levels were considered to be better, and the decrease of 30% under individual norms was decided to be a limit for exclusion of workers from active work with nerve agents. A further decrease can be considered

a significant diagnostic marker for suspect intoxication. According to the decrease, the classification of steps of intoxication can be assumed: a decrease of 30–50% indicates mild poisoning, 50–70% indicates medium poisoning, and 70–90% indicates severe intoxication. This decrease is in good agreement with results for humans and animals (Bajgar, 1992). Increased AChE activity was also observed for males (not significant) and is in agreement with other results (Augun et al., 2002). Similar results were obtained by other authors, for example, no significant differences in RBC AChE activity between males and females (Rumenjak, 1998) and a tendency to an increase in activity with age (not significant). In general, the values of RBC AChE activity are very good compared with the results of other authors (Rumenjak, 1998; Augun et al., 2002; Zhou et al., 2003).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This chapter describes the sensitivity of blood ChE as a diagnostic parameter for monitoring workers exposed to nerve agents. The sensitivity to detect changes in response to nerve agent exposure is ordered as RBC AChE > whole blood ChEs > plasma BChE. Normal ChE activity assayed with different methods is in good correlation. RBC AChE or whole blood ChE can be considered a good marker for the monitoring of nerve agent exposure.

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SECTION VIII

PROPHYLACTIC, THERAPEUTIC AND COUNTERMEASURES

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CHAPTER

66

Pharmacological Prophylaxis Against Nerve Agent Poisoning: Experimental Studies and Practical Implications

Jiri Bajgar, Josef Fusek, Jiri Kassa, Kamil Kuca and Daniel Jun

INTRODUCTION

Inhibitors of cholinesterases are very important chemicals in the group of organophosphates (OPs). These compounds are used in industry, in veterinary and human medicine, and unfortunately in military (chemical warfare agents (CWAs)/nerve agents) and terrorist activities (Japanese incidents in 1994–1995). The bulk of these compounds is used as pesticides (insecticides, acaricides, etc.). According to the World Health Organization (WHO), more than 1 million serious accidental and 2 million suicidal poisonings with insecticides occur worldwide every year, and of these approximately 200,000 deaths occur, mostly in developing countries (Eyer, 2003; Bajgar et al., 2004, 2007a). The mechanism of action, prophylaxis and treatment of intoxications with OPs, is currently a very timely topic.

The term *prophylaxis* is sometimes unclear. Generally, it is limited to medical countermeasures applied just before penetration of a toxic agent into the organism without further antidotal therapy. It is also described as a pretreatment, but usually antidotal treatment is performed. Thus, when treatment is unnecessary, it can be described as prophylaxis. Although successful prophylaxis can be observed for some OPs, full protection of the organism without postexposure treatment, especially for soman poisoning, remains open. When treatment is given after the exposure, the term *posttreatment* is used. It is obvious that when the drug is administered prior to nerve agent exposure with the aim of protecting the organism, exposure to these agents is expected, and therefore post-exposure therapy can be used; i.e., *pretreatment* could be considered the right term. The term *prophylaxis* used in this chapter is limited to medical countermeasures applied shortly before penetration of a toxic agent into the organism with the aim of protecting the organism against the toxic agent.

Prophylaxis against nerve agents has been described and summarized in various publications (Bajgar et al., 2004, 2007a, 2009, 2011, 2012, 2013; Layish et al., 2005; Patocka et al., 2006; Tuorinsky, 2008; Elsinghorst et al., 2013; Kuca et al., 2013). The basic mechanism of action of OPs is based on their ability to inhibit the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) at cholinergic peripheral and central synapses (Marrs et al., 1996; Bajgar et al., 2004; Masson, 2011; Colovic et al., 2013). In general, prophylaxis can be focused on protection of AChE against inhibition using reversible cholinesterase inhibitors. The level of OP can be diminished by using enzymes that hydrolyze these agents or enzymes that bind the agents (to specific proteins or to antibodies) and thereby reduce the OP level and inhibition of cholinesterases—AChE and BuChE (EC 3.1.1.8; the scavenger effect) in the organism; this can be described as detoxification. Another approach to prophylaxis is based on using present antidotes and other drugs (Bajgar et al., 2004, 2007a, 2009, 2011, 2012, 2013; Kuca et al., 2013). Different drugs or their combinations can also be used. A simple schema describing the fate of the agent in the organism and possible target sites for prophylaxis is shown in Figure 66.1.

In the schema, basic reactions for the effect of nerve agents are indicated—they are penetration of the agent into the organism through different ways of administration; after penetration into the blood (transport system), the agent is distributed to the places of metabolic and toxic effects. To stop or minimize the toxic effect, different possibilities can be explored. Because of inhibition of AChE

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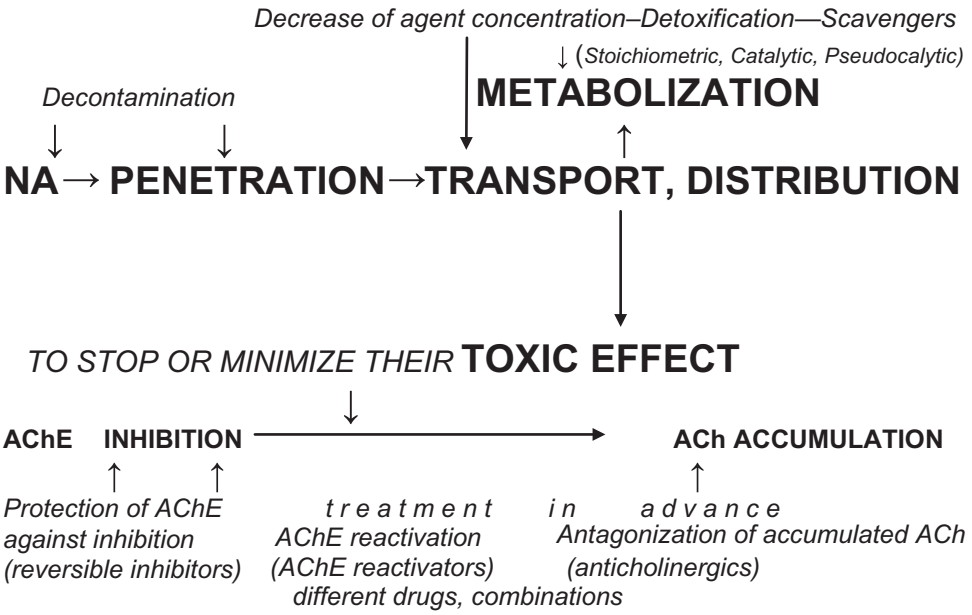

FIGURE 66.1 Four basic reactions of OP in the organism (in capitals) and possible approaches for prophylaxis (in italics). *Source: Modified from Bajgar (2004, 2009) and Gupta (2009).*

is the main, trigger, and basic pharmacodynamic factor, protection against AChE inhibition is one possible process using reversible AChE inhibitors. Treatment in advance (administration of anticholinergics and reactivators) is another possibility, and the use of different drugs minimizing the toxic action is the third possible approach for prophylaxis. Certainly, all these principles can be combined.

PROTECTION OF AChE AGAINST INHIBITION

Intact AChE is a basic requirement for normal function of the organism, and thus for effective prophylaxis. The enzyme is changed in a way that will make it resistant to OP. This can be achieved by using reversible inhibitors, preferably carbonates (CMs), which are able to inhibit AChE reversibly, and after spontaneous recovery of the activity (decarbamylation) normal AChE serves as a source of an active enzyme.

The ability of some CMs to protect an organism poisoned with an OP has been known for many years (Koelle, 1946; Koster, 1946). Physostigmine and neostigmine have been used to protect animals against DFP. The number of OPs studied for protection has been expanded, as well as the number of CMs studied. The results are very dependent on experimental conditions; nevertheless, the protective effects of physostigmine, aminostigmine, pyridostigmine, and others against AChE inhibition caused by different OPs (mostly soman) have been demonstrated (Patocka, 1989; Marrs et al., 1996; Tonkopii, 2003). It has been described that weak inhibitors are able to protect cholinesterases from strong inhibitors (paraoxon; Petroianu et al., 2005a, 2007). Non-OP inhibitors were also tested (Kassa et al., 2012; Lorke et al., 2012, 2013; Petroianu et al., 2013). It appeared from many studies that the cholinesterase inhibitor pyridostigmine was the most promising prophylactic drug, particularly against soman poisoning (Gordon et al., 1978, 2005; Patocka et al., 1991; Anderson et al., 1992; Koplovitz et al., 1992; Maxwell et al., 1993; Bajgar et al., 1994; Kassa and Bajgar, 1996; Kassa and Fusek, 1998, 2000; Tuovinen, 1998; Koupilova and Kassa, 1999; Kassa et al., 2001a,b). On the basis of these results, pyridostigmine was introduced into some military units as a prophylactic drug against nerve agents. Its prophylactic effect (like the effects of other CMs) is increased with its dose; however, with a higher dose, the side effects are also increased. This problem can be solved by adding pyridostigmine-antagonizing drugs, anticholinergics. Many anticholinergics have been tested for their ability to protect the organism against intoxication with soman (and other nerve agents). On the basis of this research, the prophylactic combination PANPAL, composed of pyridostigmine with trihexyphenidyl and benactyzine (Bajgar et al., 1994, 1996; Kassa and Bajgar, 1996; Kassa and Fusek, 1998, 2000; Fusek et al., 2000, 2006; Kassa et al., 2001a), was introduced into the Czech army, and later into the Slovak army. The presence of anticholinergics allowed for an increase in the pyridostigmine dose and, simultaneously, an increase its prophylactic efficacy (Kassa et al., 1997; Bajgar et al., 2004, 2007a; Fusek et al., 2006). Moreover, prophylaxis with PANPAL improves following post-exposure antidotal therapy with anticholinergics and oximes (Bajgar et al., 2004; Kassa,

2006). The prophylactic antidotal combination called PANPAL has no side effects, as demonstrated on volunteers: no statistically significant different changes in the actual psychic state, as well as no negative changes in the dysfunction time, were observed. An improvement of the tapping test following PANPAL administration was demonstrated. A decrease in the heart frequency 60 min following PANPAL administration lasting 480 min and returning to normal values within 24 h was demonstrated (Fusek et al., 2006).

Other CMs also have a good prophylactic efficacy, especially physostigmine and aminostigmine (due to their central effect which is contrary to pyridostigmine; Tuovinen and Hanninen, 1999; Kim et al., 2002; Tonkopii, 2003). Experimental studies with transdermal administration of physostigmine suggest a serious interest in the prophylactic use of this drug (Levy et al., 1992; Jenner et al., 1995; Walter et al., 1995; Kim et al., 2005; Cho, 2012; Cho et al., 2012). A similar approach was described with scopolamine (Che et al., 2011).

Structurally different inhibitors from the CM and OP groups were also studied. From these compounds (preferably binding to the AChE anionic site), tacrine, its 7-methoxyderivative (7-MEOTA) and huperzine A, were considered and experimentally studied with respect to prophylaxis *in vitro* and *in vivo* (Bajgar, 2004). The most interesting results were obtained with huperzine A (Lallement et al., 2002; Gordon et al., 2005; Bajgar et al., 2007b; Karasova et al., 2009; Wang et al., 2013).

SCAVENGERS

Keeping AChE intact can be achieved by eliminating the agent before its penetration into the target sites containing the enzyme. In this case, the detoxification principle can be used in two different ways: administration of enzymes splitting the OP or specific enzymes (cholinesterases) that bind the OP. The enzymes that hydrolyze nerve agents are called *catalytic* scavengers, and the enzymes that bind nerve agents are described as *stoichiometric* scavengers. OPs are bound to the exogenously administered enzyme or decomposed by the enzyme and thus the nerve agent level in the organism is decreased (the action of a "scavenger"). Enzymes, which hydrolyze OPs, were thoroughly studied by Raveh et al., (1992) and Li et al., (1995). Catalytic scavengers are enzymes displaying a turnover with OP nerve agents as substrates, allowing rapid and efficient protection (Masson et al., 1998; Ditargiani et al., 2010; Gupta et al., 2011; Kuca et al., 2013; Otto et al., 2013). Paraoxonase and similar enzymes are prospective hydrolyzing scavengers (Aharoni et al., 2004; Rochu et al., 2008; Valiyaveettil et al., 2010, 2011a,b; Trovasler-Leroy et al., 2011). On the other hand, many studies have been completed using cholinesterases as scavengers. AChE was used as a prophylaxis against OP poisoning, and a correlation has been found between protection and the blood–enzyme level in mice (Wolfe et al., 1987; Raveh et al., 1989). BuChE and AChE were observed to be very effective in protection against OP intoxication (Ashani et al., 1991; Doctor et al., 1991, 1997, 2002; Maxwell et al., 1993, 1998; Moore, 1996; Saxena et al., 1997; Bird et al., 2010). The idea to use the plasma as a source of cholinesterase to eliminate nerve agent action after peritoneal dialysis with human plasma in rats was also described (Bajgar et al., 1982), but without further development.

The administration of enzymes as scavengers seems to be very promising: the enzyme is acting at the very beginning of the toxic action, without interaction with the target tissues and without side effects (Doctor et al., 1997, 2002; Saxena et al., 2004, 2011; Huang et al., 2007). All of these features are of great interest, and they are yielding practical results—namely, isolation of the enzyme, examination for lack of an autoimmune response, and establishment of pharmacokinetic and pharmacodynamic properties (Saxena et al., 2002). Recombinant human BuChE can be produced from the milk of transgenic goats (Cerasoli et al., 2005) commercially produced as Protexia. Moreover, BuChE pretreatment also showed protective effects on AChE inhibition in the brain regions following low-level sarin inhalation exposure: the enzyme (BuChE) administered into the bloodstream does not influence erythrocyte AChE, plasma BuChE activity is increased, and the brain AChE is not affected. Following intoxication with a nerve agent, the agent is bound to plasma BuChE (now existing in excess), and proportionally bound to erythrocyte AChE; both enzymes are inhibited, however, to a lesser extent in comparison with the unpretreated situation. The part of the nerve agent bound to both enzymes represented a loss of the agent, and the diminished level of nerve agent is able to penetrate the brain. Therefore, AChE activity in the brain is protected (Sevelova et al., 2004; Bajgar et al., 2007c).

The efficacy of cholinesterases as a bioscavenger of OP can be enhanced by combining enzyme pretreatment with oxime reactivation, since the scavenging capacity extends beyond a stoichiometric ratio of cholinesterases to OP. Human BuChE has previously been shown to protect mice, rats, and monkeys against multiple lethal toxic doses of OP anticholinesterases (anti-AChEs; Maxwell, 1992; Allon et al., 1998). An interesting approach can be simultaneous administration of BuChE and reactivators; BuChE acts as a scavenger binding the nerve agents. A reactivator acting as a *pseudocatalytic* bioscavenger reactivates BuChE simultaneously, and reactivated enzyme serves as a new scavenger (Jun et al., 2007; Kovarik et al., 2010).

This approach was tested using human BuChE (Jun et al., 2011). The best reactivators were trimedoxime and

obidoxime, but only in the case of OP insecticides (paraoxon, methamidophos, and leptophos; Jun et al., 2007, 2011; Musilova et al., 2009). Thus, currently available reactivators cannot be of interest and practical use as pseudocatalytic scavengers for nerve agents.

Given our increasing knowledge of bioengineering and biotechnology, recombinant human BuChE with a good protective effect against nerve agents was obtained from transgenic animals. A connection between these two enzymes (binding and hydrolyzing) is possible, with the aim of obtaining a modified enzyme splitting OPs and simultaneously reacting with AChE as a scavenger (Broomfield et al., 1997). Antibodies against OPs are at the research stage and are more focused on the detection of OPs (Lenz et al., 1997; Miller and Lenz, 2001).

PROPHYLAXIS WITH CURRENT ANTIDOTES

The aim of this approach was to achieve a sufficient level of antidotes in the blood circulation before intoxication, so the antidotes can be tested as prophylactics. This can be characterized as "treatment in advance." The problem with their use, however, is the timing, duration, and achievement of sufficient levels of these antidotes after administration. The antidotes currently used for the treatment of OP poisoning in this context include anticholinergics, reactivators, and anticonvulsants (Bajgar et al., 2004, 2007a). The prophylactic efficacy of antidotes (as demonstrated in treatment studies) is good; administration of the antidotes typically takes place very shortly (i.e., within minutes) after intoxication. Another study showed good prophylactic efficacy of pyridostigmine or ranitidine with pralidoxime against paraoxon poisoning (Petroianu et al., 2005b, 2006). Extension of the duration of the effects of the antidote by achieving sufficient levels in the blood by oral administration is not possible (especially reactivators, as they are decomposed in the gastrointestinal tract) and therefore is excluded. Consequently, other routes of administration were sought. Transdermal administration of one of the most effective reactivators (HI-6) was shown to be the most realistic approach (Dolezal et al., 1988; Bajgar, 1991). The final result was a new prophylactic transdermal antidote TRANSANT, containing the patch impregnated with HI-6. This preparation was clinically tested (including dermal sensitivity) without any harmful effects; field testing was also successful, and TRANSANT was introduced into the Czech army and later into the Slovak army (Bajgar et al., 2004; Fusek et al., 2007). If its administration is combined with PANPAL, the two anticholinergics contained in PANPAL are able to prevent and treat symptoms of nerve agent poisoning. Therefore, a combination of TRANSANT and PANPAL administered as a pretreatment provides the best prophylactic efficacy compared to pyridostigmine or PANPAL administered alone (Bajgar et al., 2004). The anticonvulsant drugs benzodiazepines (e.g., diazepam, midazolam, alprazolam, triazolam, and clonazepam) were studied, but isolated prophylactic administration did not work to very good effect (Herink et al., 1990, 1991; Kubova et al., 1990).

PROPHYLACTIC USE OF OTHER DRUGS

Prophylactic administration of different drugs (alone or in combination) against intoxication with OPs was studied. Calcium antagonists (e.g., nimodipine), neuromuscular blockers (e.g., tubocurarine), adamantanes (e.g., memantine), and the opiate antagonist meptazinol (Galli and Mazri, 1988; Gupta and Dettbarn, 1992; McLean et al., 1992; Marrs et al., 1996; Karlsson et al., 1998; Stojiljkovic et al., 1998) were also tested, offering different results with limited practical utility. On the other hand, a positive prophylactic effect has been demonstrated with procyclidine (e.g,. antimuscarinic, antinicotinic, and the anti-NMDA receptor drug; Myhrer et al., 2002, 2003), metoclopramide (Hasan et al., 2004), clonidine (Loke et al., 2001), or procyclidine and donepezil (Haug et al., 2007). The prophylactic effect of a group of drugs with anticholinergic and/or antiglutamatergic properties (e.g., benactyzine, biperiden, caramiphen, procyclidine, and trihexyphenidyl), with respect to their anticonvulsant properties, was studied to prevent damage of the central nervous system (CNS) induced by seizures. Only procyclidine and caramiphen antagonized soman-induced seizures (Myhrer et al., 2008a; Schultz et al., 2014). Among the different drugs tested, procyclidine appears to be an effective anticonvulsant, with few cognitive side effects (Myhrer et al., 2008b). Procyclidine with physostigmine (administered transdermally) showed very good prophylactic efficacy against soman in dogs; moreover, this efficacy was increased by using antidotal therapy with HI-6 and atropine (Kim et al., 2005). Special importance can be placed on suramine (a protease inhibitor). Administration of this compound prior to soman intoxication (and followed by administration of atropine) showed good prophylactic effects (Cowan et al., 1996). However, all these studies are experimental, and they have not reached the practical output stage. The combinations of various drugs as prophylactics can be of very different character. They can be used simultaneously (a combination of different drugs) or as a pretreatment and a post-treatment with different antidotes. Administration of pyridostigmine (or other AChE inhibitors) prior to intoxication and treatment with different drugs is a typical example (Anderson et al., 1992, 1997; Kassa, 1995; Bajgar et al., 1996;

TABLE 66.1 Drugs Used in the Prophylaxis Against OP Nerve Agent Poisoning (Relatively Perspective Drugs Are in Bold)

Principle	Drug Group	Drug	Duration	Equipment of the Army	Efficacy	Comment
Protection of cholinesterase against inhibition	Carbamates	**Pyridostigmine, Aminostigmine, Physostigmine**	8 h	PYRIDOSTIGMINE BROMIDE	+++	Dose limited, side effects. Alone is not very effective, following antidotal treatment enhances its effect
		Syntostigmine, Eptastigmine, Mobam				
		Decarbofuran, Heptylphysostigmine				
	Others	**Huperzine A**				
		Tacrine, Methoxytacrine				
	Organophosphates					
		TEPP, Paraoxon				
		Ethyl-4-nitrophenylphosphonate				
	Aminophenols	Eseroline				
Simulation of treatment	Anticholinergics	**Biperidene**, **Scopolamine**, **Benactyzine** Atropine, Aprophen, Hyoscine				Transdermal administrations of scopolamine, physostigmine and HI-6 were studied
		Adiphenine, Caramiphen				
		Pentamethonium, Mecamylamine				
		Trihexyphenidyl				
	Reactivators	HI-6				
		PAM, Obidoxime, Trimedoxime, Methoxime	8 h	TRANSANT (HI-6, transdermal admiministration)	+	Alone is not effective
	Others	**Suramine** Benzodiazepines, Tubocurarine Memantine, Procyclidine Nimodipin, Clonidine				
Detoxification	Cholinesterases enzymes hydrolyzing OP monoclonal antibodies against OP	**Butyrylcholinesterase, Mutants**				Very perspective PROTEXIA®
		Acetylcholinesterase				
		Triesterase Paraoxonase				
Combinations			8 h	PANPAL (pyridostigmine, trihexyphenidyle, benactyzine)	++++	Efficacy is increased with following antidotal treatment
				PANPAL + TRANSANT	+++++	In combination, the best prophylactic efficacy

Kassa and Bajgar, 1996; Tuovinen and Hanninen, 1999; Kim et al., 2002). Aerosolized scopolamine was described to protect guinea pigs against inhalation toxicity to sarin (Che et al., 2011). There are other combinations as well, such as the administration of triesterase (Tuovinen and Hanninen, 1999; Tuovinen et al., 1999), procyclidine (Kim et al., 2002; Myhrer et al., 2002, 2003), clonidine (Loke et al., 2001), or sustained release of physostigmine and scopolamine (Meshulam et al., 2001). The results depend on experimental conditions, but this approach (administration of different drugs) has yielded some good results. However, up to now, they have been only on an experimental level.

Clearly, there is a wide range of prophylactics. The drugs used in prophylactics against nerve agents are summarized in Table 66.1. Some of them (bolded in the

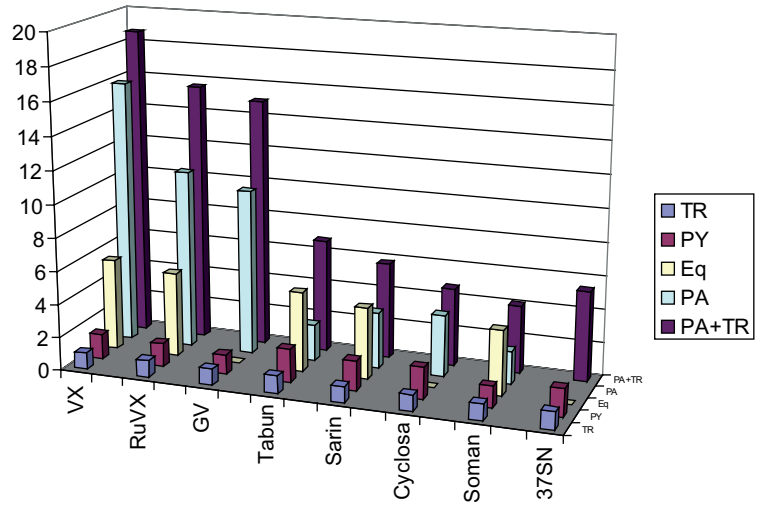

FIGURE 66.2 Efficacy (expressed as a prophylactic index) of different prophylactics (TR: TRANSANT; PY: Pyridostigmine; Eq: equine butyrylcholinesterase; PA: PANPAL; PA + TR: combination of PANPAL and TRANSANT) against selected nerve agents in rats. The results are means only. Prophylactic effect of equine butyrylcholinesterase was not tested for 2-(dimethylamino)ethyl *N*,*N*-dimethylphosphoramidofluoridate (GV), cyclosarin, and 37SN. *Source: Elaborated using data from Bajgar (2009, 2011, 2012).*

table) can be studied further. Only three prophylactics have been introduced into armies—pyridostigmine alone; PANPAL, composed of pyridostigmine, benactyzine, and trihexyphenidyl; and TRANSANT (dermal administration of HI-6). Another prophylactic drug, Protexia, can be considered as well. The efficacy of different prophylactics against nerve agents expressed as prophylactic indexes is shown in Figure 66.2.

Current prophylactics have a few problems. Reversible cholinesterase inhibitors have side effect caused by their parasympathomimetic activity. This issue can be addressed by adding anticholinergic drugs. Isolated administration of anticholinergics also can be helpful prophylactically; however, this effect can be supported by adding reversible inhibitors. The main problem of bioscavengers is immunoreaction, which can be solved by modifying the scavenger. Using the approach of the treatment in advance, reactivators are used, but the main issue is their route of administration. Further, conducting additional research into improving prophylaxes is difficult.

A comparison of features of different prophylactics is summarized in Table 66.2. Various criteria are used, including cost, side effects, and difficulty of use. The best combination of effects, cost, and accessibilty is observed when PANPAL and TRANSANT are used together. These prophylactics are shown in Figure 66.3.

It appears from these results that simple prophylaxis (without post-exposure treatment) against OP is insufficient. Therefore, pyridostigmine is important as a prophylactic drug, especially when it is combined with post-exposure antidotal treatment. For further development, it is necessary to search for novel prophylactic drugs and new routes of administration. In this context, preparations of cholinesterases are of special importance for the development of more effective prophylactics.

TABLE 66.2 Comparison of Various Prophylactics Using Different Criteria of Their Evaluation

Criterium	Pyridostigmine	PANPAL	Protexia	TRANSANT
Introduced in other armies	Yes	No	No	No
Introduced in the Czech army	No	Yes	No	Yes
Accessibility	++++	++	++	++
Cost	+	++	++++	+++
Difficulty to use	+	+	++ (i.v., i.m.)	++ (self-help)
Prophylactic effect	+	+++	+++	alone ineffective
Side effects in recommended dose	No	No	Antigenic properties	No
Duration of prophylaxis	6–8 h	6–8 h	5–8 h	8 h
Increase of effect of followed antidotal treatment	+	++	No	++

PANPAL

TRANSANT

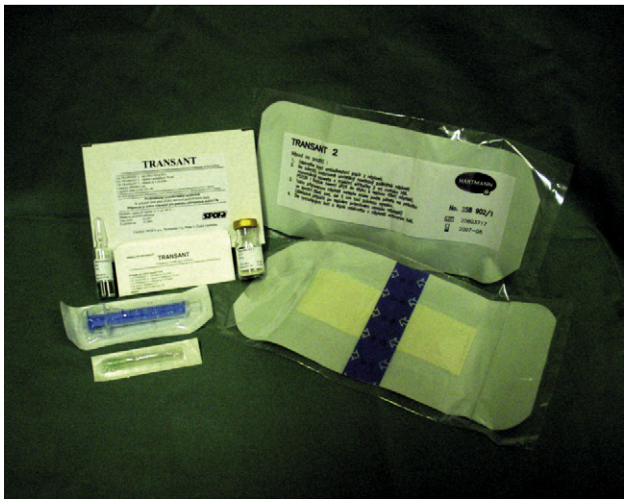

FIGURE 66.3 Two prophylactics: PANPAL (top) and TRANSANT (bottom).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

There are many drugs being tested for their prophylactic efficacy against nerve agent intoxication. However, only three of these (pyridostigmine alone, PANPAL, and TRANSANT) have been introduced into military practice. The prospective approach for the future seems to be to search for more effective drugs or their combinations using modern techniques (e.g., molecular modeling), including new routes of administration, and the use of purified enzymes, especially cholinesterases and paraoxonases produced by biotechnology.

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CHAPTER

67

Prophylactic and Therapeutic Measures in Nerve Agents Poisoning

Trond Myhrer and Aas Pål

INTRODUCTION

Nerve agents are regarded as the most toxic means among all chemical weapons. The first nerve agents were originally synthesized during the 1930s in Germany in attempts to achieve more efficient pesticides based on organophosphorus compounds. Tabun was the first one synthesized, followed by sarin and soman. VX is another type of nerve agent that was originally developed in the United Kingdom when searching for new insecticides. The organophosphorus nerve agents are highly potent irreversible inhibitors of the enzyme acetylcholinesterase (AChE) that hydrolyzes acetylcholine (ACh). Accumulation of ACh in the synaptic cleft results in overstimulation of muscarinic and nicotinic receptors. This cholinergic overactivity can affect all organ systems. The toxic signs in humans include pinpoint pupils (miosis), bronchoconstriction, hypersalivation, increased lung secretions, sweating, diarrhea, loss of consciousness, seizures, and respiratory arrest. Miosis appears to be a very sensitive index of direct exposure and can be painful for several days. Chest tightness, rhinorrea, and increased salivation can occur within seconds or minutes of inhalation of nerve agent. If exposure is substantial, then death may occur from respiratory arrest within minutes. Individuals moderately exposed usually recover completely, although Electroencephalogram (EEG) abnormalities have been reported in those severely exposed to sarin 6–8 months after the terror attack in Japan (Murata et al., 1997).

BACKGROUND

Exposure to nerve agents requires immediate medical treatment. For this purpose, military personnel are issued with autoinjectors containing countermeasures for self-administration or "buddy aid." Antidotes against nerve agents are based on drugs acting at the muscarinic receptors and Gamma-amino-butyric-acid, A-receptor ($GABA_A$) receptors (McDonough and Shih, 1997). In addition, partial protection against nerve agents can be obtained by the use of reversible (carbamate) AChE inhibitors shielding a portion of AChE from irreversible inhibition by nerve agents before nerve agent exposure. Furthermore, reactivation of any unaged AChE by an oxime is regarded as important immediate treatment after nerve agent exposure.

A number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime, 2-PAM, HI-6), an anticholinergic (atropine), and a $GABA_A$ agent (diazepam, avizafone) combined with carbamate (pyridostigmine) pre-treatment (Aas, 2003). However, such treatment regimens can reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and concomitant convulsions, unless atropine is administered early and at a high dose (McDonough and Shih, 1997). Such seizures rapidly progress to status epilepticus, a condition that is strongly associated with mortality and brain damage in experimental animals (Shih et al., 2003). Thus, there is an urgent need to search for novel strategies able to prevent or terminate nerve agent-induced seizures. For this reason, the present chapter focuses on animal studies (guinea pigs, rats, monkeys) designed to acquire novel insights into the neuropharmacological mechanisms of nerve agent-induced seizures and neuropathology.

MECHANISM OF ACTION

It has been hypothesized that several neurotransmitter systems become involved sequentially in the initiation

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and maintenance of seizures elicited by nerve agents (McDonough and Shih, 1997). The progression of events can conceptually be divided into three phases. An early cholinergic phase lasting from the time of exposure to approximately 5 min after onset of seizures is dominated by high cholinergic activity followed by a transitional phase of cholinergic and glutamatergic hyperactivity, and finally a predominantly glutamatergic phase after approximately 40 min (McDonough and Shih, 1997). In the latter study, a substantial body of evidence has been provided in support of this model.

Nerve agent intoxication results in inhibition of AChE and a tremendous buildup of ACh, leading to protracted muscarinic receptor activation. This activation seems to happen in a dose-related manner. The higher the dose of soman, the shorter the latency to onset of seizures and the greater the likelihood of early death in rats (Myhrer et al., 2006a). The brainstem respiratory drive is vulnerable to increased cholinergic input, because the function of the respiratory center is disrupted. Both muscarinic and nicotinic antagonists can protect the respiratory center against cholinergic overstimulation (Kubin and Fenik, 2004). In compliance with this notion, the nicotinic antagonist mecamylamine in high doses can prolong survival in mice intoxicated by soman (Hassel, 2006). The functional integrity of the diaphragm is not significantly compromised by soman, unless a dose of 14× LD_{50} is used in cats (Rickett et al., 1986). After a soman dose of 5× LD_{50}, the latency to seizure onset is approximately 1 min, whereas the latency is approximately 14 min when the dose of soman is 1× LD_{50} in rats (Myhrer et al., 2003; 2006a). The change in ACh levels throughout the brain has been reported to precede that of any other neurotransmitter (Fosbraey et al., 1990). The increases in glutamate occur in a number of brain regions with different time courses after seizures have started (McDonough and Shih, 1997). According to the three-phase model, efficacious pharmacological countermeasures against nerve agents should preferentially exert cholinergic and glutamatergic antagonism along with GABAergic agonism.

PROPHYLACTIC MEASURES

Animal Studies

It takes a higher dose of anticonvulsants to terminate seizures induced by soman than other classical nerve agents such as tabun, sarin, cyclosarin, and VX (Shih and McDonough, 2000). This suggests that a drug capable of stopping soman-generated seizures would be equally effective for the treatment of seizures evoked by other nerve agents. This is the reason why soman is the nerve agent most frequently used in animal models to evaluate potential anticonvulsant drugs.

Pyridostigmine does not penetrate the blood–brain barrier (BBB) to any extent and therefore provides very limited protection of the brain (Aas, 2003). For this reason, some recent investigations of prophylactic treatment in animals have focused on physostigmine as an AChE inhibitor, because this carbamate (in contrast to pyridostigmine) readily crosses the BBB (Birtley et al., 1966). Although physostigmine in optimal doses does not protect against a lethal dose of soman, it appears effective when used along with an anticholinergic drug in guinea pigs (Philippens et al., 2000). Physostigmine in combination with scopolamine has been reported to protect guinea pigs effectively against soman, sarin, and tabun (Philippens et al., 2000; Wetherell et al., 2002). Similar protective effect has also been obtained with monkeys. Pre-treatment of rhesus monkeys with physostigmine and scopolamine or physostigmine and trihexyphenidyl 25 min before exposure to 2× LD_{50} of soman results in survival without convulsions or loss of consciousness (von Bredow et al., 1991).

The cholinergic and glutamatergic antagonist, procyclidine, in combination with physostigmine, has been demonstrated to assure full protective effects against convulsant doses of soman in rats and guinea pigs without any need for post-exposure treatment (Kim et al., 2002). Because physostigmine has a rather short half-life, it will preferably require continuous administration. In a study of rats, osmotic minipumps were implanted subcutaneously, allowing sustained delivery of physostigmine and procyclidine in 3 days before exposure to a soman dose of 1.3× LD_{50}. This treatment exerted full protective effects against lethality, convulsions, learning and memory impairments, and physical incapacitation (Choi et al., 2004). In a subsequent study in beagle dogs, a matrix-type patch attached to the abdominal side of the animal delivered physostigmine and procyclidine for 2 days before being challenged with one of various doses of soman (2–10× LD_{50}). The patch delivery protected against seizures and death up to 4× LD_{50} of soman. When the patch was combined with HI-6 and atropine injected intramuscularly 1 min after soman exposure, protection was achieved up to a dose of 6× LD_{50} (Kim et al., 2005).

In a study from our laboratory, the combined effects of physostigmine and procyclidine were tested against various doses of soman. Physostigmine (0.1 mg/kg) in combination with procyclidine doses of 1, 3, or 6 mg/kg effectively prevents the development of convulsions and hippocampally monitored seizures when the doses of soman were 1.3×, 1.6×, or 2× LD_{50}, respectively (Figure 67.1). Results from [^{3}H]MK-801 binding experiments show that procyclidine inhibits the phencyclidine site at the N-methyl-D-aspartic acid (NMDA) receptor in a concentration-dependent manner (Myhrer et al., 2004a). Physostigmine (0.1 mg/kg) and procyclidine in a dose of

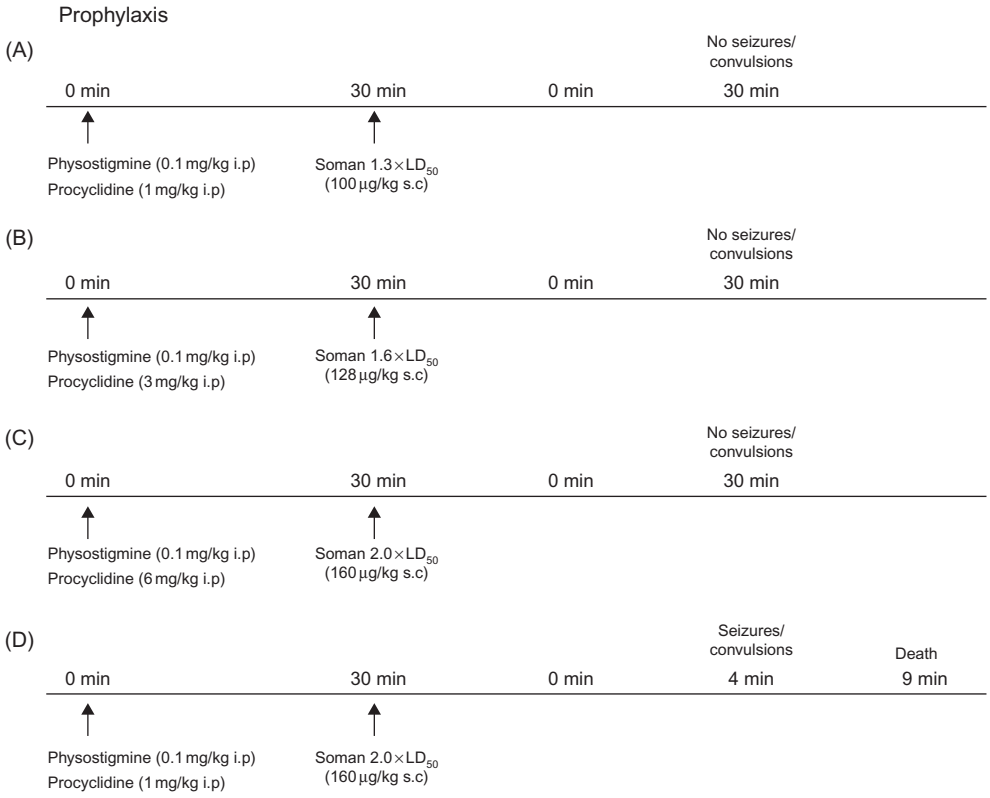

FIGURE 67.1 Experimental design for demonstration of anticonvulsant effects of a fixed dose of physostigmine combined with increasing doses of procyclidine relative to increasing doses of soman (A–C). Insufficient dose of procyclidine compared with soman results in early death (D).

1 mg/kg do not prevent convulsions/seizures when the soman dose is 1.6× LD_{50}. Subsequent treatment with scopolamine in doses of 0.5 or 1 mg/kg immediately after (3 min) seizure onset shows that only the highest dose can produce a reliable termination (Figure 67.2). When scopolamine (1 mg/kg) is given later (10 min) after onset of seizures, no effect is obtained. The sustained seizures were subsequently treated with diazepam (10 mg/kg) and pentobarbital (30 mg/kg), and finally terminated 25 min after onset. In rats given inadequate prophylaxis, both modified convulsions and seizures are seen. It is recommended that moderate doses of prophylactics should be used to avoid adverse effects on cognitive functions, because insufficient prophylaxis can be compensated for by adjunct treatment (Myhrer et al., 2004a).

To avoid the problem with a short-lasting prophylactic drug, the AChE inhibitors huperzine, donepezil, and galantamine may offer alternatives with long-lasting efficacy. Both rats and guinea pigs are well-protected against soman-induced seizures and neuropathology when pretreated with huperzine, whereas pyridostigmine does not have such effects (Lallement et al., 1997; Tonduli et al., 2001). Donepezil alone does not protect well against seizures evoked by soman, but when donepezil is combined with procyclidine, significant protection is obtained in rats (Haug et al., 2007). Galantamine in combination with atropine administered before a convulsant dose of soman can effectively protect guinea pigs against seizures (Albuquerque et al., 2006).

When pyridostigmine is combined with benactyzine and trihexyphenidyl (exerting both cholinergic and glutamatergic antagonism) in rats, effective protection is obtained against signs of neurotoxicity induced by soman. However, the combination with pyridostigmine and biperiden is reported to be even more efficacious than the former combination against soman poisoning (Kassa et al., 2003).

The results from studies of prophylactic treatment presented so far are in compliance with the notion that seizures are triggered by cholinergic hyperactivity followed by sustained high cholinergic activity for approximately 5 min after seizure onset. An exception has been seen when MK-801 given before soman can prevent the occurrence of seizures in both guinea pigs and rats (Braitman and Sparenborg, 1989; Shih et al., 1991). In the latter studies, however, MK-801 was combined with other drugs (pyridostigmine, atropine, and pralidoxime or HI-6, respectively). Hence, it is difficult to rule out the possibility that MK-801 interacted with effects of the additional drugs. HI-6 has been shown to decrease the release of ACh when hippocampal slices are exposed to soman (Øydvin et al., 2005). Microinfusions of MK-801

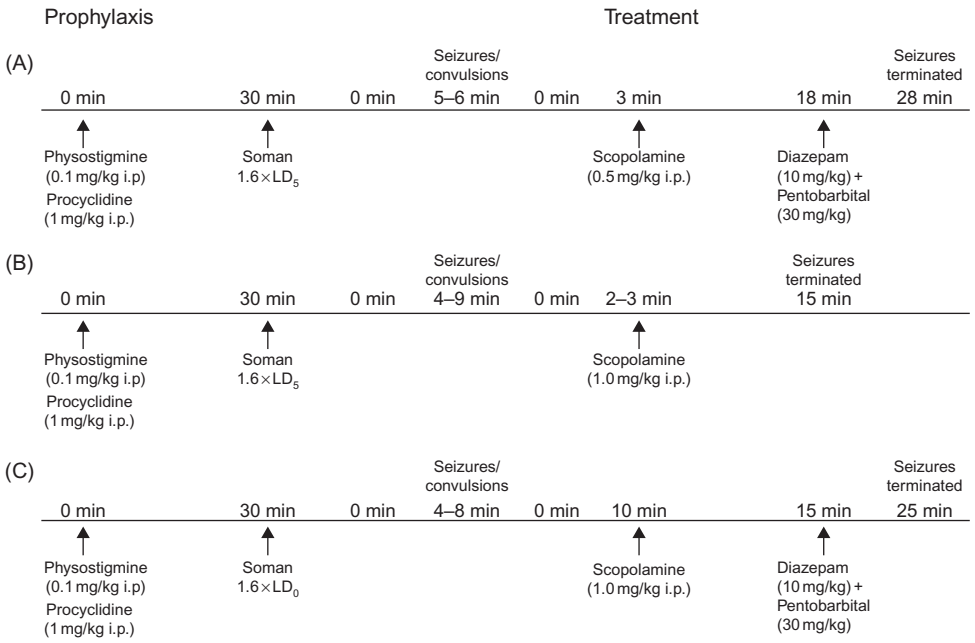

FIGURE 67.2 Experimental design for demonstration of how insufficient prophylactic treatment can be compensated for by adjunct post-exposure treatment. Low dose of scopolamine requires additional treatment (A). High dose of scopolamine is sufficient to terminate seizures (B). High dose of scopolamine given outside the cholinergic window requires additional treatment (C).

or ketamine into the seizure-controlling region, area tempestas, do not cause anticonvulsant effects against soman intoxication, whereas microinfusions of atropine or scopolamine do so (Myhrer et al., 2008a). Moreover, systemic administration of solely MK-801 or N-(1-(2-thienyl)cyclohexyl)piperidine (TCP) before soman does not prevent the occurrence of seizures (Carpentier et al., 1994; Myhrer et al., 2008a).

It is crucial that prophylactics used to protect military personnel, and possibly first responders, against lethal doses of nerve agents do not by themselves produce impairment of cognitive capability. This issue appears to be neglected in nerve agent research (Myhrer et al., 2004b). In the latter study, it was examined whether physostigmine, scopolamine, and various doses of procyclidine might reduce rats' innate preferences for novelty (Figure 67.3). When these drugs were tested separately, the results showed that physostigmine (0.1 mg/kg) and procyclidine (3 mg/kg) do not affect preference for novelty, whereas scopolamine (0.15 mg/kg) and procyclidine in a higher dose (6 mg/kg) result in a preference deficit. The combination of physostigmine and scopolamine or physostigmine and procyclidine (6 mg/kg) causes a marked deficit in preference for novelty. A much milder deficit is observed when physostigmine is combined with lower doses (1 or 3 mg/kg) of procyclidine. The latter combinations also have milder adverse impact on the animals' interests in the test environment and activity measures than the former combinations. In a more recent study, a regimen consisting of HI-6 (125 mg/kg), levetiracetam (50 mg/kg), and procyclidine (20 mg/kg) has been shown to protect effectively against a soman dose of 1.3× LD_{50} (Myhrer et al., 2013b). However, the cognitive impairment in the novelty test is similar to that seen after the combinations of physostigmine and scopolamine or physostigmine and procyclidine (6 mg/kg) (Myhrer and Enger, unpublished results).

Administration of physostigmine and procyclidine for 3 days by means of minipumps (72 and 432 µg/kg/h, respectively) in rats does not affect passive avoidance performance (Choi et al., 2004). A similar administration of physostigmine and scopolamine for 2 weeks in marmosets results in normal performance in a two-choice discrimination serial task. However, the concentration of scopolamine used is equivalent to an acute dose of 0.009 mg/kg, which is considerably lower than doses that have been shown to affect marmoset cognitive performance (e.g., 0.06 mg/kg) (Muggleton et al., 2003).

In a behavioral study of rats, potential cognitive effects of a group of anti-Parkinson drugs (with excellent anticonvulsant properties against nerve agents) were examined in the novelty test (Myhrer et al., 2008b). It was shown that benactyzine (0.3 mg/kg), caramiphen (10 mg/kg), and trihexyphenidyl (0.12 mg/kg) reduce rats' innate preferences for novelty, whereas biperiden (0.11 mg/kg) and procyclidine (3 mg/kg) do not. When benactyzine, caramiphen, or trihexyphenidyl are combined with physostigmine, the cognitive impairment disappears. This counteracting effect, however, produces changes in locomotor and rearing activity not seen by

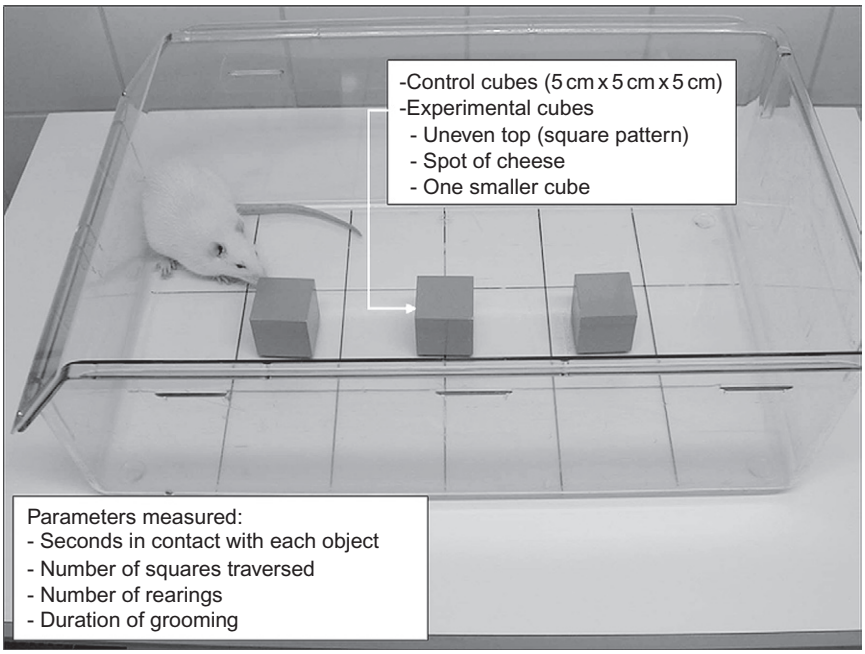

FIGURE 67.3 Novelty test measuring rats' innate preference for novelty, locomotor activity, rearing, and grooming. Testing is performed in three sessions (one per day). Each session consists of 5 min of exploring with neutral cubes present (phase 1) and phase 2 (5 min) occurs after 10 min in the home cage, during which the central cube has been replaced by a novel one. The novelty is represented by uneven top (session I), tiny bit of cheese on one side (session II), or smaller cube (session III).

each drug alone. AChE inhibitors and anticholinergics used as prophylactics can offset each other, but exceptions are observed in a previous study (Myhrer et al., 2004b) when a very potent anticholinergic (scopolamine), or a high dose of procyclidine, still results in cognitive deficits despite co-administration with physostigmine. The half-life of physostigmine is 17 min in plasma and 16 min in the brain of rats (Somani and Khalique, 1986). A crucial issue is how physostigmine is able to antagonize receptor blocking effects of anticholinergics by increasing the level of ACh and thus reduce or prevent cognitive impairment. This apparently subtle balance is probably attained in different ways for other relevant AChE inhibitors used as pre-treatment against nerve agents, such as donepezil, huperzine, and galantamine. To equalize cognitive side effects of potent anticovulsants by use of AChE inhibitors is not recommendable, because accurate adjustments for differences in half-life would be required for acute doses. Use of continuous delivery, however, may circumvent the problem with a different half-life of combining drugs.

Human Use

Pre-treatment with the carbamate, pyridostigmine, is a well-established method to protect against nerve agent intoxication in the armed forces in a number of nations (Aas, 2003). A tablet (30 mg) of pyridostigmine bromide is supposed to be taken every 8 h by the service personnel. The rationale behind this use is that carbamate occupies a portion of the available AChE (15–40% of the erythrocyte AChE) and renders it inaccessible to nerve agents in the blood, because nerve agents only bind to unprotected enzymes (Leadbeater et al., 1985). The AChE has been reversibly inhibited by pyridostigmine spontaneous decarbamoylates, and the enzyme is again able to hydrolyze ACh. Because pyridostigmine does not penetrate the BBB to any extent under normal circumstances, it only protects the peripheral nervous system. Pyridostigmine has been reported to have negligible physiological and psychological side effects (Leadbeater et al., 1985). However, in a recent meta-analysis, it is stated that carbamate can cause side effects in predisposed individuals (Golomb, 2008).

Physostigmine (a carbamate) readily crosses the BBB and has been considered a convenient replacement of pyridostigmine. Based on a series of animal studies, the combination of physostigmine and the cholinergic antagonist, hyoscine (scopolamine), has been brought to the level of clinical trials in the United Kingdom (Scott, 2007). The problem with the relatively narrow time window and potential side effects of physostigmine has been solved by continuous co-administration with hyoscine in terms of transdermal delivery. The pharmacological antagonism between these two drugs probably alleviates the potentially adverse effects produced by each

drug alone. This pre-treatment regimen is supposed to be less reliant on supporting therapy than the use of pyridostigmine (Scott, 2007).

The prophylactic combination of pyridostigmine with trihexyphenidyl and benactyzine has been introduced in the Czech Army. The presence of two anticolinergics made it possible to increase the dose of pyridostigmine and thus enhance the prophylactic efficacy (Bajgar, 2004). This combinational regimen (designated PANPAL) has been reported to have no side effects as demonstrated in volunteers; no changes in the psychic state or dysfunctions were observed. Actually, an improvement of tapping test performance was found after administration of PANPAL (Bajgar, 2004). For further details, see chapter 66.

The use of human plasma-derived butyrylcholinesterase to neutralize the toxic effect of nerve agents *in vivo* has been shown to increase survival and protect against attenuated cognitive function after nerve agent exposure. No post-exposure autoinjectors are necessary (Lenz et al., 2007). This bioscavenger is currently being produced by using outdated human blood and is ready for clinical trials. Both military personnel and potentially some first responders are relevant groups needing protection by biological scavengers.

THERAPEUTIC MEASURES

Animal Studies

Post-exposure treatment of animals consists of two categories: immediate and subsequent therapies. In the first category, drugs with anticholinergic effects will be of preference, whereas drugs with antiglutamatergic effects will be of preference in the second category according to the three-phase model. Drugs enhancing $GABA_A$ neurotransmission (diazepam and other benzodiazepines) are effective during all phases of nerve agent intoxication (McDonough and Shih, 1997). Immediate therapy also includes an oxime to reactivate nonaged inhibited AChE.

Reactivation of inhibited AChE is considered to be an important element in post-exposure treatment. Bispyridinium oximes can reactivate the phosphorylated enzyme if they are administered before the change from the reactivatable to the unreactivatable state, the process referred to as "aging." The "aging" process is fastest with soman in brain and diaphragm preparation (a few minutes) compared with other nerve agents (Sun et al., 1979). However, administration of oximes after "aging" has already occurred can enhance survival (McDonough and Shih, 1997). The oximes pralidoxime and obidoxime have been widely used, but HI-6 appears to be a promising substitute in treatment of intoxication with nerve agents. HI-6 has relatively low toxicity and is efficient in the treatment of soman intoxication (Clement, 1982; Hamilton and Lundy, 1989; Kusic et al., 1985; Lundy et al., 2011; Van Helden et al., 1992; Walday et al., 1993). A disadvantage of HI-6 is its lack of stability in aqueous solutions that require storing as powder in a separate chamber in the autoinjector (Thiermann et al., 1996).

Anticholinergic drugs and an oxime are usually applied after prophylactic treatment (pyridostigmine). Anticholinergics have optimal efficacy during the cholinergic phase (i.e., from exposure to approximately 5 min after onset of seizures). Such drugs are classified as muscarinic receptor antagonists, and they are not able to counteract the nicotinic signs of intoxication like muscle fasciculation, muscle fatigue, and weakness (McDonough and Shih, 2007). The rat brain is provided with the muscarinic receptor subtypes M1–M5. However, effective drugs against nerve agents have been shown to be anticholinergics with high specificity toward M1 (McDonough and Shih, 1997). The antinicotinic anticholinergic agent, mecamylamine, does not have anticonvulsant efficacy in rats poisoned with soman (Shih et al., 1991). The effectiveness of potent centrally active anticholinergics has been shown to be at least as good as diazepam in terminating nerve agent-induced convulsions (McDonough and Shih, 1997). Rats pretreated with HI-6 (to reduce lethality) were given a soman dose of 1.6× LD_{50} and received anticonvulsants 5 or 40 min after onset of seizures. The results show that anticholinergics (scopolamine, atropine, benactyzine, trihexyphenidyl) can effectively terminate seizure activity at 5 min, but not at 40 min after seizure onset (McDonough and Shih, 1993). These anticholinergics are as effective and potent as anticonvulsants as if they had been given as pre-treatment.

In our laboratory, we have examined the efficacy of a triple combination of drugs with adequate anticonvulsant effects and a dual combination with inadequate anticonvulsant effects followed by adjunct therapy (Myhrer et al., 2006a). The results show that combined (mixed as a single compound) intramuscular injections of HI-6 (42 mg/kg), atropine (14 mg/kg), and avizafone (3 mg/kg) administered 1, 16, and 31 min after exposure to a soman dose of 4× LD_{50} completely terminate seizures with a moderate mortality rate (25%). When the soman dose was lowered to 3× LD_{50}, the anticonvulsant effect was complete, and no rats died within 24 h. Rats challenged with 5× LD_{50} of soman all died within 10 min. Without avizafone in the combination, seizures induced by 3× or 4× LD_{50} of soman cannot be terminated unless an adjunct therapy consisting of procyclidine (6 mg/kg), diazepam (10 mg/kg), and pentobarbital (30 kg/kg) is given, and the mortality rate is comparatively high (78%). Administration of the adjunct therapy alone 6–16 min after 4× LD_{50} of soman stopped the seizure activity, but all the rats died within 24 h (Myhrer et al., 2006a). The latter study shows that administration of

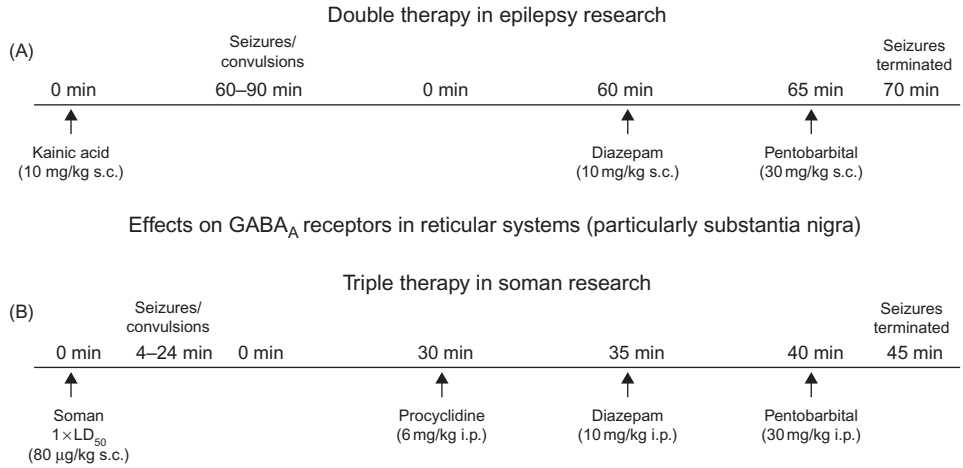

FIGURE 67.4 Experimental design revealing anticonvulsant effects of diazepam and pentobarbital in epilepsy research (A). Anticonvulsant effects achieved by triple regimen when administered 30–40 min after onset of seizures (B).

countermeasures (mimicking autoinjectors) soon after exposure to a relatively high dose of nerve agent can prevent death, even without pre-treatment of the animals.

The great challenge in treatment of nerve agent-poisoned victims seems to be the cases of long delays before treatment can start. Seizures lasting beyond 40 min are increasingly difficult to terminate (Lallement et al., 1999; Carpentier et al., 2001). Even conservative estimates suggest that it will take at least 30 min for emergency personnel to access individuals unprepared for exposure to nerve agents. Even soldiers properly provided with protective masks, gloves, and clothes may need medical help, because poor training, poor discipline, or bad luck can lead to intoxication of nerve agents (Lallement et al., 1999). Hence, there is a pertinent need to search for novel strategies capable to control nerve agent-generated seizures well after their onset.

Drugs that enhance $GABA_A$ neurotransmission have been shown to be effective in intoxicated animals during all phases of seizures by counteracting the elevated level of glutamatergic activity (McDonough and Shih, 1997). Seizures induced by kainic acid or lithium/pilocarpine in rats can be terminated 1 h after onset by the administration of diazepam and pentobarbital (Du et al., 1995). This potent combination of two $GABA_A$ agonists may create a promising anticonvulsant therapy if it is supplied with an anticholinergic agent. Procyclidine was chosen for this purpose, because this drug exerts antagonism on both muscarinic and nicotinic receptors and additionally has antagonistic effects on NMDA receptors (Kim et al., 2002). Procyclidine (6 mg/kg), diazepam (10 mg/kg), or pentobarbital (30 mg/kg) does not stop soman-induced seizures when administered separately, but both convulsions and seizure activity are terminated when these agents are given together. This triple therapy is 100% effective when administered 30–40 min (5 min between each injection) after onset of convulsions (Figure 67.4) (Myhrer et al., 2003).

In both military and civilian contexts, it would appear somewhat inconvenient for medical personnel to administer three injections of antidotes to achieve termination of seizure activity. It would be more expedient to replace diazepam and pentobarbital by a single $GABA_A$ modulator assuring corresponding anticonvulsant impact. However, systemic administration of $GABA_A$ modulators does not differentiate well between their anticonvulsant properties (Shih et al., 1999). In a study based on microinfusion of GABAergic modulators into seizure-controlling areas in the rat brain (area tempestas and substantia nigra), it was found that muscimol, ethanol, and propofol possess the best anticonvulsant potencies against soman seizures among the agents examined (Myhrer et al., 2006b). In a subsequent study, it was shown by systemic administration that procyclidine (10 mg/kg) combined with either muscimol (20 mg/kg), ethanol (3 mg/kg), or propofol (50 mg/kg) can effectively terminate soman-induced seizures when administered 5 min apart at 30–35 min after onset. Procyclidine and propofol are considered the most relevant double regimen to replace the previous triple regimen consisting of procyclidin, diazepam, and pentobarbital (Myhrer et al., 2006c).

It has been demonstrated that untreated seizures/convulsions induced by soman result in extensive neuropathology and pronounced cognitive deficits in the Morris water maze (Filliat et al., 1999; Raveh et al., 2002; 2003). In our laboratory, we investigated whether there is a relationship between the extent of neuronal pathology and degree of dysfunction in the performance of cognitive tasks after soman-induced convulsions that had lasted for 45 min (Myhrer et al., 2005). One behavioral task used was the novelty test (Figure 67.3) that

FIGURE 67.5 A three-choice simultaneous brightness discrimination test. The rats (deprived of water) learn that they receive water from the well in the positive cylinder (in this case gray, which is used for half of the rats in each group; the other half have a black cylinder). The criterion (five correct choices in succession) is obtained after 2 days of training. Thirteen days after acquisition, the rats are tested for what has been retained of the learning principle (gray or black positive cylinder).

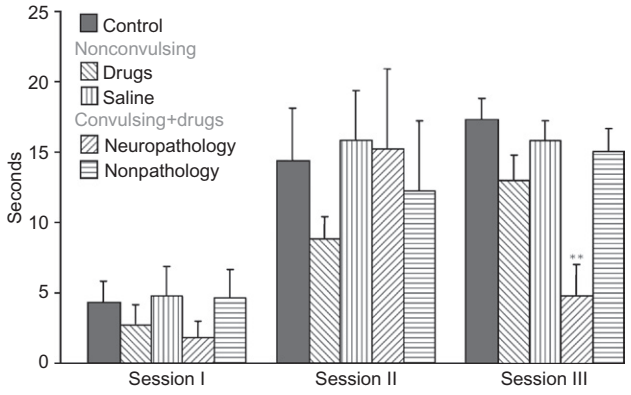

FIGURE 67.6 Mean (SEM) measures of preference for novelty 9–11 days after exposure to soman. Preference for novelty is expressed as the difference in seconds between exploring of novel versus neutral objects. Drugs given are procyclidine (6 mg/kg), diazepam (10 mg/kg), and pentobarbital (30 mg/kg). **Significantly different from all other groups ($p<0.01$).

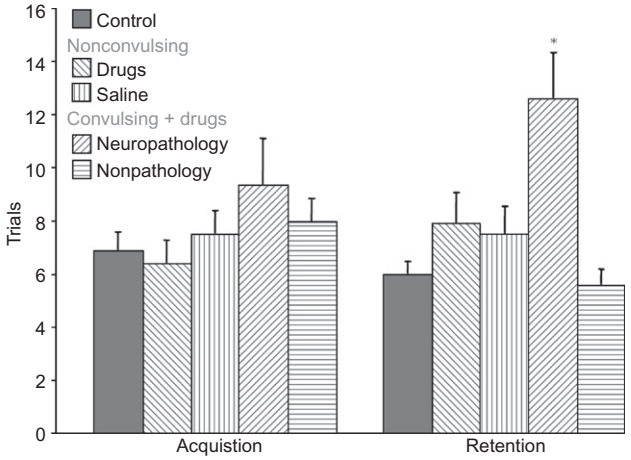

FIGURE 67.7 Mean (SEM) performances (trials to criterion) of acquisition (14–15 days after soman) in the brightness discrimination task. Drugs are as in Figure 67.6. *Significantly different from all other groups ($p<0.05$).

has proven particularly sensitive in revealing cognitive dysfunctions after selective disruptions of entorhinal projections (Myhrer, 1988, 1989). The other behavioral task used was a simultaneous brightness discrimination test (Figure 67.5) for assessing memory function. A three-choice version of this task has proven sensitive to selective hippocampal or rhinal lesions (Myhrer, 2000). When the convulsions are stopped after 45 min with the triple regimen (procyclidine, diazepam, pentobarbital), the results show that the neuronal pathology (assessed with Fluoro-Jade B staining) varies from none at all to 30% damage in the index areas (hippocampus, amygdala, piriform cortex). Cognitive deficits are seen in the novelty test (11 days after exposure) (Figure 67.6) and retention of the brightness discrimination task (28 days after exposure) among the rats with neuropathology (Figure 67.7). Furthermore, significant correlations between neuropathology scores and behavioral measures have been found for the animals that convulsed. It is concluded that early management of soman-induced convulsions is of major importance in preventing neuropathology and accompanying cognitive impairments (Myhrer et al., 2005).

In a number of studies, attempts have been made to terminate soman-triggered seizures/convulsions well after their onset. However, unlike the studies from our laboratory (Myhrer et al., 2003, 2005, 2006c), previous studies have been based on animals also treated before the exposure to soman. Marked anticonvulsant effects have been obtained in guinea pigs pretreated with pyridostigmine, administered atropine 1 min after a convulsant dose of soman, and treated with ketamine 30–120 min after poisoning (Dorandeu et al., 2005). Treatment with the combination of 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide (NBQX) and TCP 30 or 50 min after onset of soman-induced seizures has pronounced anticonvulsant impact on rats pretreated with HI-6 and atropine (Lallement et al., 1994b). When pretreated rats (HI-6) are given a single drug of a number of anticholinergics or antiepileptic agents 40 min after onset of soman-evoked seizures, the anticonvulsant efficacy is rather modest (McDonough and Shih, 1993; Shih et al., 1999).

Seizures evolving to status epilepticus (seizure activity for 30 min or more) are strongly associated with death or brain damage. The first signs of neuropathology are

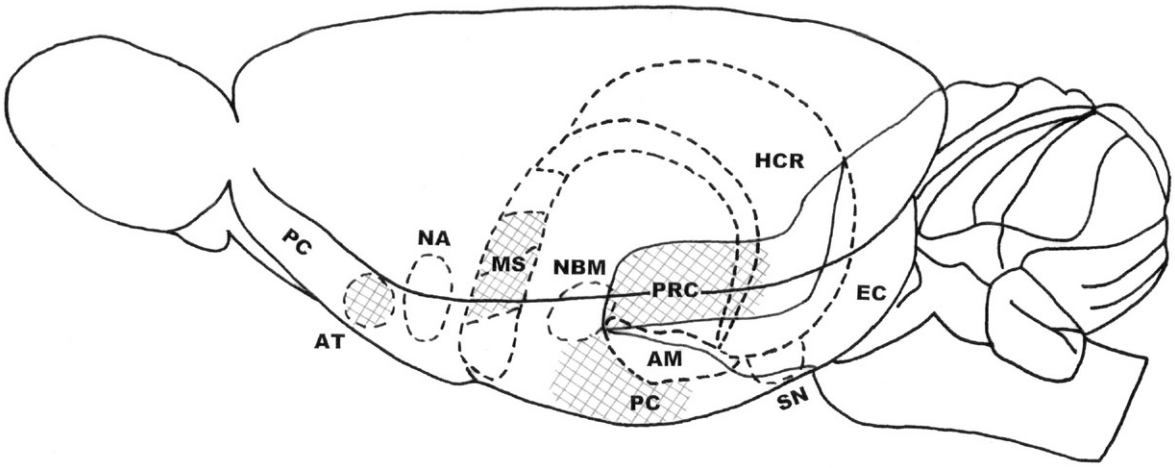

FIGURE 67.8 Lateral view of the rat brain showing neuronal target areas for nerve agents. Anticonvulsant efficacy was obtained by lesions in the area tempestas (AT), medial septum (MS), piriform cortex (PC), or perirhinal cortex (PRC) (crosshatched areas). Lack of anticonvulsant effect was seen by damaging the nucleus accumbens (NA), nucleus basalis magnocellularis (NBM), hippocampal region (HCR), amygdala (AM), substantia nigra (SN), or entorhinal cortex (EC).

usually detectable approximately 20 min after onset of soman-generated seizures in rats (Lallement et al., 1994a; McDonough et al., 1995). The mechanism behind sustained epileptiform activity may be related to increased inactivation of $GABA_A$ receptors along with an upregulation of NMDA receptors (Chen and Wasterlain, 2006). The net outcome of these changes would require comparatively high doses of antiglutamatergic drugs to control nerve agent-induced seizures well after onset. This situation appears to comprise the biggest challenge within nerve agent research.

A triple regimen consisting of procyclidine, diazepam, and pentobarbital has been shown to effectively terminate soman-evoked seizures in rats when administered 30–40 min after onset. A refinement of the triple regimen resulted in a double regimen composed of procyclidine and propofol that can stop seizures 30–35 min after they have been triggered by soman. However, both the triple and double regimens would need monitoring of vital functions, because pentobarbital and propofol can suppress normal function of the respiratory center in the brainstem. Thus, alternative approaches making it possible to design anticonvulsants predominantly affecting the forebrain have to be perfected.

Epileptiform activity does not spread randomly throughout the brain, but seems to be generated and propagated by specific anatomical routes (Gale, 1988; Löscher and Ebert, 1996). This suggests that certain propagation pathways may function as common denominators for the development of certain types of epileptiform activity, independent of the ways seizures are triggered. Within the rat brain, there are areas with control mechanisms able to attenuate all aspects of convulsive activity. The localization of such control areas have been identified in preclinical epilepsy research by use of lesion and microinfusion techniques. Our intent has been to use corresponding techniques in nerve agent research. Although the ultimate purpose is to develop pharmacological therapies that will be effective after exposure to nerve agents, a prophylactic experimental design has to be applied. Target areas for nerve agents that can act as seizure-controlling sites can be mapped by selective lesions.

If selective damage to a brain structure ensures anticonvulsant effects against nerve agent intoxication, then the constellation of receptor types in the damaged area may provide clues for designing drugs with powerful anticonvulsant properties. Primary structures are those containing large assemblies of cholinergic neurons and areas demonstrated to have control capabilities in experimental epilepsy. Aspiration lesion of the medial septum (Figure 67.8) causes prevention or increased latency to onset of convulsions, whereas damage to the nucleus basalis magnocellularis or nucleus accumbens does not have anticonvulsant effects in rats exposed to a convulsant dose of soman (Myhrer et al., 2007). Rats with aspiration lesion of the seizure-controlling area tempestas in the anterior piriform cortex display marked anticonvulsant behavior in response to soman, whereas such effect is not seen when the substantia nigra is destroyed (Myhrer et al., 2007). Similar lesions made in the perirhinal cortex or posterior piriform cortex produce anticonvulsant efficacy against soman intoxication, but anticonvulsant impact is not achieved when lesions are made in the entorhinal cortex, hippocampal region, or amygdala (Myhrer et al., 2008c).

Pharmacological agents with potential anticonvulsant efficacy in the brain areas that have been identified as

target sites for generation of seizures (the area tempestas, medial septum, perirhinal cortex, posterior piriform cortex) have been used in microinfusion studies (Myhrer, 2010). In the area tempestas, cholinergic, but not glutamatergic, antagonism is likely the active property of the anti-Parkinson agents, caramiphen and procyclidine, because the NMDA antagonists ketamine and MK-801 do not have anticonvulsant effects (Myhrer et al., 2008a). The $GABA_A$ modulators muscimol, ethanol, and propofol produce anticonvulsant effects, whereas diazepam and pentobarbital do not (Myhrer et al., 2006b). In the medial septum, only muscarinic receptors seem to be the effective ones, because ketamin and muscimol do not produce anticonvulsant effects (Myhrer et al., 2009). In the perirhinal cortex, both muscarinic and glutamatergic receptors have to be blocked simultaneously (procyclidine) to achieve anticonvulsant effect. Neither scopolamine alone nor ketamine alone causes anticonvulsant effects (Myhrer et al., 2010a). The positive effect of NBQX suggests that alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are critical for anticonvulsant impact in the perirhinal cortex (Myhrer et al., 2010a). Furthermore, modulation of metabotropic glutamate receptors (mGluRs) in the perirhinal cortex also has anticonvulsant effects. Injection of 2-methyl-6-(phenylethynyl)pyridine (MPEP) (antagonizing mGluR5) or DCG-IV (agonistic effect on both mGluR2 and mGluR3) results in marked anticonvulsant efficacy (Myhrer et al., 2010b). In a more recent study, double infusions of anticonvulsants were used to reach a larger area of the perirhinal cortex. With such a technique, MPEP protects 67% of the rats from having soman-induced seizures, whereas for DCG-IV protection is 83% (Myhrer et al., 2013a). These findings suggest that there is an early increase of glutamatergic activity in the perirhinal cortex. In the posterior piriform cortex, modulators of muscarinic receptors (scopolamine) and $GABA_A$ receptors (muscimol) seem to be the effective ones (Myhrer et al., 2010a). According to the results cited, efficient anticonvulsant pre-treatment has to be based on pharmacological agents affecting a number of subreceptors.

Powerful anticonvulsant efficacy can be obtained by microinfusion of drugs into seizure-controlling brain sites in rats before exposure to nerve agent. However, focal administration of anticonvulsants after seizure onset has modest impact. Repeated infusions of atropine into the medial septum after a comparatively low dose of soman (0.9× LD_{50}) can finally stop seizure activity (Lallement et al., 1992). Similarly, repeated infusions of atropine into the area tempestas after onset of seizures induced by soman (1.3× LD_{50}) have evident anticonvulsant impact, but the seizure activity recurs 10–15 min after each injection (Myhrer and Enger, unpublished data). Thus, there are reasons to believe that large parts of the neuronal network must be affected systemically by anticonvulsants to stop ongoing seizures. Successful termination of seizures well after their onset probably requires a combination of drugs, each with optimal effects in some of the seizure-controlling brain areas.

Procyclidine emerged as the most potent anticonvulsant drug tested in the microinfusion studies (Myhrer, 2010). Enhancement of procyclidine's excellent anticonvulsant efficacy in the seizure-controlling sites of the forebrain would allow a novel and interesting approach. A drug with such enhancing properties has been developed in preclinical epilepsy research. Levetiracetam (Keppra®) is an antiepileptic drug that strongly enhances the anticonvulsant effects of compounds affecting either glutamatergic or GABAergic neurotransmission (Kaminski et al., 2009). It has also been reported that levetiracetam may both reduce release of ACh and reduce postsynaptic responsiveness in cholinergic synapses (Oliveira et al., 2005). Levetiracetam (50 mg/kg) combined with either procyclidine (20 mg/kg) or caramiphen (20 mg/kg) terminate seizure activity, but the survival rate is considerably higher for levetiracetam and procyclidine than for levetiracetam and caramiphen. Both therapies can also save the lives of rats that were about to die 5–10 min after seizure onset. Prophylactic use of the combination therapies 20 min before exposure to soman prevent onset of seizures more effectively when levetiracetam is combined with procyclidine than caramiphen (Myhrer et al., 2011).

We have performed a study in which effects of a physostigmine regimen (HI-6, scopolamine, physostigmine) were compared with those obtained by our procyclidine regimen (HI-6, levetiracetam, procyclidine) against various doses of soman at different times after intoxication. The prophylactic potencies of the regimens were also examined (Myhrer et al., 2013b). The results show that both regimens administered two times (1 and 5 min after exposure to 3×, 4×, or 5× LD_{50} of soman) effectively prevent or terminate epileptiform activity within 10 min. Both regimens also protect against death, and the rats recovered well. However, when the regimens were administered 10 and 14 min after a soman dose of 1.6× LD_{50}, only the procyclidine regimen terminates seizure activity and protects all rats against death. When rats pretreated with pyridostigmine received treatment 20 and 24 min after a soman dose of 1.3× LD_{50}, only the procyclidine regimen successfully stopped seizures and prevented death among all rats. When the regimens were given as prophylactic treatment 20 min before a soman dose of 1.3× LD_{50}, both the physostigmine and procyclidine regimens prevented convulsions, but only the procyclidine regimen yielded neuroprotection (Myhrer et al., 2013b).

Not all combinations derived from microinfusion studies will work equally well. For example, the potency of the metabotropic glutamate modulators DCG-IV and

TABLE 67.1 Anticonvulsant and Life Preserving Effects of Four Treatments Regimens on Soman-Induced (1.3× LD_{50}) Seizures 20 min After Onset in Rats Pretreated with Pyridostigmine (0.1 mg/kg)

Drugs	Dose mg/kg	*N*	Antiseizure Response		Lethality Response (24 h)	
			Ratio	Percent	Ratio	Percent
HI-6	125					
Scopolamine	1	6	0/6	0	6/6	100
Physostigmine	0.1					
HI-6	125					
Levetiracetam	50	6	6/6	100	0/6	0
Procyclidine	20					
HI-6	125					
Procyclidine	20	8	6/8	75	3/8	35
DCG-IV	4					
HI-6	125					
Procyclidine	20	7	5/7	71	2/7	29
MPEP	30					

Based on results from Myhrer et al. (2013b,c).

MPEP is very powerful in the perirhinal cortex (Myhrer et al., 2013a). However, when administered systemically together with procyclidine and HI-6, both combinations exert effective anticonvulsant impact, but they do not reach the same high level as the procyclidine regimen (Table 67.1). The multifunctional properties of procyclidine (antiglutamatergic and anticholinergic) seem to profit from the enhancing effects of levetiracetam, whereas the single function of DCG-IV and MPEP (antiglutamatergic) does not result in increased anticonvulsant capacity when combined with levetiracetam and HI-6 (Myhrer et al., 2013c).

Human Use

Oximes should be used as soon as possible after exposure to nerve agents, because of the short time window of the aging process. The human *in vitro* half-life for soman-inhibited AChE is 1.3 min, for sarin-inhibited AChE it is 3 h, and for tabun-inhibited AChE it is 13 h (Vale et al., 2007). When aging has occurred, new AChE has to be synthesized. It is the extremely short aging time of soman that led to the development of carbamate (pyridostigmine) prophylaxis for nerve agent poisoning. A number of nations use the oxime obidoxime (e.g., Finland, Germany, Norway, the Netherlands), whereas others use P2S (United Kingdom), 2-PAM (United States), or HI-6 (Canada, Sweden) (Aas, 2003). To date, there is no single oxime available that has high efficiency in reactivating AChE inhibited by any known nerve agent. However, HI-6 is considered by most countries to be a very promising oxime after exposure to most nerve agents (Aas, 2003).

Most nations have issued their military personnel with two to three autoinjectors containing atropine and an oxime. Additionally, some nations have provided their soldiers with one autoinjector containing diazepam or avizafone (Aas, 2003). Atropine has to be administered at relatively high doses (2 mg or more) because of its competitive nature (competes with ACh). The use of autoinjectors speeds the uptake of the drug because of a spraying effect as the needle plunges into the muscle, resulting in the distribution of drug within a larger muscle area, allowing for more rapid drug uptake than by using conventional needle and syringe (Sidell et al., 1974). However, atropine in too high doses can result in inhibition of sweating and the potential for inducing heat casualties. Even a dose of 2 mg combined with heavy work in a hot environment may induce a core temperature that is too high.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This chapter has focused on the development of novel countermeasures against nerve agents to improve existing prophylactic and post-exposure treatments. Effective prophylactic treatment can be achieved by using a fixed dose of physostigmine in combination with varying doses of procyclidine. Increased lethal doses of soman can be counteracted by corresponding increases in the dose of procyclidine. However, the higher the doses of procyclidine, the more pronounced the cognitive side effects. Moderate doses of procyclidine should be preferred, because insufficient prophylaxis can be compensated for by adjunct treatment in terms of diazepam and pentobarbital. Because a slight cognitive impairment might be inevitable with effective prophylactics, reduced reliance on pre-treatment appears preferable.

Immediate treatment with atropine and an oxime is very efficacious against nerve agent intoxication. However, subsequent treatment well after onset of seizures represents a huge problem in nerve agent research, because of the refractory nature of the epileptiform activity. A medical therapy has been developed through studies of microinfusions of anticonvulsants into seizure-controlling sites in the forebrain of rats. From these studies, procyclidine was revealed as the most potent anticonvulsant, and its potency was further enhanced when combined with the antiepileptic levetiracetam during systemic administration. The regimen is very effective against supralethal doses of soman (3 × , 4 × , 5× LD_{50}) when given 1 and 5 min after intoxication. When the treatment was administered 10 and 14 min or 20

and 24 min after soman exposure, the procyclidine regimen was able to terminate seizures and preserve lives. When used as prophylactic therapy, the regimen has powerful effect against seizures and provides effective neuroprotection. The procyclidine regimen has apparent capacities to serve as a universal therapy against soman intoxication in rats. In a microinfusion study, the importance of determining the unique pharmacological thresholds for producing anticonvulsant responses in the specific brain structures activated during nerve agent-induced seizures has further been emphasized (Skovira et al., 2012). The ultimate aim should be the development of well-tolerated anticonvulsants that can be administered by the soldiers themselves regardless of the time after nerve agent intoxication.

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CHAPTER

68

Medical Management of Chemical Toxicity in Pediatrics

Elora Hilmas and Corey J. Hilmas

Opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by Nemours/Alfred I. DuPont Hospital for Children or the US Food and Drug Administration.

INTRODUCTION

There are millions of chemical compounds known to humanity, but only a limited number are weaponized by conventional militaries. The Organization for the Prohibition of Chemical Weapons (OPCW), the 184-member watchdog agency enforcing the guidelines of the Chemical Weapons Convention (CWC), has identified 55 chemical agents and their precursors that can be used as weapons (OPCW, 2005). Although some of the chemicals are well known [e.g., sarin, soman, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), mustard], other less obvious choices for chemical terrorism include industrial chemicals such as chlorine and toxic precursors, which are considered "weapons of opportunity."

In the hands of terrorists, chemical warfare agents (CWAs) and toxic industrial chemicals (TICs) pose significant threats to civilian populations. A 2002 report to the US Congress by the Central Intelligence Agency (CIA) reported that terrorist groups "have expressed interest in many toxic industrial chemicals—most of which are relatively easy to acquire and handle—and traditional chemical agents, including chlorine and phosgene" (DCI, 2002). While traditional chemical warfare nerve agents are attractive to terrorist groups, they require a significant degree of financial resources and capital knowledge to manufacture. Furthermore, the United States and remaining signatory members of the CWC have pledged nonproliferation of CWAs (OPCW, 2005). Unfortunately, millions of tons of TICs continue to be manufactured annually in the United States alone. While they support the wide variety of products generated on a daily basis, including dyes, textiles, medicines, solvents, plastics, paints, and insecticides, they are lethal compounds in the hands of terrorists.

Chemical terrorism is the intentional use of toxic chemicals to inflict mass casualties on an unsuspecting military or civilian population, including children. Such an incident could quickly overwhelm local and regional public health resources and emergency medical services. In addition to the use of CWAs and TICs, an act of chemical terrorism may involve the targeting of industrial factories, tanker cars, or vehicles containing toxic substances with conventional explosives near residential communities or schools. Regardless of the methods used, the release of toxic chemicals by terrorists embodies a real and serious threat to US national security and public health. They can quickly incapacitate those who are exposed and can lead to mortality if not recognized and treated promptly. Moreover, the toxicity of these agents can be enhanced in children due to pediatric vulnerabilities. It is imperative to recognize the different ways that children may react to toxicity compared to adults.

BACKGROUND

Even though many efforts have been made to protect the United States from terror threats, it remains paramount for the scientific community to continue building the knowledge base regarding CWAs and better understand the toxicities that can occur when children are exposed. Unfortunately, pediatric treatment recommendations are often extrapolated from adult data,

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even though it is well recognized that pediatric patients should not be regarded as miniature adults. Children present unique vulnerabilities to these chemicals, and special considerations should be taken.

Due to the possibility of pediatric casualties from chemical agent attacks, several pediatric advocacy groups, such as the American Academy of Pediatrics (AAP), have commented on the urgent need for pediatric chemical casualty research (Blaschke and Lynch, 2003). The Committees on Environmental Health and Infectious Diseases have provided the following consensus statement regarding children and chemical–biological threats (CEH/CID, 2000).

> Because children would be disproportionately affected by a chemical or biological weapons release, pediatricians must assist in planning for a domestic chemical–biological incident. Government agencies should seek input from pediatricians and pediatric subspecialists to ensure that the situations created by multiple pediatric casualties after a chemical–biological incident are considered.

After the terrorist attacks of September 11, 2001, the AAP initiated a number of initiatives to address the need to prepare for terrorism against children. For example, the AAP created a Task Force on Terrorism, a comprehensive web resource to disseminate information on terrorism and its impact on children. In addition, the AAP has published several reports and policy statements to provide guidance for healthcare practitioners preparing for a mass chemical casualty event. These efforts were augmented by the passage of several key federal legislative acts aimed at improving public health emergencies and their response to chemical terrorism. Finally, the Centers for Disease Control and Prevention (CDC) created the Strategic National Stockpile (SNS) site as a national repository of antibiotics, chemical antidotes, and antitoxins. The SNS contains a pediatric formulary, along with compounding materials that would assist clinicians in creating dosage forms appropriate for pediatric administration of chemical antidotes (CEH/CID, 2006) in the event of chemical terrorism.

Indeed, chemical terrorism on US soil is a very sobering possibility in the future. A significant subset of casualties from a mass chemical exposure will be comprised of vulnerable populations such as children and the elderly. In the hopes of better understanding the impact of such an event, it is necessary to learn from historical incidents and case studies where children were exposed to toxic chemicals and treated.

In this chapter, the CWAs and "weapons of opportunity" most likely to be used by terrorists to inflict casualties will be examined, along with a brief historical account and discussion of the unique challenges of managing pediatric chemical casualties. The sections focusing on each chemical agent will highlight the pediatric-relevant vulnerabilities and guidelines for medical management. The final two sections will discuss decontamination of children and recommendations to help prepare healthcare managers and providers in the event of a chemical event. It is hoped that this compilation will provide the necessary guidance and treatment recommendations on how best to treat children involved in a chemical attack.

HISTORY OF PEDIATRIC CHEMICAL CASUALTIES

Historically, chemical attacks were limited to the battlefield, and casualties were predominantly military personnel. In turn, the majority of our knowledge concerning management of chemical casualties has come from the experiences of treating members of the military. Today, the threat of chemical use is extended to civilian populations as state and nonstate sponsored terrorists target innocent civilians. The risk of chemical and biological terrorism is more tangible since the events of September 11, 2001 and the intentional spread of anthrax through the US Postal Service. Terrorists expanded their scope and threat to inflict mass civilian casualties on a scale never before seen. The threat from attack has moved from the traditional battlefield to the home front. Innocent civilians, including children, are now prime targets for groups to foment terror and destabilize governments. Even before the attack on the World Trade Center towers, the twentieth century witnessed numerous instances where civilian populations were exposed to toxic chemicals or targeted on a grand scale.

During the flurry of chemical barrages across trench lines in World War I, children from bombed towns in France and Belgium were treated at British, French, and American "gas" hospitals as a result of CWA exposure. Numerous reports of civilian casualties from mustard, chlorine, and phosgene are well documented in British archives (Thomas, 1985). After the final tally of civilian casualties from gas warfare was complete, participants saw how ill prepared their civilians were against such weapons. School-age children learned the importance of protective measures against chemicals through donning gas masks and evacuating contaminated areas (Figure 68.1).

Civilians have been unintended and, in some cases, intended targets of CWAs since World War I. While cyanide was used on Jewish prisoners in World War II, chemical weapons would not be used again during combat on civilian populations until the Iran–Iraq War. In the spring of 1987, Saddam Hussein bombed the Iranian city of Sardasht with mustard munitions, resulting in thousands of civilian casualties (Foroutan, 1996a,b, 1998a–c).

Following the attack on Sardasht, Iraq attacked Kurd settlements in early 1988, leading to the infamous attack

on Kurdish residents of Halabja in March. Thousands of innocent civilian ethnic Kurds perished during the chemical attack, and 75% of them were women and children. Mustard and nerve agents were dropped on civilians from helicopters and planes, and eyewitnesses reported large smoke clouds causing great morbidity and mortality among children (Hay and Roberts, 1990).

FIGURE 68.1 School-age girl with US civilian noncombatant gas mask MI-I-I, child size. *Source: Photograph courtesy of the US Army Research Development and Engineering Command, Historical Research and Response Team, Aberdeen Proving Ground, MD.*

While the list of nations who have not signed the international treaty to ban chemical weapons is short, it is not surprising that one of those countries has recently been in the international spotlight for stockpiling and deploying chemical weapons in its ongoing civil war. In July 2012, Syria admitted to possessing stocks of chemical weapons. Rebels in northern Syria employed chemical weapons in the town of Khan al-Assal, and opposition forces reported use of chemical weapons (phosphorus bombs) in the Damascus suburb of al-Otaybeh in March 2013. Similar attacks were reported on March 24 in the town of Adra, and on April 29 in the city of Saraqeb. Escalating tensions led to the worst use of chemical weapons since Saddam Hussein's ethnic cleansing of Kurds from northern Iraq in 1988. Another attack happened in the early morning hours on August 21, 2013, in multiple civilian locations on the outskirts of the Syrian capital of Damascus. Concluding a monthlong investigation by the United Nations, Secretary-General Ban Ki-Moon confirmed that sarin nerve gas was indeed the culprit in this rocket assault (Figure 68.2). Sarin gas was deployed in either opposition-held or contested regions containing significant numbers of civilians. Many of the reported casualties were women and children, arriving with constricted pupils, profuse salivation, and hypotonia. News agencies posted numerous photographs

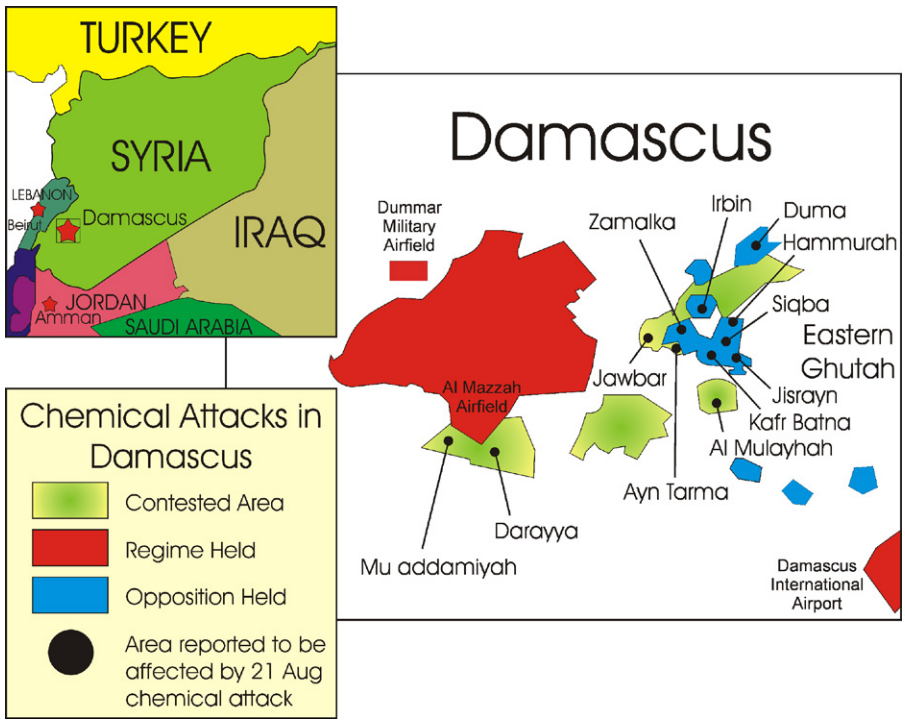

FIGURE 68.2 Areas in and surrounding Damascus reportedly affected by sarin rocket attacks on August 21, 2013.

involving pediatric casualties in the aftermath of the sarin rocket attack. While final numbers of the actual civilian death toll from sarin are unknown, Damascus field hospitals and clinics reported up to 1,300 dead, with pediatric numbers climbing into the hundreds. The full extent of morbidity among sarin survivors of the attack is also unknown, but it is clear that hospitals were unprepared to handle the mass influx of casualties with sufficient stockpiles of nerve agent antidotes. Damascus is a reminder of the horror and threat posed by nerve agents.

These events confirm the devastating reality that chemical threats pose to our unprotected population today. A military and civilian response to the use of chemical weapons on American soil may not be a matter of if, but rather when. These events underscore the need for all pediatric-related healthcare workers to prepare for a mass casualty incident involving CWAs or TICs.

CHALLENGES TO MANAGING PEDIATRIC CHEMICAL CASUALTIES

Overview

Managing the pediatric victims of chemical terrorism is an especially difficult challenge. In addition to the obvious physiologic and anatomic differences in children compared to adults (Table 68.1), there are important psychological and behavioral differences that put children at risk (Rotenberg and Newmark, 2003). Anecdotal reports have claimed that children are likely to be the first to manifest symptoms, to develop more severe manifestations, and to be hospitalized for other related illnesses. In fact, it is anticipated that children will be overrepresented among the initial index cases in a mass civilian exposure to toxic chemicals. Children have many characteristics that make them vulnerable to toxic exposures. The smaller mass of a child automatically reduces the dose of toxic agents needed to cause observable or lethal effects. Studies involving organophosphates (OPs), compounds related to nerve agents, have shown that immature animals have greater vulnerability. Some OPs produce the same degree of lethality in juveniles at a fraction of the dose producing lethality in adults (Rotenberg and Newmark, 2003). Children exhibit an exceptional vulnerability to both the acute and chronic effects of chemicals and are disproportionately more susceptible than adults. The increased toxicity seen in children compared to adults from various routes of exposure can be attributed to a wide variety of factors (shown in Exhibit A). These unique anatomical and physiological considerations described next cause the rates of absorption, distribution, metabolism, and excretion of toxic chemicals and drugs to differ in children with respect to adults.

TABLE 68.1 Summary Chart of Pediatric Vulnerabilities and Implications for Clinical Management

Unique Vulnerability in Children	Implications and Impact from Chemical Toxicity
BODY COMPOSITION	
• Larger BSA/body mass • Lower total lipid/fat content	• Greater dermal absorption • Less partitioning of lipid soluble components
VOLUME STATUS	
• More prone to dehydration • Chemical agents lead to diarrhea and vomiting	• Children can be more symptomatic and show signs of severe dehydration
RESPIRATORY	
• Increased basal metabolic rate/greater minute volume	• Enhanced toxicity via inhalational route
BLOOD	
• Limited serum protein-binding capacity • Greater cutaneous blood flow	• Potential for greater amount of free toxicant and greater distribution • Greater percutaneous absorption
SKIN	
• Thinner epidermis in preterm infants • Greater cutaneous blood flow	• Increased toxicity from percutaneous absorption of chemical agents
ORGAN SIZE/ENZYMATIC FUNCTION	
• Larger brain/body mass • Immature renal function/ lower renal function • Immature hepatic enzymes	• Greater CNS exposure • Slower elimination of renally cleared toxins, chemicals and metabolites • Decreased metabolic clearance by hepatic phase I and II reactions
ANATOMICAL CONSIDERATIONS	
• Short stature: breathe closer to ground where aerosolized chemical agents settle • Smaller airway • Greater deposition of fine particles in the upper airway • Higher proportion of rapidly growing tissues	• Mustard significantly affects rapidly growing tissues • Exposure to chemicals can have significant impact on bone marrow, developing CNS • Increased airway narrowing from chemical agent-induced secretions
CNS	
• Higher BBB permeability • Rapidly growing CNS	• Increased risk of CNS damage
MISCELLANEOUS	
• Immature cognitive function • Unable to flee emergency • Immature coping mechanisms	• Inability to discern threat, follow directions, and protect themselves • High risk for developing PTSD

EXHIBIT A

ANATOMICAL AND PHYSIOLOGICAL CONSIDERATIONS UNIQUE TO CHILDREN

- Differences in anatomy
- Allometric scaling factors (e.g., increased surface area-to-volume ratio)
- Cardiovascular status
- Permeability of the pediatric blood–brain barrier (BBB)
- Dermatologic factors (e.g., increased cutaneous blood flow; Simonen et al., 1997; Fluhr et al., 2000)
- Increased skin pH (Behrendt and Green, 1958; Fluhr et al., 2004)
- Plasma protein binding
- Volume of distribution (V_d)
- Organ size and maturity
- Pharmacokinetic maturity (e.g., metabolic differences; Fairley and Rasmussen, 1983)

Respiratory Vulnerability

Inhaled doses in young children may be greater than in adults. Some studies have demonstrated a twofold increase in respiratory tract exposure per unit surface area compared to adults (Bennett and Zeman, 1998). Deposition of fine particles is higher in young children (ages 7–14) than adults when the data are normalized by lung surface area (Bennett and Zeman, 1998) and an even greater deposition has been modeled for younger children (Martonen et al., 2000). The higher respiratory rate and minute volumes per respiratory surface area of a child means that they will inhale a greater dose of a toxic chemical vapor (Rotenberg and Newmark, 2003). Also, children can become intoxicated simply through breathing air that is closer to the ground. Many toxic chemicals display a high vapor density, causing them to distribute closer to the ground (CSMC, 2003). This may lead to greater toxicity for a child than an adult. In addition, children have less endurance than adults in terms of using their respiratory accessory muscles, putting them at risk for respiratory failure.

Children are especially susceptible from toxic chemicals due to their unique airway anatomy (Figure 68.3). These differences include a greater degree of subglottic narrowing, diminished airway diameter, tendency for nose-breathing, and large tongue size relative to the mouth (Rotenberg and Newmark, 2003). OP nerve agents induce bronchospasm and bronchoconstriction during a cholinergic crisis. In comparing the effect of a nerve agent on adult and pediatric airways, Figure 68.3 illustrates that a similar change in airway diameter results in a greater percentage increase of airway resistance in children. In addition, copious glandular secretions during a cholinergic crisis may further restrict airflow through an already narrow airway. Therefore, children are at higher risk for toxicity from inhalational chemical exposure.

Volume Status Vulnerability

The circulatory system of children can be severely affected by chemical attacks (Rotenberg and Newmark, 2003).

Children have lower fluid reserves than adults, so small fluid volume losses can cause significant effects. For example, a 5-kg child experiencing severe dehydration (15% body weight loss) loses 750 mL of fluid. A significant loss of fluid from the gastrointestinal tract as a result of chemical-induced glandular secretions can affect intravascular volume. Also, children are more prone to vomiting and diarrhea than adults. Therefore, children may dehydrate faster during a chemical event (CSMC, 2003).

Neurological Vulnerability

The immature central nervous system (CNS) of children can lead to greater toxicity (Rotenberg and Newmark, 2003). Toxic agents can often transverse the immature BBB in children. Infants and children are at greater risk of seizures than adults. This is concerning because seizures are common in cases of moderate to severe nerve agent intoxication. Infants are at the highest risk of toxicity due to their susceptibility to imbalances of neurotransmitter systems. Prolonged seizures, or status epilepticus, can cause neuronal injury and deficits of normal brain development in children.

Dermatological Vulnerability

Barrier thickness, cutaneous blood flow, surface-to-volume ratio, temperature, hydration, and skin pH are important factors to consider in the assessment of pediatric dermatological vulnerabilities. The skin of newborns, while appearing vulnerable, has the same histologic features of adult skin with some differences, including immaturity of collagen, hair follicles, and sebaceous glands. While newborns and young children are often described as having thinner skin than adults, the stratum corneum, the most superficial layer of the skin, is thinner in premature infants than in full-term infants, children, and adults (Rutter and Hull, 1979; Harpin and Rutter, 1983; Nopper et al., 1996). The skin of a child, however, does not differ significantly compared to adults, as evidenced by similar measurements of skin physiological parameters

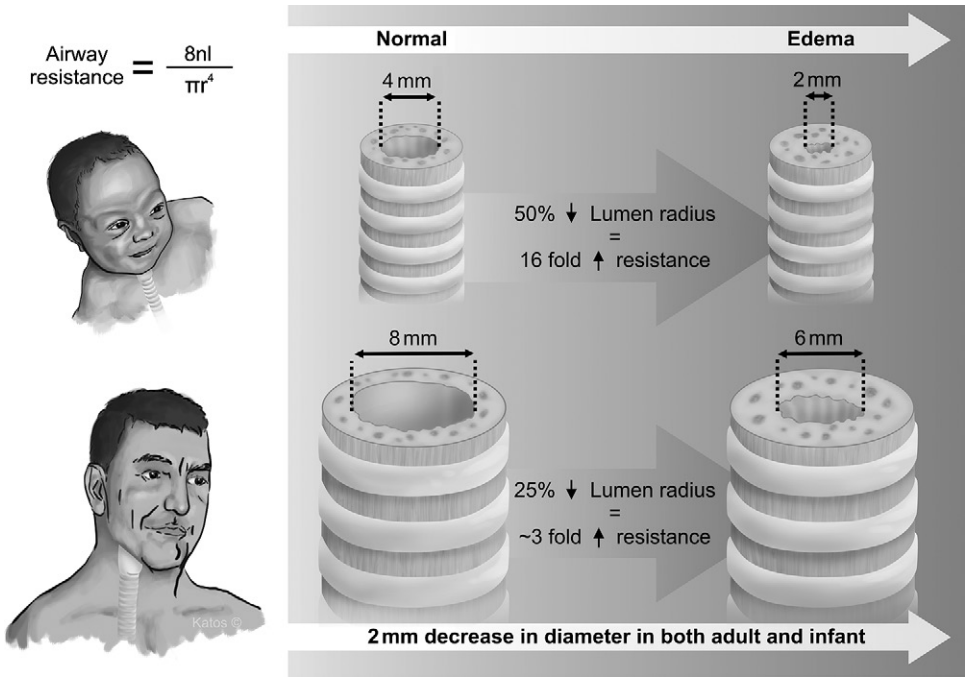

FIGURE 68.3 Comparison of pediatric and adult airways. The most important factor to consider in pulmonary toxicity from cholinesterase inhibition is the airway resistance through the conducting portion of the respiratory system. Airflow from the trachea and mainstem bronchi to the small bronchioles can be characterized as airflow through a series of straight tubes or laminar flow (West, 1995; Guyton and Hall, 2005). Poiseuille's law provides a relationship between flow rate and radius of the tube ($F = P\pi r^4/8nl$), where n is the coefficient of viscosity, P is the pressure difference across the length l of the airway section, r is the radius of the airway, and F is the airway flow rate. Since the resistance to flow R is driving pressure P divided by flow F, using the analogy of Ohm's law, the following relationship for airway resistance R can be derived ($R = 8nl/\pi r^4$). The effect of a 2-mm change in airway diameter on airway resistance is illustrated for both an infant and an adult. *Bottom panel* (adult scenario): a 2-mm reduction in the airway radius as a result of nerve agent-induced bronchoconstriction results in a 25% reduction and a corresponding increase in airway resistance by approximately threefold. *Top panel* (infant scenario): a similar reduction in a child decreases the airway lumen by 50% and increases airway resistance by 16-fold. *Source: Illustrations are copyright-protected and printed with permission by Alexandre M. Katos.*

(e.g., transepidermal water loss, skin pH, and stratum corneum capacitance and conductance; Fluhr et al., 2000). Children 3 months old have the same abdominal skin stratum corneum thickness as 11-year-olds and adults (Fairley and Rasmussen, 1983).

Moreover, children have larger surface area-to-volume (mass) ratios, resulting in greater absorption of chemicals. The skin surface area of infants and toddlers is very large compared to their body weight. An increase in the surface area-to-body weight ratio increases their potential absorption to dermal exposures of chemicals. For instance, a typical infant weighs a fraction (1/20) of a 70-kg male adult, but an infant's surface area is only 1/8 as great. The total skin surface area that is exposed per kilogram of body weight, therefore, is 2.5 times higher for infants than adults (Lynch and Thomas, 2004). Burns with extensive skin loss, as seen with certain chemical exposures, can cause significant water loss and toxicity in children (Lynch and Thomas, 2004). Despite the few prospective scientific studies on skin vulnerability in the pediatric population, it can be summarized that the thin, immature skin of preterm infants and the unique dermatological properties of children put them at higher risk for toxicity from percutaneous chemical exposure (CSMC, 2003).

Plasma Protein Binding, Volume of Distribution, and Organ Maturity

Children may be at an increased risk of chemical toxicity due to having lower levels of plasma proteins. One factor affecting the amount of free chemical/drug in the circulation is the fraction bound to plasma protein. Neonates have a low protein-binding capacity for albumin and alpha-1-glycoprotein (Besunder et al., 1988; Kearns and Reed, 1989; Clewell et al., 2002) and a decreased ability to conjugate and excrete bilirubin, which binds to plasma proteins. This can lead to a smaller pool of available protein-binding sites in plasma (Ginsberg et al., 2004). A lower serum protein-binding capacity equates to a greater fraction of free chemical available in the circulation, and therefore increased toxicity.

The volume of distribution or V_d (liters per kilogram of body weight) of chemicals/drugs is an important factor to consider in pediatrics. Because of the expanded water content in early life (newborns and infants), water-soluble chemicals may tend to have a larger volume of distribution. Toxic lipophilic agents, on the other hand, will be decreased in their partitioning to fat because of the lower

body lipid content in young children compared to older children and adults (Kearns and Reed, 1989; Morselli, 1989; Clewell et al., 2002). It can be argued that due to these lower fat stores, lipophilic agents such as nerve agents will reach higher concentrations in the plasma, leading to an increased chance of chemical toxicity.

Another factor affecting tissue distribution of chemicals in children is organ size per body weight. The brain is disproportionately large in young children. This fact, combined with an immaturity and permeability of the BBB in young children, leads to higher brain concentrations of some chemicals and the potential for enhanced neurotoxicity (Saunders et al., 2000). Liver mass per body weight is greatest in the early postnatal period and other tissues (liver, kidney, lung, and brain) undergo rapid growth during the first 2 years of life (NRC, 1993). These organs are at increased risk for toxicity in children due to their disproportionately larger size per body weight.

Organ maturity in the pediatric population is another factor affecting clearance of toxic agents and therapeutics. In particular, renal clearance is diminished in children compared to adults. Glomerular filtration rate and transporter (secretory) systems in the proximal convoluted tubule are decreased at birth (Kearns and Reed, 1989; Morselli, 1989). In addition, cardiac output, while higher in children, has a lower percentage reaching the kidneys (Ginsberg et al., 2004). This will tend to decrease renal clearance even more, leading to even greater plasma levels of toxic agent. A consequence of immature kidney function and reduced clearance in children can be seen with nerve agents since the parental forms of nerve agents and their metabolites undergo hydrolysis with predominantly renal elimination.

One might consider that renal clearance is faster due to allometric scaling differences in children compared to adults. According to the rules of allometric scaling, smaller organisms have greater respiratory rates, cardiac output, nutrient and oxygen demands, and basal metabolic rates than larger organisms. This appears true for children because respiratory rate, cardiac output, and liver mass are greater per body weight than adults. However, faster metabolic rates are not seen in neonates because of immaturity of hepatic enzymes and reduced hepatic clearance, leading to a prolonged toxic agent/drug half-life and longer duration of action.

Metabolic Vulnerability

Children are unable to detoxify toxic agents as efficiently as adults because they have less mature metabolic systems (Rotenberg and Newmark, 2003). In particular, phase I oxidative systems, phase II conjugating systems, and miscellaneous other systems (e.g., serum esterases, hydrolases, and dehydrogenases) are all immature in children compared to adults. Neonates and children up to 1 year are most affected in their maturing enzymatic function with the greatest effect seen in the first 2 months of life. This leads to slower metabolic clearance of many drugs, toxic chemicals, and activated metabolites, leading to significant toxicity in this age group (Ginsberg et al., 2004). In addition, several authors have reported a reduced activity of AChEs, pseudocholinesterases, and arylesterases (paraoxonase) in premature and full-term newborns (Stead, 1955; Lehmann et al., 1957; Augustinsson and Brody, 1962; Ecobichon and Stephens, 1973). These levels do not reach adult levels until 1 year of age (Morselli, 1976). In addition, newborns possess levels of paraoxonase, the enzyme that detoxifies OP pesticides, that are half of those found in the general adult population (Rotenberg and Newmark, 2003). Other studies suggest that newborns have paraoxonase levels fourfold lower and activities threefold lower than their mothers (Holland et al., 2006).

Traumatic Injury Vulnerability

Another special challenge to managing pediatric patients is the fact that trauma and injury often accompany chemical attacks (Abraham et al., 2002). Chemical exposures are often dispersed through explosive devices. Traumatic injury patterns differ in children compared to adults. Due to the smaller size of children, multiple trauma occurs more frequently. Compared to adults, children often sustain more head trauma due to their relatively large head size and weaker supportive musculature. Also, their more compliant skeletal system provides less protection to internal organs, leading to greater internal injuries without overlying fractures.

Neurobehavioral Vulnerability

Immature cognitive function can put children at risk during a chemical attack (Rotenberg and Newmark, 2003). Children lack the ability to discern threat, to protect themselves, or to follow directions. Infants, toddlers, and young children do not have the motor skills to flee from the site of an incident (CEH/CID, 2000). This can adversely affect their avoidance of a contaminated area and decontamination in the event of exposure. During decontamination, procedures for children who have been separated from their caregivers must be taken into consideration. Without guidance, children may not be able to follow directions for the decontamination process (Wheeler and Poss, 2003).

Psychological Vulnerability

Children have fewer coping skills when sustaining or witnessing injuries such as parental or sibling death (Henretig et al., 2002b). These events can produce either short- or long-term psychological trauma. It is not

unusual for children involved in attacks to suffer from posttraumatic stress disorder (PTSD) related to what they have experienced (CEH/CID, 2000; ARC, 2002). During the management of a chemical event, there are certain behaviors that make the management of children difficult. Children are often influenced by the emotional state of caregivers, requiring providers to remain calm. Also, fear or discomfort may cause children to disobey or act out against providers of care (see Table 68.1) (Blaschke et al., 2003).

Even beyond the behavior of children, there are other barriers to emergency management. The high-pressure hoses and cold water that are used to decontaminate victims can expose children to significant additional risk (CSMC, 2003). Use of these items can result in hypothermia and skin damage. Also, emergency care providers often need to wear bulky full protective suits when treating victims. These suits make it difficult to treat very small children who might need intricate procedures such as blood draws. One constant challenge that is consistent with the management of children is the lack of pediatric formulations of specific therapeutics (e.g., autoinjectors containing oxime). Antidotes for chemical agents are often not available in ready-to-administer pediatric dosages, although some progress has been made in this area. In the event of a true chemical event, there is a risk that pediatric centers would be overwhelmed and the ability to expand the number of pediatric hospital beds may be limited (CEH/CID, 2000). Finally, most healthcare workers are not fully aware of the management or presentation of toxic signs and symptoms of chemical agent exposure. This problem is exacerbated because children typically present differently than adults.

Other Vulnerabilities

In addition to the vulnerabilities listed previously, other factors can put children at greater risk for toxicity from chemical agent attacks. For instance, the fluid and food intakes of children differ significantly from adults, with greater water and milk consumption per weight. Children ingest about 100 mL/kg per day of water compared to the 40–60 mL/kg per day ingested by adults. Therefore, if water or milk supplies become contaminated, children would feel a greater impact than adults. Also, the diets of children include greater consumption of foods that can be contaminated, such as fruits and vegetables (CEH/CID, 2006).

Medical Response Vulnerability

Due to the myriad of factors outlined here that make the management of pediatric chemical exposures challenging, it is not surprising that healthcare practitioners often lack the knowledge or are not sufficiently trained to handle a mass influx of pediatric casualties. This deficiency was clearly documented in a study done by Schobitz et al. (2008), when pediatric and emergency medicine residents were tested on the medical management of pediatric victims of biological and chemical terrorism (Schobitz et al., 2008). A test containing essential content was developed and validated by experts. This test was given to volunteer residents and was re-administered 5 months after a lecture on the content. The 34 pediatric residents and 15 emergency medicine residents who took the exam scored a median of 65% and 73%, respectively. The authors investigated the benefit of the lecture and found that the 16 residents who attended the lecture and completed the posttest achieved a median score of 70%. For the 20 residents who did not attend the lecture but completed the posttest, a median score of 66.6% was recorded. The authors concluded that there are significant knowledge deficits among pediatric and emergency medicine residents in their abilities to handle pediatric victims of biological and chemical terrorism. A suggestion was made to incorporate educational curriculum on preparedness into residency curriculums (Schobitz et al., 2008).

EXHIBIT B

PEDIATRIC CASE HISTORY—NERVE AGENTS

Nerve Agent Exposure in Nazhmar, Iran

One victim of an attack on the village of Nazhmar on March 22, 1988, was a young child of unreported age and weight. He presented immediately with marked miosis and was comatose. Breathing was irregular, and foamy secretions were protruding from his mouth and nose. The patient was working very hard to breathe and noted to be using his accessory muscles of respiration. Wheezing was obvious on auscultation, and he showed obvious difficulty on exhalation. Upon suction removal of oral and nasal secretions, the patient was noted to have progressively rigid extremities, to the extent that venous access became difficult. The secretions were noted to become bloody. Over a 15-min period, a total of 7.5 mg of atropine was administered in three treatments. The patient was noted to improve, with eye opening, moaning, and speaking two-word phrases. As his muscle tone decreased, his breathing improved, but wheezing was still evident. The child was decontaminated after treatment and subsequently discharged after an hour. At the time of discharge, secretions were not completely dried up, but his pupils were fully dilated and reactive to light (Foroutan, 1998c).

EFFECTS OF SPECIFIC AGENTS

Nerve Agents

Introduction

Nerve agents pose a real threat to our unprotected civilian population. They can quickly incapacitate those who are exposed and can lead to mortality if not recognized and treated promptly. The toxicity of these agents can be enhanced in children due to pediatric vulnerabilities. Also, it is important to recognize the different ways that children may react to toxicity compared to adults.

The major nerve agents are the G-series (tabun, sarin, cyclosarin, and soman) and V-series (VX) compounds, which are clear, colorless, tasteless, and in most cases, odorless. They have been demonstrated to penetrate normal clothing and skin. Also, these agents are highly toxic, as evidenced by the fact that as little as 10 mg of VX on the skin is considered to be an LD_{50} in adults (Rotenberg and Newmark, 2003). In addition, these agents produce toxicity rapidly compared to biological agents. Most G-series nerve agents are highly volatile, and can be dispersed into aerosols that are inhaled by victims. One of the G-series agents, sarin, is volatile and may sink close to the ground (in undisturbed air) where children breathe. Nerve agents may also be disseminated in liquid form. Treatment for dermal exposure begins with rapid topical decontamination.

Although our military experience managing toxicity from nerve agent exposure is limited, exposures to related chemicals such as the OP class occur commonly each year in the United States. In 2006, there were a total of approximately 5,400 OP exposures across the United States (Bronstein et al., 2007). OPs, such as malathion, are commonly used as pesticides. OP toxicity manifests in a similar fashion as toxicity from nerve agents; however, this chemical class is considerably less toxic. One case series of 16 children who experienced poisoning with OPs confirmed that pediatric patients present with toxicity differently than adults (Lifshitz et al., 1999). These children often did not manifest the classic muscarinic effects (such as salivary secretions and diarrhea) seen in adults.

Mechanism of Toxicity

Nerve agents cause toxicity by inhibiting esterase enzymes, especially acetylcholinesterase (AChE; Rotenberg and Newmark, 2003). When nerve agents bind to AChE, they prevent hydrolysis of acetylcholine (ACh). When ACh accumulates in the synaptic space of neurons, this leads to overstimulation of muscarinic and nicotinic receptors that is often termed *cholinergic crisis*. Also, it is important to note that the nerve agent–AChE bond undergoes a reaction called *aging* (Dunn and Sidell, 1989). Once this process is complete, the enzyme becomes irreversibly inactivated. This aging process dictates the need for prompt therapy to prevent irreversible toxicity.

Clinical Presentation

The signs and symptoms of a cholinergic crisis can be remembered using the mnemonic BAG the PUDDLES (Figure 68.4); these range in severity from lacrimation and urination to seizure activity (Rotenberg and Newmark, 2003). The manifestations of cholinergic crisis seen in a particular individual depend on the dose and route of exposure, as well as the duration of exposure. If death occurs from nerve agents, it is primarily attributed

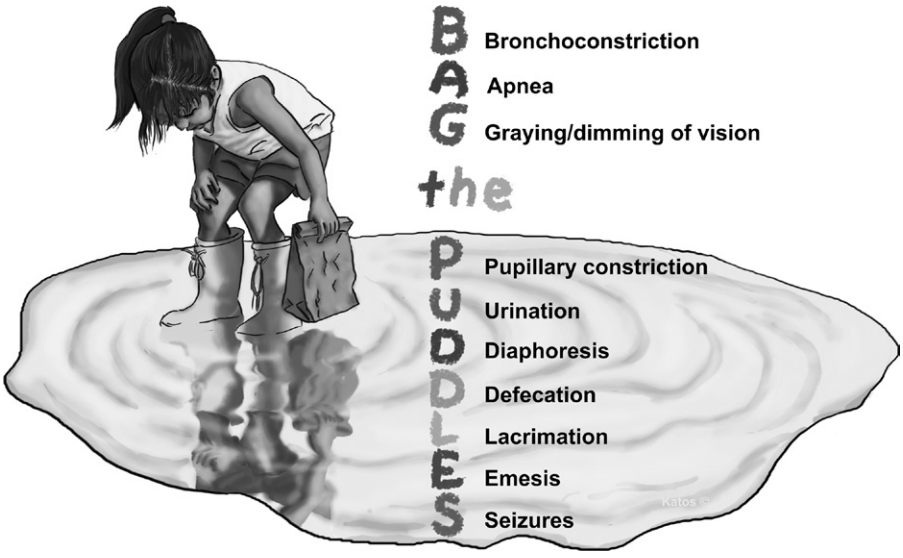

FIGURE 68.4 Helpful mnemonic for cholinergic crisis (BAG the PUDDLES). *Source: Illustrations are copyright-protected and printed with permission by Alexandre M. Katos (Rotenberg and Newmark, 2003).*

to respiratory failure. Nerve agents affect the respiratory system by causing central apnea, flaccid neuromuscular paralysis, bronchoconstriction, and profound glandular secretions (Hilmas et al., 2006).

Children present a clinical picture that can be very different to that observed in adults. Children in cholinergic crisis may not necessarily manifest with miosis (constriction of pupils) (Rotenberg and Newmark, 2003). In fact, one case series demonstrated absence of miosis in 43% of pediatric victims. Many of the pediatric case reports involving nerve agent exposure in children in Halabja and Damascus involved mydriasis (pupil dilation), so children may not always follow a classic picture of cholinesterase inhibition with regard to pupillary findings. Studies involving pediatric exposure to OPs have suggested the appearance of isolated CNS effects (such as stupor and coma) in the absence of peripheral muscarinic effects. Pediatric victims of OP intoxication display significant muscular weakness and hypotonia in the absence of glandular secretions in 70–100% of cases involving moderate to severe levels of exposure (Rotenberg and Newmark, 2003). For adults, a presentation of central intoxication (weakness and hypotonia) from OPs without peripheral muscarinic signs and symptoms would be extremely atypical.

Unfortunately, there are no data on the long-term effects of nerve agent poisoning in children, and the effects must be extrapolated from what has been discovered in the adult population (Rotenberg and Newmark, 2003). Surveillance studies performed on victims of the sarin attacks in Japan revealed a wide range of sequelae, such as continued respiratory problems, vision disturbances, headache, and fatigue. Neuropsychiatric problems were also reported as a delayed effect.

Laboratory Findings

Use of cholinesterase levels is limited, especially for confirmation of exposure (Rotenberg and Newmark, 2003). Treatment should not be delayed for these levels to return. Levels should be used after exposure only to confirm diagnosis (after treatment has begun), to monitor recovery, or for forensic investigation.

Pediatric Vulnerability

Children have several vulnerabilities, putting them at risk of increased toxicity from this class of chemical agents. A child's smaller mass alone reduces the dose needed to cause symptoms or lethality. For volatile nerve agents, children are especially at risk for respiratory toxicities due to their anatomic differences compared to adults. Their smaller airways can become compromised by the large amount of secretions and the bronchospasm caused by the agents. Also, a greater dose of nerve agent will be inhaled in children due to their higher respiratory rate and minute volumes.

Treatment

The overall treatment approach to nerve agent exposure focuses on airway and ventilatory support, aggressive use of antidotes (atropine and pralidoxime), prompt control of seizures, and decontamination as necessary (Henretig et al., 2002a). Atropine is used for its antimuscarinic effects, and oxime is used to reactivate AChE. The combination of atropine and pralidoxime chloride (2-PAM Cl) is recommended for the prompt treatment of all serious cases. The timing of atropine and 2-PAM Cl administration is critical. In short, the sooner these antidotes are given, the better the outcome. Oxime therapy is rendered ineffective if given after the enzyme aging process has been completed (Dunn and Sidell, 1989). This fact has led to the use of autoinjectors because of their ability to rapidly administer intramuscular doses of these medications. However, there are no current Food and Drug Administration (FDA)–approved pediatric autoinjectors for 2-PAM Cl. Other administration routes and methods include intravenous (IV) or intraosseous (IO) for atropine and slow IV or continuous infusion for 2-PAM Cl. Data show that peak plasma concentrations of medications administered from autoinjectors are achieved in less than 5 min versus 25 min for intramuscular (IM) administration using a needle and syringe (Rotenberg and Newmark, 2003). The mainstay of adult therapy includes the use of autoinjector technology containing atropine and 2-PAM. Recently, a dual-chambered autoinjector called the Antidote Treatment Nerve Agent Autoinjector (ATNAA) has received FDA approval for the military and Duodote for civilian emergency medical technicians and first-responders (see Figure 68.5). Meridian also produces the older Mark I kit (Figure 68.6), which is composed of separate autoinjectors for atropine and 2-PAM.

These products are provided by Meridian Medical Technologies, which has partnered with the US Department of Defense to be the only FDA-approved supplier of nerve agent antidotes. The Mark I kit and the single autoinjector devices deliver 600 mg of 2-PAM Cl and 2 mg of atropine (AtroPen) in seconds. This kit was developed originally for administration to soldiers, not for children, and with the approval of the Duodote system, the Mark I kit will most likely become antiquated. The autoinjector technology incorporates a spring-loaded needle to disperse medication in an all-or-nothing fashion. It is impossible to give partial doses of an autoinjector to children. Drug dosing of atropine and 2-PAM Cl in pediatrics is primarily weight based, so a standard dose cannot be used. Pediatric versions of the Mark 1 kit are available overseas but are not currently available in the United States (PEAP, 2004). In June 2003, the FDA approved pediatric doses of the AtroPen (atropine autoinjector) to respond to the lack of pediatric-specific therapy (Meadows, 2004). Meridian's AtroPen

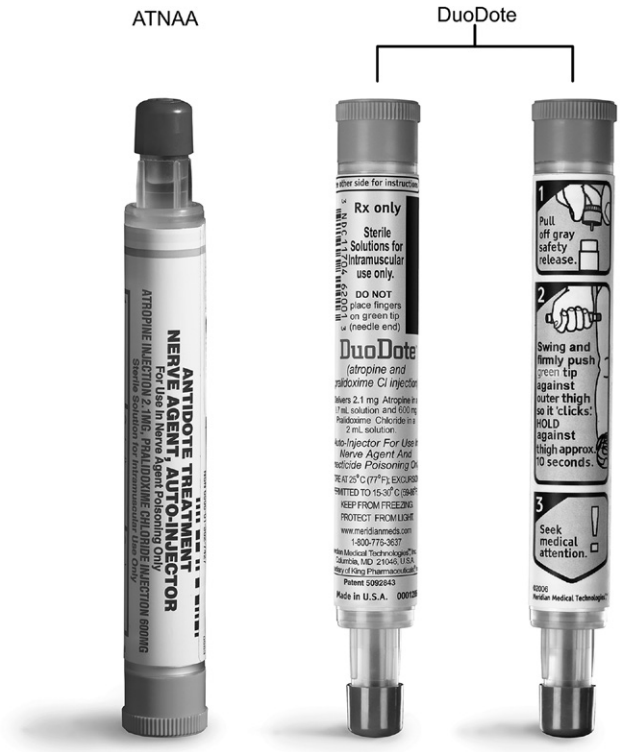

FIGURE 68.5 ATNAA and Duodote. *Source: Photo reproduced with permission from Meridian Medical Technologies.*

FIGURE 68.6 MARK 1 kit. *Source: Photo reproduced with permission from Meridian Medical Technologies.*

is now available in four dosages, 0.25, 0.5, 1, and 2 mg (Figure 68.7). The 0.25-mg dose should be used for infants weighing less than 7 kg, the 0.5 mg treats patients weighing 7–18 kg, the 1 mg treats patients weighing 18–41 kg, and the 2 mg dose should be used for children and adolescents who weigh more than 41 kg. The needle length

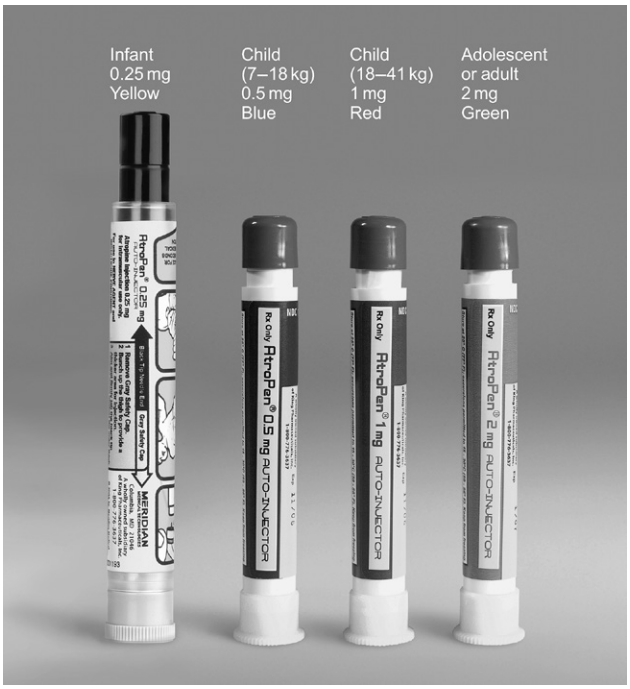

FIGURE 68.7 Atropen pediatric autoinjectors. Dose sizes: 0.25 mg—infant, 0.5 mg—child (7–18 kg), 1 mg—child (18–41 kg), 2 mg—adolescents and adults. *Source: Photograph reproduced with permission from Meridian Medical Technologies.*

for these autoinjectors is 0.8 inches, with a needle gauge of 22. The administration technique of autoinjectors in children is shown in Figure 68.8. Since the AtroPen delivers only atropine and not 2-PAM Cl, there continues to be a limitation to the prompt treatment of children. This fact has caused groups such as the pediatric expert advisory panel from the National Center for Disaster Preparedness to recommend the use of the Mark 1 kit before using the AtroPen (PEAP, 2004). Information on how to use the Mark 1 kit for children is given in Table 68.2. The use of adult dose-based autoinjectors in children has been addressed. Amitai et al. (1999) reviewed 240 instances of accidental pediatric atropine injections using adult dose-based autoinjectors. A low incidence of toxicity was found, with no seizures, arrhythmias, or death. Subsequently, several pediatric guidelines have suggested adult-dose atropine and 2-PAM Cl autoinjectors can be safely used in children larger than 13 kg and inserted 0.8 in.

Administration of atropine and 2-PAM Cl must be done cautiously (Rotenberg and Newmark, 2003). Atropine can cause increased heart rate, dry mouth and skin, and near vision can be affected for up to 1 day. Because sweating is prevented, elevated temperatures and heat stress may be observed. Exposure to 2-PAM Cl can cause double or blurred vision and dizziness (Anon, 2002). Doses must be reduced with renal insufficiency. If a medication is given too quickly as an IV

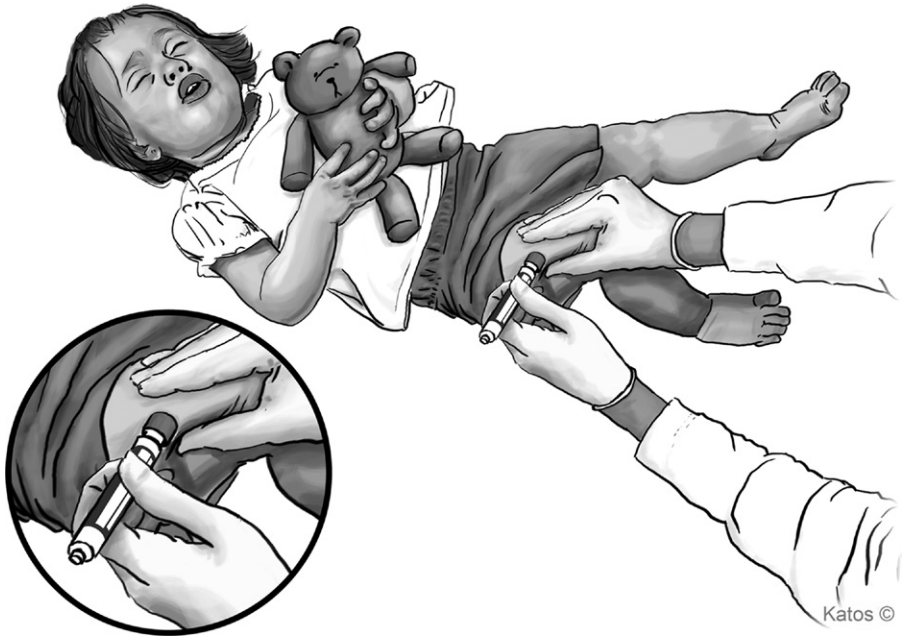

FIGURE 68.8 Technique for using the Atropen pediatric autoinjector.

TABLE 68.2 Dosing of the Mark 1 Kit for Children with Severe, Life-Threatening Nerve Agent Toxicity[a]

Approximate Age (Years)	Approximate Weight (kg)	Number of Mark 1 Kit Autoinjectors to Use	Atropine Dosage Range (mg/kg)	Pralidoxime Dosage Range (mg/kg)
3–7	13–25	1	0.08–0.13	24–46
8–14	26–50	2	0.08–0.13	24–46
>14	>51	3	0.11 or less	35 or less

Source: PEAP (2004).
[a]*If an adult MARK I kit is the only available source of atropine and pralidoxime, it should not be withheld from even the youngest child (i.e., <3 y.o.).*

FIGURE 68.9 Diazepam autoinjector. *Source: Photograph reproduced with permission from Meridian Medical Technologies.*

injection, laryngospasm and rigidity can occur. Higher doses can cause hypertension while lower doses can cause mild electrocardiogram (ECG) changes (Rotenberg and Newmark, 2003).

Although benzodiazepines are not considered to be an antidote, their use in the treatment of nerve agent exposure is critical (Rotenberg and Newmark, 2003). Status epilepticus can often occur as the nerve agent crosses the BBB and causes irritation. Benzodiazepines are the only effective agents that have been proven to treat nerve agent-induced seizures. This group of medications should be used for both prevention and treatment. It is recommended that if more than one organ is impaired, there is impaired consciousness or muscle twitching, and benzodiazepines should be quickly administered. In choosing a specific medication, various agents can be used. Our military department uses the medication diazepam that is administered as an autoinjector (Figure 68.9). In Israel, there is a move toward using midazolam for their population. Some physicians are recommending the use of lorazepam in the pediatric population. Regardless of which medication is used, repeat doses may be needed. For the pediatric population, benzodiazepines should be considered if there is any suspicion of seizure. Nonconvulsive status and subtle seizures are common in infants and children, making it difficult for healthcare providers to recognize nerve agent toxicity.

TABLE 68.3 Management of Mild/Moderate Nerve Agent Exposures

Nerve Agents	Severity of Symptoms	Management: Antidotes[a] Age	Antidotes[a] Dose	Benzodiazepines (If Neurological Signs) Age	Benzodiazepines (If Neurological Signs) Dose
Tabun, sarin, cyclosarin, soman, VX	**Mild/moderate symptoms** • Localized sweating • Muscle fasciculations • Nausea • Vomiting • Weakness/floppiness • Dyspnea • Constricted pupils • Blurred vision • Rhinorrhea • Excessive tears • Excessive salivation • Chest tightness • Stomach cramps • Tachycardia • Bradycardia	Neonates and infants <6 mo	**Atropine** 0.05 mg/kg IM/IV/IO to max 4 or 0.25 mg AtroPen® and **2-PAM** 15 mg/kg IM or IV slowly to max 2 g/h	Neonates	**Diazepam** 0.1–0.3 mg/kg/dose IV to a max dose of 2 mg OR **Lorazepam** 0.05 mg/kg slow IV
		Infants (6 mo–4 yrs)	**Atropine** 0.05 mg/kg IM/IV/IO to max 4 or 0.5 mg AtroPen® and **2-PAM** 25 mg/kg IM or IV slowly to max 2 g/h	30 days–5 yrs	**Diazepam** 0.05–0.3 mg/kg IV to a max of 5 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV not to exceed 4 mg
		Children (4–10 yrs)	**Atropine** 0.05 mg/kg IV/IM/IO to max 4 or 1 mg AtroPen® and **2-PAM** 25–50 mg/kg IM or IV slowly to max 2 g/h	5 yrs and older	**Diazepam** 0.05–0.3 mg/kg IV to a max of 10 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV not to exceed 4 mg
		Adolescents (>10 yrs) and adults	**Atropine** 0.05 mg/kg IV/IM/IO to max 4 or 2 mg AtroPen® & **2-PAM** 25–50 mg/kg IM or IV slowly to max 2 g/h	Adolescents and adults	**Diazepam** 5–10 mg up to 30 mg in 8 h period OR **Lorazepam** 0.07 mg/kg slow IV not to exceed 4 mg

Source: Anon (2002) and Rotenberg and Newmark (2003).

[a]*In general, pralidoxime should be administered as soon as possible, no longer than 36 h after the termination of exposure. Pralidoxime can be diluted to 300 mg/mL for ease of IM administration. Maintenance infusion of 2-PAM at 10–20 mg/kg/h (max 2 g/h) has been described. Repeat atropine as needed every 5–10 min until pulmonary resistance improves, secretions resolve, or dyspnea decreases in a conscious patient. Hypoxia needs to be corrected as soon as possible.*

For each of the medications used to treat nerve agent toxicity, there are weight-based dosing recommendations for pediatric patients. The exact dose to utilize for a specific patient will depend on two critical factors: the severity of the exposure and the weight or age of the patient. Pediatric-dosing recommendations for medications used to treat mild to moderate nerve agent exposure are displayed in Table 68.3. Dosing recommendations to treat severe nerve agent exposure are displayed in Table 68.4.

Perioperative Care of Children with Nerve Agent Intoxication

As mentioned earlier, it is not uncommon for chemical exposures and trauma to occur at the same time, requiring surgery. It is important to realize that many drugs used for perioperative management can exacerbate the side effects encountered with nerve agent exposure. Nerve agents can cause drug interactions with medications typically used for resuscitative efforts (Abraham et al., 2001). Anesthetics, such as sodium pentothal and propofol, cause cardiac depression, an effect exacerbated by the excessive muscarinic activity induced by nerve agents. Doses of these drugs may need to be reduced. Use of volatile anesthetics may be preferable because they bronchodilate and reduce the need for nondepolarizing drugs. When nondepolarizing drugs are used, they are often reversed by the use of neostigmine, which affects AChE activity. Halothane should be avoided in infants because the cardiac side effects can be accentuated in the presence of nerve agents. Depression of the cardiovascular system by halothane may cause further bradycardia, hypotension, and reduction in cardiac output. In general, the use of muscle relaxants is not recommended in the setting of nerve agent toxicity. Nerve agents provide a depolarizing block, and in the presence

TABLE 68.4 Management of Severe Nerve Agent Exposures

Nerve Agents	Severity of Symptoms	Management: Antidotes[a] Age	Antidotes[a] Dose	Benzodiazepines (If Neurological Signs) Age	Benzodiazepines (If Neurological Signs) Dose
Tabun, sarin, cyclosarin, soman, VX	**Severe symptoms** • Convulsions • Loss of consciousness • Apnea • Flaccid paralysis • Cardiopulmonary arrest • Strange and confused behavior • Severe difficulty breathing • Involuntary urination and • Defecation	Neonates and infants <6 mo	**Atropine** 0.1 mg/kg IM/IV/IO or 3 doses of 0.25 mg AtroPen® (administer in rapid succession) and **2-PAM** 25 mg/kg IM or IV slowly OR 1 Mark 1 kit (atropine +2-PAM) if no other options exist	Neonates	**Diazepam** 0.1–0.3 mg/kg/dose IV to a max dose of 2 mg (PDH) OR **Lorazepam** 0.05 mg/kg slow IV
		Infants (6 mo–4 yrs)	**Atropine** 0.1 mg/kg IV/IM/IO or 3 doses of 0.5 mg AtroPen® (administer in rapid succession) and **2-PAM** 25–50 mg/kg IM or IV slowly or 1 Mark 1 kit (atropine +2-PAM) if no other options exist	30 days–5 yrs	**Diazepam** 0.05–0.3 mg/kg IV to a max of 5 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV (not to exceed 4 mg)
		Children (4–10 yrs)	**Atropine** 0.1 mg/kg IV/IM/IO or 3 doses of 1 mg AtroPen® (administer in rapid succession) and **2-PAM** 25–50 mg/kg IM or IV slowly OR 1 Mark 1 kit (atropine +2-PAM) up to age 7, 2 Mark 1 kits for ages >7–10 yrs	5 yrs and older	**Diazepam** 0.05–0.3 mg/kg IV to a max of 10 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV (not to exceed 4 mg)
		Adolescents (>10 yrs) and adults	**Atropine** 6 mg IM or 3 doses of 2 mg AtroPen® (administer in rapid succession) and **2-PAM** 1,800 mg IV/IM/IO OR 2 Mark 1 kits (atropine +2-PAM) up to age 14, 3 Mark 1 kits for ages >14 yrs	Adolescents and adults	**Diazepam** 5–10 mg up to 30 mg in 8 h period OR **Lorazepam** 0.07 mg/kg slow IV (not to exceed 4 mg)

Source: Anon (2002) and Rotenberg and Newmark (2003).

[a]*In general, pralidoxime should be administered as soon as possible, no longer than 36 h after the termination of exposure. Pralidoxime can be diluted to 300 mg/mL for ease of IM administration. Maintenance infusion of 2-PAM at 10–20 mg/kg/h (max 2 g/h) has been described. Repeat atropine as needed every 5–10 min until pulmonary resistance improves, secretions resolve, or dyspnea decreases in a conscious patient. Hypoxia needs to be corrected as soon as possible.*

of inhibited AChE activity, drugs such as succinylcholine can have longer effects than expected (Rotenberg, 2003b).

Careful use of analgesia is important when caring for victims of nerve agent exposure (Abraham et al., 2001). In general, opioids are considered safe to use because they do not act on the cholinergic system directly. However, some side effects of the drugs, such as histamine release and rare muscle rigidity, can cause difficulty in patient management. Careful dose titration and monitoring for side effects is critical. However, there is one opioid that can have an interaction with nerve agents. Remifentanil, a potent opioid, contains an ester linkage susceptible to hydrolysis because it is partially metabolized by plasma cholinesterase. This is the same enzyme that is inactivated by nerve agents, resulting in a prolonged duration of action for remifentanil. Therefore, use of remifentanil in the postoperative care of nerve agent-exposed victims is not recommended, as other analgesics are available (Rotenberg, 2003b). Compared to other CWAs, patients exposed to nerve agents pose unique challenges for medical and surgical management.

Summary

Nerve agent exposures must be handled quickly and efficiently. When children are exposed, it is important to remember that antidote dosing will be determined by the patient's weight and the severity of exposure. Progress has been made to provide pediatric-specific autoinjectors; however, since 2-PAM Cl is not yet available in a pediatric autoinjector form, adult autoinjectors can be used (but carefully) to manage pediatric patients.

Carbamates/Organophosphates

Introduction

Carbamates and OPs are chemicals that are often used as fungicides, insecticides, or pesticides and possess actions similar to nerve agents. These compounds are considered weapons of opportunity since their primary use is not by conventional militaries. In the United States, toxicity from these compounds is fairly rare. In 2006, there were approximately 1,200 cases of carbamate exposures and 1,500 OP exposures documented for children 19 years old and younger (Bronstein et al., 2007).

Although there were a few fatalities reported in 2006 from these substances, these fatalities occurred only in older individuals.

In general, the toxicity of these compounds resembles that of nerve agents, but it is less severe. Pediatric cases of toxicity reported in the literature are often due to accidental poisoning, when a young child ingests chemicals placed in unsecured or unlabeled containers. Exposure also can come by consuming foods that have been sprayed with pesticides. Most of the writings on pediatric toxicity from these agents includes retrospective reviews coming out of Israel, where use of these substances as a pesticide for the home and agriculture is common. A retrospective review of 37 cases from the United States has also been published (Zwiener and Ginsburg, 1988).

Mechanism of Toxicity

These compounds inhibit the hydrolysis of the neurotransmitter ACh by the enzyme AChE within the mammalian nervous system (Zwiener and Ginsburg, 1988). This inhibition causes ACh levels to rise, thus causing cholinergic hyperstimulation at muscarinic and nicotinic receptors. There are important differences in the way carbamates and OPs bind to AChE, as well as their ability to affect the CNS. Carbamates are reversible inhibitors of cholinesterase enzymes. Carbamates create a reversible bond to the cholinesterase enzyme through carbamylation, which can spontaneously hydrolyze and reverse the toxicity. Carbamate poisoning produces toxicity similar to that of OPs; however, the toxicity is usually of a shorter duration and less severe in nature (Lifshitz et al., 1994). In contrast, OPs inhibit cholinesterase via an irreversible bond of phosphate radicals to the active esteratic site of the enzyme (Lifshitz et al., 1999). Thus, the toxicity is more severe.

Clinical Presentation

OPs and carbamates have different receptor activities in the mammalian nervous system. OPs have effects on muscarinic and nicotinic receptors and can cause neurological effects in the CNS (Levy-Khademi et al., 2007). Carbamates are thought to cause only parasympathetic muscarinic effects, with limited nicotinic and CNS effects (Sofer et al., 1989). However, there are case reports in children that have revealed the presence of CNS effects with carbamate exposures (de Tollenaer et al., 2006). One pediatric case series stated that the signs and symptoms from carbamate poisoning were indistinguishable from OP exposures, with severe CNS depression, and stupor and coma occurring in eight cases (Sofer et al., 1989).

Muscarinic hyperstimulation leads to a clinical presentation of miosis, lacrimation, salivation, bradyarrhythmia, urinary incontinence, and intestinal hypermotility (Levy-Khademi et al., 2007). Nicotinic hyperstimulation leads to fasciculations, weakness, and paralysis of skeletal muscles. CNS effects include depression and agitation with coma and seizures occurring in the most severe cases for adults. Generalized tonic–clonic seizures have been seen in several pediatric exposure cases reported in the literature (Zwiener and Ginsburg, 1988).

Additional toxicities that have been reported in children include diarrhea, pulmonary edema [which was associated with OP exposure but not carbamate exposure (Lifshitz et al., 1999)], acute pancreatitis, hyperglycemia (Weizman and Sofer, 1992), dyspnea, sweaty cold skin (Sofer et al., 1989), respiratory distress or failure, lethargy, and tachycardia (Zwiener and Ginsburg, 1988).

Laboratory Findings

Key findings that have been reported include significant hypoxia, acidosis, and carbon dioxide retention (Sofer et al., 1989). Also, hyperglycemia, hypokalemia, and leukocytosis were observed in a case series of OP exposures (Levy-Khademi et al., 2007). A study done on 17 children with typical OP or carbamate poisoning looked at laboratory abnormalities that are associated with acute pancreatitis. Five of the patients (30%) had laboratory values consistent with pancreatitis with elevated immunoreactive trypsin, amylase, and serum glucose. None of the patients had hypocalcemia, renal dysfunction, or acidosis, and all experienced complete recovery of pancreatic function. The authors concluded that acute pancreatitis, due to anticholinesterase (anti-AChE) intoxication, is not uncommon in the pediatric population (Weizman and Sofer, 1992). Pancreatitis has been described in adult exposures, and the association has been investigated in animal studies (Weizman and Sofer, 1992).

Another laboratory value that is often obtained in these exposures is serum pseudocholinesterase. Serum pseudocholinesterase activities are often assessed as normal in children because the reference standards may not be reliable when assessing them. To add to the complexity, the normal range of serum cholinesterase activity is wide (Sofer et al., 1989). Researchers have described the limitations of this measurement in determining therapy for children. In fact, it is recommended that a therapeutic and diagnostic trial of atropine should be given whenever there is any possibility of intoxication with these chemicals (Sofer et al., 1989).

Additional laboratory abnormalities that have been reported in children are cardiac disturbances. Prolonged QTc intervals were reported in a few children exposed to OPs. However, there was spontaneous resolution, with no evidence of ventricular dysrhythmia on ECG (Levy-Khademi et al., 2007).

Pediatric Vulnerabilities

The clinical picture of anti-AChE intoxication in children is very different than that of adults. Often, clinicians have difficulty in diagnosing the exposure in pediatric

EXHIBIT C

PEDIATRIC CASE HISTORIES—CARBAMATES/ ORGANOPHOSPHATES

Carbamate Exposure in a Child

A 7-year-old female, previously healthy, was exposed to an unknown quantity of the fungicide pesticide manganese ethylene-bis-dithiocarbamate (maneb). She was admitted to the pediatric intensive care unit (PICU) with status epilepticus. She had experienced abdominal pain with nausea, vomiting, and headache for approximately 3 days prior to admission. On the day of admission, she was pale, unconscious, staring, and hypotonic. Upon examination, it was determined that she also had severe hypothermia (32.5°C) and hypoventilation. In addition, she was found to have a combined metabolic and respiratory acidosis and elevated blood glucose levels. Routine diagnostic investigations such as blood counts, electrolytes, liver/renal function, cerebrospinal fluid (CSF), and blood cultures, were normal. Urine/blood toxicology screens for barbiturates and benzodiazepines were negative. Liquid chromatographic-mass spectrometry confirmed the presence of maneb in her blood. Upon admission, the patient was intubated and her convulsions were treated with benzodiazepine. Within 24 h, there was a complete recovery of all neurological signs. Repeat physical and neurological exams were normal at 48h. She was discharged 3 days after admission in good condition (de Tollenaer et al., 2006).

Case History: OP Exposure in a Child

A 2-year-old, previously healthy male ingested approximately 10 mL of the OP insecticide demeton-*S*-methyl. Upon discovery of the ingestion, the child was disrobed and washed in the bathtub and then taken to the local hospital. Upon admission, which was approximately 30 min following the ingestion, the child was vomiting and salivating. Atropine was administered. He was then transferred to a larger hospital. During transport, he continued to have excessive salivation and experienced bradycardia. These episodes were treated with additional doses of atropine. Upon admission to the larger hospital, he was salivating, vomiting, and experiencing bronchial hypersecretion. At that time, his pupils were dilated, with no reaction to light, and he was tachycardic, with a pulse rate of 150 bpm. At the hospital, gastric lavage was performed and additional doses of atropine administered. He was intubated prior to the completion of gastric lavage and given diazepam for sedation. Bradycardia (<100 bpm) caused the physicians to administer additional atropine. Due to the complexity of the case, the patient was transferred again, to a hospital better equipped to handle his case. Upon that admission, the patient's blood pressure was 110/60 mm Hg, pulse 100 bpm, temperature 37.6°C, and his pupils were still dilated and unresponsive. Due to continued bronchial hypersecretion, additional atropine was administered. Obidoxime was administered twice, once at 9.5 and 11.5 h after the ingestion. After the oxime therapy, his condition stabilized, and he was able to be extubated approximately 13 h after ingestion. Over the next 4 days, atropine was administered periodically to control mild bronchial hypersecretion. Electrolytes and chest X-rays were normal during the entire course of illness. A low hemoglobin and high alkaline phosphatase were the only abnormal labs. The patient was discharged 8 days after the ingestion in good condition. Plasma cholinesterase levels were initially decreased (<400 U/L), but they rose into the normal range by discharge. A 12-month follow-up revealed that the child had no signs of neurological sequelae (Rolfsjord et al., 1998).

patients. In fact, in a retrospective review of OP/carbamate toxicity cases that were admitted to a children's medical center in the United States, the transfer diagnosis was incorrect in 80% of the patients (Zwiener and Ginsburg, 1988). Patients were misdiagnosed with a wide variety of disease states that ranged from head trauma to cranial aneurysm to diabetic ketoacidosis. It was noted that the difficulty in identifying nicotinic and muscarinic signs in children may have contributed to the high misdiagnosis rate. For example, it may be difficult to distinguish normal infant crying from excessive lacrimation (Zwiener and Ginsburg, 1988).

The lack of classic muscarinic effects does not exclude the possibility of cholinesterase inhibitor poisoning in young children with CNS depression. In one case series, tearing and diaphoresis were not observed in pediatric patients (Lifshitz et al., 1999). Miosis was absent in a number of pediatric patient cases reported in the literature with 27% of children in one case series lacking miosis on admission (Zwiener and Ginsburg, 1988). The percentage was 20% in another case series of pediatric patients (Sofer et al., 1989).

Adult literature states that the most important signs of OP toxicity are fasciculations and miosis. In one

published pediatric case series, fasciculations were quite infrequent, occurring in only 16% of cases (Sofer et al., 1989). Another pediatric case series verified this result, with the frequency of fasciculations being 22% (Zwiener and Ginsburg, 1988).

Another difference is the cardiac manifestations that are seen in adults compared to children. In one pediatric case series, cardiopulmonary manifestations were the least common, with tachyarrhythmias being more common than the bradyarrhythmias that are typically seen in adults (11 patients versus 1 patient) (Levy-Khademi et al., 2007).

Compared to adults, neurological manifestations of toxicity were the most common in children. In one case series, significant hypotonia and muscle weakness were observed in all children (Lifshitz et al., 1999). In addition, severe CNS depression with stupor and coma occurred in all the cases (Lifshitz et al., 1999). In another case series, coma occurred in 54.8% and seizures in 38.7% of children who were accidentally exposed to OPs (Levy-Khademi et al., 2007). It can be theorized that the more permeable BBB in young children permits penetration of the toxic agents into the brain, thus causing CNS depression. Another theory is that in young children, cholinesterase inhibitors have a stronger affinity to AChE in the CNS and less affinity to cholinergic synapses in the autonomic ganglia (Lifshitz et al., 1999). Accumulation of toxic compounds or their metabolites in the brain could also result in severe CNS dysfunction. Another thought is that the hypoxemia that has been observed in several pediatric cases could contribute to CNS depression (Sofer et al., 1989).

Treatment

In all pediatric cases, supportive care was used to balance electrolyte disturbances and oxygen was administered for hypoxic episodes. Often, patients were intubated and given mechanical ventilation due to the excessive salivation and bronchial hypersecretion. Gastric lavage was utilized in one case of OP ingestion (Rolfsjord et al., 1998). The treatment of OP intoxication mimics that of nerve agent exposures. Atropine and an oxime (such as pralidoxime) are the agents of choice for OP exposures. Atropine therapy alone is recommended for carbamate exposure because carbamates reversibly inhibit AChE, so there is little need for an agent such as an oxime, which reactivates the enzyme. In cholinesterase inhibitor poisoning, atropine will alleviate most of the muscarinic signs, few of the CNS symptoms, and almost none of the nicotinic symptoms (Lifshitz et al., 1999).

Although atropine use is standard, clinicians are sometimes faced with the dilemma of administering atropine to a pediatric patient with an elevated heart rate. Due to the fact that children may manifest tachycardia with toxic exposures and the fact that the chronotropic effects of atropine may be minimal in infants and small children compared with healthy young adults, one group of authors suggests that atropine should not be withheld or administered in subtherapeutic doses in tachycardic infants and children with OP or carbamate exposure. Their experience with pediatric patients showed that for patients with tachycardia at the time that atropine was administered, their heart rate decreased and none of the patients developed cardiac arrhythmias (Zwiener and Ginsburg, 1988).

Use of oximes is well accepted for OP exposures, but their role in carbamate poisoning is controversial. Animal studies have shown oxime use can increase toxicity when treating carbaryl exposure (Lifshitz et al., 1994). Therefore, there is a general guideline that oximes should be avoided if a carbamate exposure is suspected. One case series reported the routine use of oxime therapy for carbamate exposures in children (Lifshitz et al., 1994). Marked clinical improvement was observed in all patients, regardless of whether they were exposed to an OP or a carbamate. In addition to the retrospective review of cases, the authors completed an *in vitro* study of oxime use with carbamate toxicity and discovered that oximes play a minor role in direct reactivation of human carbamylated AChE. Due to this finding, the authors concluded that the current guideline to avoid oxime use in a carbamate exposure is valid.

Fortunately, in most cases of OP or carbamate toxicity, pediatric patients recovered fully if they were diagnosed rapidly and appropriate treatments were administered in a timely fashion.

Summary

There is a limited amount of literature available describing the toxicities of OP and carbamate exposure in pediatrics. What literature is available describes major differences between the manner that children manifest toxicity compared to adults. It is critical to understand these differences so that patients are not misdiagnosed and appropriate therapy is not delayed. In general, the CNS toxicities are greater in children than in adults with coma, stupor, and seizures being common. It is important to recognize that if therapy is given in a timely manner, complete recovery is often the outcome for children exposed to these toxic agents.

Vesicants

Introduction

Blister agents or vesicants are chemicals that cause blister or vesicle formation upon dermal contact. Agents such as mustards or lewisite have been used as CWAs in the past (Yu et al., 2003). Although these agents have less toxicity than nerve agents, they cause prolonged

morbidity. There are two types of mustard: sulfur mustard (HD) and nitrogen mustards (HNs). HD caused more casualties in World War I than any other chemical weapon. It caused a significant number of casualties, both civilian and military, during the Iran–Iraq War of the 1980s. HD vapor is the route most likely to be used by terror groups (Yu et al., 2003). It affects multiple organ systems including skin, eyes, respiratory and gastrointestinal tracts, and bone marrow (Yu et al., 2003). Nitrogen mustards, on the other hand, have never been used on the battlefield and will not be discussed further.

Lewisite, a vesicant with HD-like properties, causes a similar constellation of signs and symptoms involving the skin, eyes, and airways, as well as systemic effects (e.g., increased capillary permeability) after absorption. However, it does not produce immunological suppression like mustard. Another difference is that the management of lewisite toxicity includes an antidote, British anti-lewisite (BAL) (Yu et al., 2003).

Mechanism of Toxicity

HD rapidly penetrates cells and generates a highly toxic reaction that disrupts cell function and eventually causes cell death (Sidell et al., 1997). It is classified as an alkylating agent, targeting poorly differentiated and rapidly reproducing cells (Yu et al., 2003). Death is a result of massive pulmonary damage complicated by infection.

Clinical Presentation

Mustard can cause local effects on skin, airways, and eyes; however, large doses can cause fatal systemic effects (Yu et al., 2003). In a study of clinical findings among children exposed to vesicants, ocular, cutaneous, and respiratory signs were the most prevalent (Azizi and

EXHIBIT D

PEDIATRIC CASE HISTORIES—VESICANTS

Mustard Gas Exposure in 14 Children and Teenagers from Halabja, Iraq

Mustard gas was used on the civilian population during the Iraq–Iran War (1980–1988). A case series of 14 children and teenagers affected by mustard gas was reported by Momeni and Aminjavaheri (1994). They found that facial involvement was the most frequent disorder (78%), followed by genital (42%) and trunk and axillar lesions (both 14%). The most prominent laboratory abnormality was eosinophilia (in 12% of patients). As far as the time course of toxicity, skin lesions appeared 4–18 h after exposure, and then erythema developed within 20–30 h. After the erythema, blisters appeared. The authors concluded that the time of onset of toxicity was shorter and more severe in children and teenagers compared with adults (Momeni and Aminjavaheri, 1994).

Clinical Cases from Mofid Medical Center (Mustard Exposure Following the Halabja Attack on March 17, 1988)

A 3-year-old male presented to Mofid Medical Center 8 days after the Halabja chemical attack with fever (39.5°C), tachycardia (140 bpm), and tachypnea (respiratory rate (RR) 60). Cutaneous skin lesions were mild, but erythema and edema covered 45% of his skin surface area. Ocular and respiratory findings were as previously described. Laboratory findings were unremarkable except for a mild anemia. Chest roentograms revealed hilar congestion and consolidation bilaterally. The fever continued despite antibiotic therapy. On day 10 of admission (18 days after exposure), the patient developed leukocytosis with 82% polymorphonuclear neutrophils (PMNs) and worsening respiratory distress. The patient finally died 21 days after exposure.

An 8-year-old Iranian male presented at 5:30 p.m. with fever (40°C), severe agitation, delirium, and somnolence 24 h after exposure to chemical agents the previous day in Halabja. BP was 110/70 mm Hg, and the patient was notably tachycardic (120 bpm), and tachypneic (RR 42). The patient was noted to have serious dermatologic, ocular, and respiratory impairment. Erythema, vesicles, erosions, bullae, ulcerations, and edema were present on 35% of the body. Ocular manifestations included conjunctivitis and palpebral edema. At this point, the patient was working hard to breathe, as evidenced from accessory muscles of respiration (sternocleidomastoid). On physical examination of the lungs, wheezing and crepitation were noted throughout the lung field. Laboratory findings were the following: Na^+ 139, K^+ 4.1 mEq/L, BUN 25 mg/dL, calcium 7.3 mg/dL, and WBC 9,900/mm^3, with 90% neutrophils. Arterial blood gases (ABGs) were as follows: pH 7.30, pCO_2 31, pO_2 65, and HCO_3 15.1. Chest roentograms showed bilateral infiltrates. The patient died 24 h after admission and 48 h after exposure, despite receiving supportive care (Azizi and Amid, 1990).

Amid, 1990). Ocular findings consisted of the following: conjunctivitis (94%), palpebral edema (81%), eye closure (63%), keratitis (38%), blepharospasm (25%), corneal ulceration (19%), and chemosis (6%). Cutaneous signs included erythema (94%), hyperpigmentation (75%), ulceration (69%), erosion (63%), blister (56%), edema (50%), vesicles (31%), and hypopigmentation (13%). Respiratory signs included dyspnea (63%), crepitation (50%), and wheezing (25%).

Other pediatric signs of mustard exposure were photophobia, lacrimation, ophthalmalgia, and eye burning (94%). Dry cough (81%), dermal pain, and burning (94%) were also frequent complaints. Less frequent complaints were diplopia, itchy eyes, sore throat, sneezing, nasal secretions, dyspnea, burning sensation of the upper respiratory tract, suffocation, and dysphonia (Azizi and Amid, 1990).

Initial dermal signs of toxicity consist of erythema, occurring 4–8 h after exposure. Pruritus can occur with or prior to erythema (Azizi and Amid, 1990; Yu et al., 2003). Over the next 24 h, large yellowish blisters form in areas of thin skin, such as the groin and underarms. Eye damage can occur, ranging from pain and irritation to blindness. Mustard also causes clinical effects that can be delayed for hours (Azizi and Amid, 1990; Sidell et al., 1997; Yu et al., 2003). This causes victims not to recognize toxicity until well after exposure. During this time lag, sulfur mustard works to subclinically damage the skin. This latent period is significant because the shorter the latent period, the more severe the exposure and the worse the outcome.

The CNS and bone marrow can also be affected, as displayed by symptoms of fatigue, headache, and depression (Sidell et al., 1997). HD can also lead to pneumonia, the cause of death for many HD casualties in World War I due to lack of antibiotics. A leukopenic pneumonia usually occurs between 6 and 10 days after HD exposure. The manifestation of leukopenia (specifically lymphopenia) results from the myelosuppressive effects of mustard agents.

Laboratory Findings

While there is no diagnostic confirmatory test for mustard exposure, some laboratory tests can prove useful. As described previously, inflammation and infection will show up as fever and leukocytosis. Erythrocyte sedimentation rate (ESR) has been shown to be elevated in patients after mustard exposure (Motakallem, 1988). Some of these patients were in the pediatric age range of 0–18 years. Complete blood count (CBC) determinations may show abnormalities depending on the severity of the vapor inhalation or exposure (Azizi and Amid, 1990; Yu et al., 2003). The CBC may show a low hematocrit and leukopenia if the exposure was severe. There may be only a transient decrease in white blood cells (WBCs), with subsequent recovery. In pediatric cases of HD vapor exposure, decreases in hematocrit and/or WBC were likely to occur in the first 2 weeks, with the lowest levels of Hb, Hct, WBC, and neutrophil count observed in the 6th- to 10th-day samples after exposure (Azizi and Amid, 1990).

These pediatric patients also suffered from clear signs of hypoxemia and renal failure (Azizi and Amid, 1990). Unfortunately, serum creatinine and renal function tests (RFTs) were not found in the charts. ABGs may provide useful information, but they may show a varied picture. In one pediatric study of mustard casualties, most cases (43%) showed simple metabolic acidosis. The other groups showed the following:

- Mixed metabolic acidosis and respiratory alkalosis (29%)
- Simple respiratory alkalosis (14%)
- Mixed metabolic and respiratory acidosis (7%)
- Mixed metabolic alkalosis and respiratory acidosis (7%).

Blood urea nitrogen can be elevated with mustard exposure, but it does not necessarily predict outcome or mortality. Blood urea nitrogen was significantly elevated in severe mustard exposure cases in the Azizi study; three of the four pediatric mustard victims that died showed very high blood urea nitrogen (Azizi and Amid, 1990). While elevations in blood urea nitrogen were found in many of the pediatric casualties from mustard exposure, blood urea nitrogen returned to normal levels soon afterward in survivors.

Pediatric Vulnerability

The effects of sulfur mustard on children are more severe than on adults (Yu et al., 2003). Premature infants have thinner skin, and the dermal–epidermal junction is not fully developed in children (Rutter and Hull, 1979; Harpin and Rutter, 1983; Nopper et al., 1996; Seidenari et al., 2000; Mancini, 2004); therefore, the time between exposure and onset of blisters is shortened, and the number and severity of blisters will be more severe (Yu et al., 2003). In fact, lesions in children exposed to mustard have been shown to be more severe. Initial index cases of mass casualties are typically children. Eye findings tend to be greater in children because of their inability to protect themselves and tendency to rub their eyes (Azizi and Amid, 1990; Yu et al., 2003). Children are also shown to be more susceptible to pulmonary injury for reasons previously discussed. One case report looked at the long-term effects of mustard exposure in a child (Dompeling et al., 2004). This child suffered an acute, severe chemical pneumonia; the long-term consequence was chronic bronchiolitis. Finally, signs of gastrointestinal toxicity may be greater in children secondary to fluid losses in combination with lower intravascular volume reserves (Yu et al., 2003).

TABLE 68.5 Management of Vesicant Exposures

Vesicant Agents	Symptoms	Antidotes/Treatment
Mustard	• Skin erythema and pruritis • Development of large yellow blisters leading to ulcers • Eye damage • Inhalational damage: hoarseness and cough, mucosal necrosis, toneless voice, nausea, vomiting	**Decontamination:** soap, water, no bleach Copious water irrigation for eyes **Pulmonary management:** cough suppressants, throat lozenges **Skin management:** topical agents used for burns (1% silver sulfadiazine), antibiotics for secondary infections (Neosporin®), antihistamines for itching (diphenhydramine 1 mg/kg/dose orally q6–8 h max 300 mg/day, hydroxyzine 0.5 mg/kg/dose orally q6–8 h) **Immune system management:** G-CSF (filgrastim) 5–10 micrograms/kg/day subcutaneous for neutropenia
Lewisite	• Shock • Pulmonary injury • Blisters	**Decontamination:** soap, water, no bleach **Antidote:** BAL-dimercaprol may decrease systemic effects of lewisite **Pulmonary management:** BAL 3–5 mg/kg deep IM q4 h × 4 doses (dose depends on severity of exposure and symptoms) **Skin management:** BAL ointment **Eye management:** BAL ophthalmic ointment

Source: Momeni and Aminjavaheri (1994) and Yu et al. (2003).

While the decision to evacuate and hospitalize HD casualties is based on the extent [total body surface area (TBSA) >5%], severity of the skin lesions, and the recognition of multiple organ involvement (Graham et al., 2005), the threshold to hospitalize children with HD injuries should be lower. One reason is that vapor mustard used by terrorists may cause extensive pulmonary involvement while producing mild skin blisters.

Treatment

While decontamination and supportive therapy are the mainstays of treatment, antidotes to counteract HD vapor, aerosol, or liquid exposures do not exist (Yu et al., 2003). Adult decontamination may include bleach solutions; however, this method can cause greater toxicity in children. Soap and water are the preferred agents to use for decontamination in children. Supportive care consists of the management of pulmonary and skin manifestations, such as the use of cough suppressants and topical silver sulfadiazine for burns (Azizi and Amid, 1990; Sidell et al., 1997; Yu et al., 2003). Pediatric dosage and treatment recommendations for vesicant exposure are displayed in Table 68.5.

There are currently no standardized guidelines of casualty management or drugs available to prevent HD effects on skin and mucous membranes (Sidell et al., 1997; Graham et al., 2005). The mainstay of treatment is prompt decontamination, blister aspiration or deroofing (epidermal removal), physical debridement, irrigation, topical antibiotics, and sterile dressing application for cutaneous HD injuries. Current treatment strategies rely on symptomatic management to relieve symptoms, prevent infections, and promote healing. The general recommendations are described in the *Medical Management of Chemical Casualties Handbook* (USAMRICD, 2000a), the *Field Management of Chemical Casualties Handbook* (USAMRICD, 2000b), and other references (Graham et al., 2005). We will discuss the aspects of treatment that relate to the pediatric population. Most pediatric casualties will involve multiple organ systems (e.g., skin, ocular, gastrointestinal, bone marrow, and respiratory), as documented by Iranian physicians treating pediatric casualties of HD vapor during the Iran–Iraq War (Azizi and Amid, 1990).

Dermatological Management

Managing mustard skin lesions is especially challenging in the pediatric population. The goal of blister management is to keep the patient comfortable, keep the lesions clean, and prevent infection. Children especially will be extremely anxious at the sight of bullae and erythema, in addition to the burning, pruritis, and allodynia associated with HD blisters (Sidell et al., 1997). Anxiolytics may be appropriate to calm them down and prevent them from picking at bullae. Burning and itching associated with erythema can be relieved by calamine or soothing lotions and creams such as 0.25% camphor, menthol corticosteroids, antipruritics (i.e., diphenhydramine),

and silver sulfadiazine cream (Azizi and Amid, 1990; Sidell et al., 1997). Pain and discomfort can be relieved with systemic analgesics. Systemic analgesics such as morphine should be given liberally before manipulation of the burned area.

Vapor mustard typically causes a first- or second-degree burn, while liquid mustard produces damage similar to a third-degree burn. In either case, tense bullae are the hallmark of HD injuries. Bullae are typically dome-shaped, thin-walled, 0.5–5.0 cm in diameter, superficial, translucent, yellowish, multiloculated, honeycombed (Moradi et al., 1986), and surrounded by erythema (Sidell et al., 1997). Preventing children from breaking the blisters can be a challenge, especially when constant friction from clothing and blankets is irritating to the skin. These areas should be wrapped in protective dressings. Graham et al. (2005) have made an important point about the existence of a reservoir of unbound HD in human skin following a vapor (Logan et al., 2000) or liquid exposure, leading to an off-gassing period. They suggested that this off-gassing period can last for 24–36 h, whereby application of an occlusive dressing is not beneficial to prevent vapor buildup (Graham et al., 2005).

It is recommended that small blisters (<1 cm) should be left alone on the child, but the immediate area should be cleaned, irrigated daily, and covered with topical antibiotic (Sidell et al., 1997). Petroleum gauze bandage dressings should be wrapped around these unbroken blisters and changed every few days. Larger blisters (>1 cm) should be unroofed and irrigated several times a day with saline, sterile water, clean soapy water, or Dakin's solution and covered with topical antibiotic cream or ointment. It should be noted that blister fluid does not contain mustard (Buscher and Conway, 1944) and therefore does not represent a hazard to the healthcare worker. Options for topical antibiotic creams in children include silver sulfadiazine, and triple-combination antibiotic (bacitracin, neomycin sulfate, and polymyxin B sulfate; Sidell et al., 1997) but not mafenide acetate, which can cause toxicities in children (Ohlgisser et al., 1978; Geffner et al., 1981). These topical antibiotics should be applied to the area of bullae and surrounding areas of erythema. There is no information comparing the use of this combination (triple-antibiotic topical ointment) in children with use in other age groups.

While skin healing can take months to complete, pigment changes (hyperpigmentation or hypopigmentation) can persist (Sidell et al., 1997; Graham et al., 2005). It is also important to note that not all burn injuries require treatment at a burn center. Patients will require aggressive pain management and close observation for the systemic effects of HD exposure. Skin grafting, although rare, has been successfully used for deep burns (Ruhl et al., 1994).

Ophthalmology

The objective for any ophthalmology consultation on pediatric HD injuries involving the eye is the prevention of scarring and infection (Sidell et al., 1997). Eyes exposed to HD should be irrigated to remove traces of vesicant. Severe ocular involvement requires topical antibiotics (such as tobramycin OD) applied several times a day. Topical steroids may be useful in the first 48 h after exposure. Temporary loss of vision may also occur after mustard exposure (Motakallem, 1988; Azizi and Amid, 1990; Sidell et al., 1997). The patient should be reassured that vision loss is not permanent and is due to palpebral edema, not corneal damage.

Respiratory System

The conducting and ventilation portions of the respiratory tract are affected with HD vapor, necessitating a pulmonary examination (Azizi and Amid, 1990; Sidell et al., 1997; Dompeling et al., 2004). Bronchodilators are useful to diminish hyperreactive airways and should be used if a prior history of asthma or hyperreactive airways is documented. Further support with humidified oxygen may be required. Ventilatory support may be required for severe cases of HD vapor exposure before laryngeal spasm makes intubation difficult. Bronchoscopy is critical for diagnosis, therapeutic dilation against HD-induced tracheobronchial stenosis, and removal of pseudomembranes that cause airway obstruction.

Since the toxic bronchitis produced by HD is nonbacterial, antibiotic therapy should not be given during the first 3–4 days (Sidell et al., 1997). Continuous monitoring of sputum for Gram's stain and culture growth is necessary to identify the specific organism responsible for the late-developing superinfection. The presence of leukopenia in children, a grave sign of HD exposure, will require aggressive support with combination antibiotic treatment.

Gastrointestinal Tract

Atropine or common antiemetics can be given to provide relief from nausea and vomiting, early signs of HD intoxication (Yu et al., 2003). Excellent choices for pediatric-specific antiemetics include medications such as promethazine, metoclopramide, and ondansetron (Sidell et al., 1997). Persistent vomiting and diarrhea are later signs of systemic toxicity requiring prompt fluid replacement.

Bone Marrow Suppression

As a radiomimetic, HD affects rapidly dividing tissues like bone marrow in addition to the gastrointestinal tract (Sidell et al., 1997; Graham et al., 2005). HD destroys hematopoietic precursor cells; WBCs have the shortest life span and decrease in number first, followed by red

blood cells (RBCs) and thrombocytes. The bone marrow suppression that is sometimes seen can be treated with filgrastim injections. This medication stimulates the bone marrow to create and release WBCs.

Other Treatment Considerations

Fluid status, electrolytes, and urine output should be monitored in the HD-intoxicated patient. Tetanus prophylaxis should also be administered because fatal tetanus may occur even after a small, partial-thickness burn (Marshall et al., 1972).

Summary

Pediatric exposures to vesicants can be quite toxic; however, in contrast to nerve agent exposures, HD causes significantly greater morbidity than mortality. While mustard did not cause many deaths in World War I, death from HD exposure is usually due to massive pulmonary damage, complicated by infection (bronchopneumonia) and sepsis. Children often show a quicker onset and greater severity of toxicity. Skin and eye toxicity occurs in the form of blisters or irritation that can result in blindness for the most severe cases. Except for lewisite, vesicant exposures must be managed with supportive care and rapid decontamination.

Pulmonary Agents

Introduction

In January 2002, a CIA report stated that terrorist groups may have less interest in biological materials than in chemicals such as cyanide, chlorine, and phosgene (DCI, 2002), which are able to contaminate food and water supplies (Sidell et al., 1997; Graham et al., 2005). The targeting of children has the potential to destabilize governments and create widespread terror. Industrial chemicals, such as chlorine and phosgene, have advantages that make them potential candidates to be used by terrorists in the future. Both chlorine and phosgene are fairly easy to manufacture and handle, prompting national concern over their future use. In the United States alone, millions of tons of chlorine and phosgene are produced annually toward the manufacture of various products (Burklow et al., 2003). A detailed discussion of the general mechanisms of chlorine and phosgene toxicity can be found elsewhere in this book and will not be explored further here.

Clinical Presentation

Pediatric signs and symptoms of chlorine gas exposure include predominantly ocular, nasal, oropharyngeal, and pulmonary irritation of membranes (Burklow et al., 2003). The hallmark of intoxication by these choking agents involves respiratory complaints. Minor chlorine exposures can lead to burning of the eyes and throat, which indicate mucous membrane irritation. More severely exposed patients may complain of cough, choking, sore throat, shortness of breath, chest tightness, difficulty breathing, and other respiratory-related complaints. Clinical findings may also include lacrimation, rhinorrhea, laryngeal edema, hoarseness, aphonia, stridor, expiratory wheezing, tracheitis, and cyanosis (Güloğlu et al., 2002; Traub et al., 2002). Tachypnea may develop as a direct result of pulmonary irritation, and tachycardia has been demonstrated in some studies. Many pediatric patients with a prior history of reactive

EXHIBIT E

PEDIATRIC CASE HISTORY—PULMONARY AGENTS

Chlorine Gas Exposure in an Adolescent

A 14-year-old male, previously healthy except for a history of asthma, was exposed to chlorine gas by mixing household bleach with vinegar. Immediately, he began to cough and have difficulty breathing. His symptoms worsened over the next hour, leading to an admission to the local emergency room. Upon admission, the physical exam revealed that the patient was in respiratory distress, with bilateral crackles and diffuse wheezing. He also had conjunctival irritation. Vital signs were pulse 100 bpm, blood pressure 130/80 mm Hg, respiratory rate 20 breaths/min, and temperature 97°F. His initial oxygen saturation while breathing room air was 92%. A venous blood gas suggested mild CO_2 retention with a pH of 7.35, PCO_2 of 53 mm Hg, and a PO_2 of 33 mm Hg. Chest radiograph showed bilateral alveolar infiltrates with a normal heart size, which is indicative of acute lung injury. Sinus tachycardia was demonstrated on the ECG. The patient was treated with oxygen, multiple doses of nebulized albuterol, and oral prednisone. Despite these measures, his overall respiratory status continued to decline, and a repeat pulse oximetry while on 50% oxygen showed a saturation of 85%. Due to his worsening condition, he was intubated and transported to another hospital with a PICU. Upon intubation, it was noted that the patient had copious secretions. After admission to the PICU, the patient developed acute respiratory distress syndromes (ARDS) and needed ventilatory management for 19 days, along with additional doses of albuterol and methylprednisolone. After extubation, he was placed on a prednisone taper and discharged with no evidence of residual pulmonary dysfunction (Traub et al., 2002).

airway disease are at increased risk of chlorine-induced bronchospasm (Burklow et al., 2003).

Pulse oximetry may indicate low oxygen saturation (Traub et al., 2002). While ABGs usually indicate hypoxemia, carbon dioxide levels have been shown to be decreased, increased, or normal (Güloğlu et al., 2002; Traub et al., 2002). A hyperchloremic metabolic acidosis may show up on blood chemistries due to systemic absorption of hydrochloric acid.

Pulmonary edema, the most significant morbidity from pulmonary agents, can be seen on chest roentograms (Burklow et al., 2003). Pulmonary edema may develop as early as 2–4 h after exposure; radiographic evidence typically appears later. Pulmonary edema may progress to the point of producing Kerley B lines on chest x-rays. These lines are often described as rungs of a ladder running perpendicular to the lateral margin of the lungs beginning at the costophrenic angle. Chest radiographs will often show opacities of acute lung injury. Pneumomediastinum has also been reported in chlorine gas exposure (Traub et al., 2002).

Pulmonary function tests (PFTs) are not helpful (Pherwani et al., 1989; Traub et al., 2002) in this circumstance. A study of schoolchildren exposed to a chlorine gas leak reported a predominantly obstructive pattern on PFTs. This could be explained by congestion and edema narrowing the central airways rather than smaller ones.

Pediatric Vulnerability

Chlorine is a pungent green–yellow gas, twice as heavy as air (Güloğlu et al., 2002) and settles near the ground (Traub et al., 2002; Burklow et al., 2003). This poses a problem for children, leading to increased exposure for this population in the event of release, either accidentally or via an act of terror. Children can be exposed as a result of the following activities: inhaling chlorine vapors at swimming pools (Burklow et al., 2003), mixing of household bleach (sodium hypochlorite) with acidic cleaning agents (Traub et al., 2002), and industrial accidents (Pherwani et al., 1989). Phosgene, a dense gas heavier than air, is a more lethal pulmonary agent than chlorine. While the smell of chlorine is associated with swimming pools, phosgene odor is described as smelling like freshly mown hay (Burklow et al., 2003).

Initially, both agents cause intense irritation of the mucosal membranes (Burklow et al., 2003) and coughing (Güloğlu et al., 2002; Traub et al., 2002). This is typically followed by a feeling of suffocation (Burklow et al., 2003). Morbidity from pulmonary agents is the direct result of pulmonary edema, appearing 2–4 h after chlorine exposures. Since children have a smaller fluid reserve (Rotenberg and Newmark, 2003), pulmonary edema can cause rapid dehydration or even shock (Burklow et al., 2003). Due to the higher respiratory rates and minute volumes of children (Rotenberg and Newmark, 2003), exposure to pulmonary agents will be greater (Burklow et al., 2003). Concerning the effects on children exposed to pulmonary agents and subsequent treatment, there are many documented clinical case studies in the literature looking at accidental exposures and industrial accidents (Pherwani et al., 1989; Güloğlu et al., 2002; Traub et al., 2002).

Treatment

The first line of treatment for children exposed to pulmonary agents is decontamination. Decontamination can be as simple as removing the victim from the source to fresh air, followed by the removal of contaminated clothing (Burklow et al., 2003). Supportive care includes administration of humidified air, supplemental oxygen, water irrigation, and high-flow oxygen delivered via positive pressure for pulmonary edema (Traub et al., 2002; Burklow et al., 2003). Further treatment may include surgical debridement and supportive care with medications, such as albuterol for bronchospasm, corticosteroids for inflammation, and antibiotics for any secondary bacterial infections. Antidotes or specific postexposure treatments do not exist for this class of agents. Supportive treatment recommendations are shown in Table 68.6.

Summary

Chlorine and phosgene are two chemicals that can cause severe pulmonary toxicity due to pulmonary edema and direct damage to the lungs. Treatment involves decontamination and supportive care. Special care needs to be provided for exposed children because they are at higher risk for toxicity because of their unique vulnerabilities.

Cyanide

Introduction

Cyanide is used in plastic processing, electroplating of metals, metal tempering, extraction of gold and silver, fumigants, and photographic development (Baskin and Brewer, 1997; Rotenberg, 2003a). It is also found in vehicle exhaust, tobacco smoke, certain fruit pits, and bitter almonds. The major cyanide-containing compounds used by the military in World War I were hydrogen cyanide, cyanogen chloride, and cyanogen bromide. Cyanide is also liberated during the combustion or metabolism of nitrogen-containing polymers of natural and synthetic origin (Riordan et al., 2002). Cyanides can cause lethality through the inhalation of cyanide vapor or ingestion (Prajapati et al., 1992). Cyanide poisoning leads to death in minutes, but it can be effectively treated with antidotes if diagnosed quickly. Pediatricians, medical first-responders, and firefighters need to recognize victims of cyanide poisoning

TABLE 68.6 Management of Pulmonary Agent Exposures

Pulmonary Agents	Symptoms	Treatment
Chlorine	• Lacrimation • Rhinorrhea • Conjunctival irritation • Cough • Sore throat • Hoarseness • Laryngeal edema • Dyspnea • Stridor • ARDS • Pulmonary edema	**Decontamination:** Copious water irrigation of the skin, eyes, and mucosal membranes to prevent continued irritation and injury **Symptomatic care (no antidote):** Warm/moist air, supplemental oxygen, positive pressure O_2 for pulmonary edema **Bronchospasm:** Beta-agonists (albuterol) **Severe bronchospasm:** Corticosteroids (prednisone) (also used for PTS with H/O asthma but use unproven) **Analgesia and cough:** Nebulized lidocaine (4% topical solution) or nebulized sodium bicarbonate (use unproven)
Phosgene	• Transient irritation (eyes, nose, throat, and sinus) • Bronchospasm • Pulmonary edema • Apnea • Hypoxia	**Decontamination:** Wash away all residual liquid with copious water, remove clothing **Symptomatic care:** ABCs, hydrate, positive pressure O_2 for pulmonary edema **Bronchospasm:** Beta-agonists (albuterol), corticosteroids INH/IV, furosemide contraindicated **Hypoxia:** Oxygen

Source: Burklow et al. (2003).

in order to initiate immediate intervention (Baskin and Brewer, 1997; Rotenberg, 2003a). Cyanide is one of the few chemicals for which an effective antidote exists.

Mechanism of Toxicity

The cyanide ion kills aerobic organisms by shutting down oxidative phosphorylation in the mitrochondria, and therefore the utilization of oxygen in cells (Baskin and Brewer, 1997; Riordan et al., 2002). Cyanide has a propensity to affect certain organs (e.g., brain, heart, and lungs) more than others (Baskin and Brewer, 1997; Rotenberg, 2003a). Significant exposure can lead to central respiratory arrest and myocardial depression. Cyanide also acts as a direct neurotoxin (Rotenberg, 2003a), disrupting cell membranes and causing excitatory injury in the CNS (Baskin and Brewer, 1997; Riordan et al., 2002).

EXHIBIT F

PEDIATRIC CASE HISTORY—CYANIDE

Case History: Cyanide Exposure in a Child

A 2-year-old, previously healthy, 12-kg male ingested an unknown quantity of an acetonitrile-containing sculptured nail remover. The product contains an aliphatic nitrile that releases inorganic cyanide upon human metabolization. The child was brought into the emergency room because of lethargy approximately 10 h after the ingestion. Although the child was acting normally at the time of ingestion, 8 h later he was found to be moaning, poorly responsive, and having just vomited. In the emergency room, he was pale and lethargic, responding only to deep pain. Abdomen and neck exam was normal, and the lung exam revealed bilateral coarse breath sounds with a normal chest roentgenogram. Extremities were mottled and cool, with a delayed capillary refill time. Vital signs showed a temperature of 36.9°C, pulse of 140 bpm, respiratory rate of 56/min, and blood pressure of 70/30 mm Hg. ABG measurements showed a pH of 6.95, PCO_2 of 11 mm Hg, and PO_2 of 114 mm Hg. His electrolytes revealed the following levels: sodium of 137 mmol/L, potassium of 5.1 mmol/L, chloride of 114 mmol/L, bicarbonate of 4 mmol/L, serum creatinine of 70.7 μmol/L, glucose of 15.8 mmol/L, and blood urea nitrogen of 5 mmol/L. The WBC count was 9.5×10^9/L, and the hematocrit was 31%. Sinus tachycardia, at a rate of 160 bpm, with normal intervals and axis, was observed on the ECG. Serial whole-blood cyanide levels were obtained, with the initial level being 231 μmol/L (600 μg/dL) 12 h after exposure. The patient was given oxygen, sodium bicarbonate, and fluid resuscitation. Electrolyte and acid–base disturbances were corrected, and no antidotal therapy was administered due to prompt response on supportive therapies. The patient was discharged from the hospital 3 days after admission in good condition (Caravati and Litovitz, 1988).

Clinical Presentation

Cyanide is an uncommon cause of childhood poisoning. In 2006, there were only 12 reported cases of cyanide exposure in the pediatric population (<19 years) (Bronstein et al., 2007). Since the signs of toxicity (see Exhibit F) are so similar to carbon monoxide poisoning, which accounts for the largest group of poisoning deaths among children, clinicians must have a high index of suspicion to make the diagnosis (Prajapati et al., 1992; Riordan et al., 2002). Rotenberg describes a typical toxidrome induced by cyanide (Rotenberg, 2003a), which includes a rapid progression from hyperpnea, anxiety, restlessness, unconsciousness, seizures, apnea, and finally reaching death. Skin, blood, and fundi may be cherry red upon physical examination due to the inability of mitochondria to extract oxygen. In reported cases of accidental cyanide ingestion by children, other signs of toxicity included nausea, vomiting, abdominal pain, headache, lethargy, slurred speech, ataxia, stupor, coma, and respiratory depression. In addition, delayed vomiting occurred due to the slow metabolization of the chemical compound acetonitrile to cyanide, a process that can take 6–14 h after the ingestion (Geller et al., 2006).

Laboratory Findings

ABGs can provide clues of cyanide exposure. Classic cases are presented with severe metabolic acidosis, elevated anion gap, and high lactate concentrations (Rotenberg, 2003a). Impaired cellular respiration will lead to a high oxygen content in venous blood (Riordan et al., 2002; Rotenberg, 2003a). Thus, a reduced arterial-venous oxygen saturation difference suggests this diagnosis. Blood cyanide levels are confirmatory (Baskin and Brewer, 1997; Riordan et al., 2002; Rotenberg, 2003a) but will only delay the diagnosis, which must be based on the initial clinical presentation. Immediate therapeutic intervention with provision of 100% supplemental oxygen and administration of specific antidotes is paramount. An almond-like odor on the breath may alert a clinician that a person may have been exposed to cyanide, but up to 40% of the general population is unable to detect this odor.

Pediatric Vulnerability

Children are especially vulnerable to cyanide attacks (Rotenberg, 2003a). A larger exposure to cyanide vapor occurs due to the higher respiratory rates and higher surface-to-volume ratios in children. Cyanide liquid causes greater and more rapid absorption when it comes against the immature skin barrier of children. Lower body mass and immature metabolic processes can render children more susceptible than adults to toxicity from cyanide exposure. It has also been noted that children seem more susceptible to ingestion poisoning, as demonstrated by various cassava and apricot pit exposures where the severity of toxicity was greater than that seen in adults who were also ingesting these products. In fact, it has been theorized that due to children's higher gastric acidity, which leads to greater absorption, they experience more severe toxicity than adults when cassava is ingested (Geller et al., 2006). A case report of potassium cyanide ingestion among 10 children reported that the initial symptoms included abdominal pain, nausea, restlessness, and giddiness (Prajapati et al., 1992). Cyanosis and drowsiness were also noted, but the signature cherry-red skin color was not reported. Postmortem examination of the 2 children that died showed bright red blood and congested tissues. These 2 children consumed powder packets of potassium cyanide mixed in water, while the other 8 children licked the powder, leading to less toxicity. The survivors were managed with aggressive supportive care, including gastric lavage, oxygen, and IV fluids.

EXHIBIT G

MNEMONIC FOR RECOGNIZING CYANIDE TOXICITY

F-A-T R-E-D C-A-T-S
Flushing of skin
Almonds (bitter almond smell)
Tachycardia
Red (Red/pink skin, bright red retinal vessels)
Excitation of nervous system
Dizziness, **D**eath, recent **D**epression history
Confusion, **C**oma, **C**onvulsions
Acidosis (metabolic or lactic), **A**nion gap
Tachypnea
Soot in nose

Treatment

The mainstay of treatment in cases of cyanide toxicity in the United States consists of supportive treatment and use of a multistage antidote kit (Baskin and Brewer, 1997; Riordan et al., 2002; Rotenberg, 2003a). Table 68.7 details pediatric doses used for the medications in this kit, which contains amyl nitrite, sodium nitrite, and sodium thiosulfate. Antidotes should be provided only for significantly symptomatic patients, such as those with impaired consciousness, seizures, acidosis, hypotension, hyperkalemia, or unstable vital signs (Goldfrank et al., 1998). Even when patients are rendered comatose by the inhalation of hydrogen cyanide gas, antidotes may not be necessary if the exposure is rapidly terminated, the patient has regained consciousness on arrival to the

TABLE 68.7 Management of Cyanide Exposures

		Antidotes/Treatment			
		Multistage Antidote Kit (for Unconscious Patients)			
Agent	**Severity of Symptoms**	**Age**	**Amyl Nitrite Ampules**	**Sodium Nitrite (for Hb = 12)**	**Sodium Thiosulfate (for Hb = 12)**
Cyanide	• Tachypnea • Restlessness • Anxiety • Flushing • Tachycardia • Dizziness • Apnea • Respiratory failure • Seizures • Coma	Child <30 kg	1. Crush 1 amp. in gauze close to the mouth and nose of breathing victim 2. Inhale for 15 s, rest for 15 s 3. Replace pearls every 30 s until sodium nitrite can be administered	1. 0.19–0.39 mL/kg not to exceed 10 mL of 3% solution slow IV over less than 5 min or slower if hypotension develops 2. For every 1 g/dL increase or decrease change in Hb, change dose by approximately 0.03 mL/kg accordingly 3. May repeat dose at 1/2 original dose in 30 min if needed	1. 0.95–1.95 mL/kg not to exceed 50 mL of 25% solution IV over 10–20 min 2. For every increase or decrease change in Hb of 1 g/dL, change sodium thiosulfate by 0.15 mL/kg accordingly 3. May repeat dose at 1/2 original dose in 30 min if needed
		Adult	See above	10 mL of 3% solution slow IV over no less than 5 min or slower if hypotension develops	50 mL of 25% solution IV over 10–20 min

Source: Berlin (1970), Hall and Rumack (1986), and Anon (1998).
Other treatment: evacuation, decontamination, 100% O_2, and correction of acidosis, hypovolemia, and seizures.

hospital, and there is no acidosis or abnormality of the vital signs (Peden et al., 1986).

Supportive Therapy

Regardless of the antidote available, treatment will always consist of supportive therapy (Rotenberg, 2003a). Supportive therapy alone may reverse the effects of cyanide even in the face of apnea (Peden et al., 1986; Baskin and Brewer, 1997; Rotenberg, 2003a). Supportive therapy includes decontamination, which includes gastric lavage and administration of activated charcoal if appropriate, oxygen, hydration, and anticonvulsants. Decontamination measures should take place prior to patient transport to a medical center. First-responders and healthcare professionals should in turn take precautions not to intoxicate themselves through direct mouth-to-mouth resuscitative efforts (Riordan et al., 2002). They must also wear personal protective equipment when transporting the victims to areas with adequate ventilation (Rotenberg, 2003a). Clothes are an obvious source for recontamination of the victim, so they must be removed. Subsequently, the skin should be flushed with copious amounts of water. The temperature of the water becomes a major consideration for children, who may not tolerate the extremes. Depending on the hospital size, antidote kits may or may not be available. Therefore, the time when supportive care is implemented becomes extremely important.

Antidotal Therapy

The US standard cyanide antidote kit uses a small inhaled dose of amyl nitrite followed by IV sodium nitrite and sodium thiosulfate (Anon, 1998; Rotenberg, 2003a). This antidote converts a portion of the hemoglobin's iron from ferrous iron to ferric iron, converting the hemoglobin into methemoglobin. Cyanide is more strongly drawn to methemoglobin than to the cytochrome oxidase of cells, effectively pulling the cyanide off the cells and onto the methemoglobin (Berlin, 1970; Baskin and Brewer, 1997). Once bound with the cyanide, the methemoglobin becomes cyanmethemoglobin (Anon, 1998). Therapy with nitrites is not innocuous, since methemoglobin cannot transport oxygen in the blood. The doses given to an adult can potentially cause a fatal methemoglobinemia in children or may cause profound hypotension. Treatment of children affected with cyanide intoxication must be individualized based upon their body weight and hemoglobin concentration. Once an ampule of amyl nitrite has been broken one at a time into a handkerchief, the contents should be held in front of the patient's mouth for 15 s, followed by 15 s of rest. This should be reapplied using this interrupted schedule until sodium nitrite can be administered. Continuous use of amyl nitrite may prevent adequate oxygenation. Taylor Pharmaceuticals, the manufacturer of the kit, recommends the dose for children of sodium nitrite to be 6–8 mL/m^2 (approximately 0.2 mL/kg body weight), but not to exceed an adult dose of 10 mL of a 3% solution (approximately 300 mg). While excessive sodium nitrite can cause methemoglobinemia, it should be noted that in the 70-year history of using the kit, the only reported fatality of such toxicity from using the kit involved a child without serious cyanide poisoning who was given two adult doses of sodium nitrite (Berlin, 1970; Hall and Rumack, 1986). In fact, the scientific literature recommends pediatric dosing based on monitoring hemoglobin levels. The next

TABLE 68.8 Variation of Sodium Nitrite and Sodium Thiosulfate dose with Hemoglobin Concentration

Hemoglobin (g/dL)	Initial IV Dose Sodium Nitrite 3% (mL/kg) ∗Do Not Exceed 10 mL Total Dose∗	Initial IV Dose Sodium Thiosulfate 25% (mL/kg) ∗Do Not Exceed 50 mL Total Dose∗
7	0.19	0.95
8	0.22	1.10
9	0.25	1.25
10	0.27	1.35
11	0.3	1.50
12	0.33	1.65
13	0.36	1.80
14	0.39	1.95

Source: Berlin (1970), Hall and Rumack (1986), and Anon (1998).

part of the cyanide antidote kit is sodium thiosulfate, which is administered intravenously (Hall and Rumack, 1986; Baskin and Brewer, 1997; Anon, 1998; Rotenberg, 2003a). The sodium thiosulfate and cyanmethemoglobin become thiocyanate, releasing hemoglobin; thiocyanate is excreted by the kidneys. Table 68.8 provides a dosing chart for the safe dosing of sodium nitrite and sodium thiosulfate, with continuous monitoring of hemoglobin levels. Before treating pediatric patients with nitrites, it is imperative that prescribers inquire about conditions that may predispose a victim to anemia and, if there are concerns, doses should be decreased. Methemoglobin levels must be monitored sequentially in children and should not exceed 20% (Rotenberg, 2003a). Due to concerns about the excessive methemoglobinemia, along with the complicated administration procedures associated with the cyanide antidote kit, experts have suggested that alternative therapies, such as hydroxocobalamin, may be preferable to use in children (Geller et al., 2006).

Alternative Strategies

Alternative methods of treating cyanide intoxication are used in other countries. For example, the antidote used primarily in France is hydroxocobalamin (a form of vitamin B_{12}), which combines with cyanide to form the harmless vitamin B_{12a} cyanocobalamin (Baskin and Brewer, 1997; Rotenberg, 2003a). In France, this medication is used for children at a dose of 70 mg/kg. A study of 41 French children with fire smoke inhalation showed a prehospital mortality rate of 4% for those given hydroxocobalamin and not found in cardiac arrest (Geller et al., 2006). The authors of the study noted that for those children found in cardiac arrest by paramedics, administration of hydroxocobalmin did not prevent mortality. Another case series detailed 8 pediatric patients exposed to cassava, where 4 of the most severely affected children were given the cyanide antidote kit, while the others were given 500 mg of hydroxocobalamin. All the children improved regardless of which therapy they were given and were discharged from the hospital with no sequelae. This medication appears to have a good safety profile; adverse effects reported such as transient reddish-brown discoloration of the urine and mucous membranes. Some elevations in blood pressure and rash have also been reported (Geller et al., 2006). The FDA approved hydroxocobalamin for use in the United States in December 2006 to treat cyanide exposure victims in a product called Cyanokit, manufactured by EMD Pharmaceuticals, Inc. The package insert for this medication provides adult dosing and a statement that the safety and effectiveness of Cyanokit has not been established in the pediatric population. However, there is a reference to the 70 mg/kg dose that is used in Europe (Anon, 2006).

Summary

Cyanide is found in a wide variety of industrial processes and has been explored by Al Qaeda for use as a weapon of terror (Rotenberg, 2003a). Whether ingested or inhaled, cyanide is very lethal. Cyanide produces toxicity through impairment of mitochondrial enzymes, disrupting the electron transport chain, and preventing their utilization of oxygen. The mainstay of treatment of cyanide toxicity consists of the use of a multistage antidote kit. The management of children with cyanide toxicity should include appropriate antidote dose adjustments and proper monitoring to prevent fatal methemoglobinemia. Another antidote, hydroxocobalamin, may gain favor over time as the treatment of choice for pediatric cyanide exposures, due to its preferable safety profile and its ease of administration (Geller et al., 2006).

DECONTAMINATION OF CHILDREN

Decontamination after a chemical terrorist attack needs to be well-planned, efficient, and cognizant of the special needs of children. It is well-recognized that the unique vulnerabilities of children may lead to a disproportionate number of pediatric victims after a chemical attack. Without proper planning and consideration as to how children will be decontaminated, the potential for preventable pediatric casualties is increased due to time loss and confusion. It is highly recommended for pediatricians to be involved in the development of each hospital's plans for decontamination. Over the last several years, many advances have been made in the management of the critically injured child. In fact, studies have shown that children managed in a PICU have better

outcomes than children managed in an adult intensive care unit (ICU; Wheeler and Poss, 2003). Not all hospitals have the resources to have their own PICU, but they need to be able to provide the initial resuscitation and stabilization of pediatric victims of a terrorist attack. It is highly recommended that predetermined, written transfer agreements exist between emergency departments in community hospitals and centers that specialize in pediatric care. These agreements will allow the rapid transport of critically injured children to the sites that can ensure the best outcomes.

The first step in the decontamination process is the appropriate triage of patients (Burklow et al., 2003). If this step is done quickly and accurately, patients will be appropriately managed and outcomes will improve. The key to triage is the ability to ration care when resources are limited. Victims are usually classified into tiered categories. The classic categories that have been used on the battlefield include minimal, delayed, immediate, and expectant. Patients in the minimal category have minor injuries that may not require medical care or can be managed with self-care. However, it should be noted that it is difficult for children to manage themselves, in addition to the fact that the category they are placed within can change more rapidly than for adults. The delayed category describes patients who have injuries that will require medical intervention, but the injuries are not immediately life-threatening. Logically, the immediate category describes patients who are critically injured and need medical intervention to save life or limb. Finally, the expectant category describes those patients who are so critically injured that they are not expected to survive. The expectant category poses a special challenge to civilian healthcare workers, who are used to expending vast resources and personnel to maximize survival.

In a mass casualty event, clinicians need to come to grips with the fact that the most ill may not be treated. Although the classic categories of triage are fairly well known, they are not consistently used among hospitals. Some categories are developed specific to chemical attacks. An example of this are triage categories that separate patients as "exposed" and "not exposed." At the University of Maryland Medical Center, the biochemical response triage categories differentiate between exposed and not exposed individuals. Furthermore, recognizing that not all exposed individuals will necessarily be symptomatic but may still need to be isolated, the categories differentiate between those who are exposed and symptomatic, exposed and asymptomatic, and those with unrelated emergent conditions. Regardless of what categories get utilized, triage must focus on the fact that the best outcome is achieved for the greatest number of victims. To achieve this outcome, appropriate identification of the causative agent is critical. This can be a challenge because often, full identification is delayed. To protect those involved in triage, full personal protective equipment is highly recommended. Working in full personal equipment can be cumbersome and uncomfortable, but when triage is done correctly, unnecessary decontamination can be avoided.

After triage, the decontamination process should begin (Wheeler and Poss, 2003). All workers who are involved in this process must be appropriately protected with butyl rubber aprons and gloves, double layers of latex gloves, waterproof aprons, and chemical-resistant jumpsuits. Personal protective equipment should also include an appropriately selected air-purifying or atmosphere-supplying respirator, depending upon how well the threat environment has been categorized. It is important to note that this equipment often needs to be changed to prevent healthcare worker exposure.

The setup and use of the decontamination area must be carefully thought out. Often, the area is split into different zones (Rotenberg et al., 2003). At a minimum, there must be a dirty contaminated zone and a clean decontaminated zone. It is critical to emphasize that traffic must go one way between zones. This will eliminate the possibility of a cleaned patient becoming cross-contaminated or an exposed patient entering a healthcare facility before being decontaminated. Security personnel must be utilized to make sure that patients do not consciously or unconsciously violate the rules. A secondary triage will be needed as patients enter the clean zone to allow patients to receive antidotes or be referred for further care. Keep in mind that for severely ill patients, antidote administration may precede decontamination.

The selection of the appropriate decontamination agent is important. The gold standard for decontamination is plain water (Rotenberg et al., 2003). Other agents that have been used for decontamination include carbonaceous adsorbent powder, dilute (0.5%) hypochlorite solution, water with soap, and dry decontaminants such as flour or talcum powder. For children, the use of water or water with soap is preferred. In addition to agents used to decontaminate, other cornerstones to management include exposure to fresh air (when patients have been exposed to chemicals in the gaseous form), a change of clothing, and showers.

Conducting decontamination in children can be especially difficult. At every step of the process, special considerations need to be addressed (Rotenberg et al., 2003). Starting at triage, clinicians need to understand how chemical toxicities manifest in children; also, an understanding of what normal vital signs should be for a child will be critical. Pediatric-specific triage tools often consider different vital signs, such as heart rate

and respiratory rate parameters and the varying ability of patients to communicate. It is important for the triage to include the examination of a child's mouth and eyes because of frequent hand-to-mouth and hand-to-eye activity. If antidote administration is needed, pediatric references should be readily available, and an understanding of pediatric doses will be needed. When there is a lack of experience with managing children, the otherwise efficient decontamination process can get bogged down. Some hospitals have decided to set up pediatric-specific areas to address the specific needs of children.

Clinicians may need to handle uncooperative or nonverbal children. This becomes especially challenging when an IV line needs to be started. Placing a line in a child while in full protective equipment is no small feat. Also, keep in mind that the unfamiliar presence of a clinician in full personal protective equipment can cause fear and distress in a child. Children undergoing decontamination will benefit from a parent or guardian to guide them through and reassure them. For those children who present alone, a guardian will need to be appointed and a system for parental identification will be needed. Hospitals will need to plan for this extra resource. In fact, one Israeli hospital has employed social workers to participate in their disaster preparation to help manage patient/family needs and psychological distress (Rosenbaum, 1993). It is recommended not to separate parents and children during a time of crisis. Plans should be made for the decontamination and treatment of parent–child pairs (Rotenberg et al., 2003).

A range of specially sized supplies is needed to appropriately manage children, which range from pediatric-sized emergency equipment to basic needs, such as formula for feeding and diapers. Since decontamination often includes disrobing, child-sized clothing would be needed. For children who may need to be observed for hours, toys will be needed.

Also, the agents used to decontaminate children should be carefully selected. Bleach or hypochlorite solutions are not recommended for use with children due to the possibility of skin irritation or damage (Rotenberg et al., 2003). Water is the gold standard for decontamination. When employing water in decontamination, the temperature of the water must be considered. Children, especially newborns and infants, are prone to hypothermia and hemodynamic instability from cold water. Water at a comfortable temperature is recommended, along with a good supply of blankets that can be used to quickly warm up pediatric patients after water decontamination. In some situations, indoor sprinkler systems have been used when outdoor conditions were inhospitable.

PREPARATION FOR A CHEMICAL EVENT

Understanding chemical agents used for terrorism and knowing how to manage toxicity is just the first step in preparing for a chemical event. Appropriate training on how to manage pediatric patients in these scenarios is critical. Pediatricians are uniquely trained to participate in the management of pediatric casualties and to advocate for children so that their needs are addressed in emergency planning (Bradley et al., 2003). Many hospitals have held emergency exercises to see how prepared they are for these situations. Beyond this, the assessments should identify deficits and should be used to forge partnerships and relationships and share assets in the community to manage every possible scenario (Blaschke et al., 2003). Healthcare facilities responsible for treating pediatric victims in a chemical–biological event could be easily strained and overwhelmed. Often large-scale chemical–biological incidents necessitate the use of alternative areas to triage patients such as auditoriums and arenas. These alternative triage areas need to know how to manage pediatric victims (CEH/CID, 2000). Planning for an attack begins with the development of local health resources. Unfortunately, with chemical releases, clinical effects can occur extremely quickly, limiting the amount of time available to borrow resources from nearby communities. First-responders must be educated to recognize pediatric signs and symptoms from each chemical agent, how to wear protective gear in the face of persistent agents, handle pediatric patients, and be able to manage field decontamination. It is critical that adequate supplies of protective gear are available. When planning for decontamination procedures, pediatric vulnerabilities and challenges need to be considered, such as the temperature of the water and the ability of children to follow directions.

Since children spend the majority of the day at school, community preparation for a threat must include the local educational system. Development of a rapid evacuation plan and the establishment of in-school shelters are critical. Schools can play a valuable role for the management of pediatric casualties.

Another key element to appropriate preparedness is the development of a pharmaceutical cache of antidotes, antibiotics, and vaccines. This cache will play a key role in the initial management of a chemical attack. Even though the SNS is now in place throughout the United States, it may be several hours before it reaches a hospital and the supply is divided among several sites. The SNS has made efforts to include pediatric-ready medications, such as suspensions and solutions. Efforts must be made for local pharmaceutical caches to address pediatric needs. An example of a pediatric pharmaceutical cache is displayed in Table 68.9.

TABLE 68.9 Example Pediatric-Specific Hospital Emergency Drug Cache

Drug	Strength	Dosage Form	Pediatric Dosing	Therapy or Prophylaxis	Disease
Albuterol MDI	17 gm	INH	2–4 puffs q4h	Respiratory distress from chemical agents	Chemical exposure
Amoxicillin oral suspension	400 mg/5 mL 100 mL	Oral suspension	15 mg/kg q8h—up to 40 kg, >40 kg 500 mg q8h	Chemoprophylaxis	Anthrax
Atropine	1 mg/mL	Injection	See dosing table	Chemotherapy	Nerve agent exposure
Ciprofloxacin oral suspension	250 mg/5 mL 100 mL	Oral suspension	20–30 mg/kg/day divided q12h for 60 days	Chemoprophylaxis	Anthrax, plaque
Clindamycin	600 mg/NS 50 mL	IVPB	30 mg/kg/day q8h (max 4.8 g/day)	Chemotherapy	Anthrax
Cyanide antidote package	1 kit	Kit	See dosing table	Chemotherapy	Cyanide poisoning
Diazepam IV	5 mg/mL × 2 mL	Injection	See dosing table	Seizures post chemical exposure	SZ postchemical exposure
Doxycycline oral suspension	25 mg/5 mL 60 mL	Oral suspension	2.5 mg/kg q12h—up to 40 kg, >40 kg 100 mg q12 h for 60 days	Chemoprophylaxis	Anthrax, cholera, brucellosis, plague
Oseltamivir suspension	12 mg/mL 25 mL	Suspension	For children ≥1–12 yrs: ≤15 kg: 2 mg/kg/dose (max 30 mg) BID × 5 days, >15–23 kg: 45 mg/dose BID × 5 days, >23–40 kg: 60 mg/dose BID × 5 days, >40 kg 75 mg/dose BID × 5 days	Chemotherapy	Avian influenza
Potassium iodide	65 mg	Tablet	4–18 yrs: 65 mg, 1 mo–3 yrs: 32.5 mg, <1 mo: 16.25 mg	Chemotherapy	Radiation emergency
Pralidoxime	1 gm/20 mL SDV	Powder for injection	See dosing table	Chemotherapy	Nerve agent exposure
Ribavirin solution	40 mg/mL 100 mL	Solution	LD 30 mg/kg followed by 15 mg/kg/day BID × 10 days	Chemotherapy	Viral hemorrhagic fever

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Much progress has been made in understanding how pediatric patients need to be managed when they are affected by chemical agents. Several pediatric organizations, such as the AAP, have given guidance on how best to handle these situations. It is a special challenge to gather information regarding pediatric chemical casualties because our experience is so limited. Further research and resources are needed to fully understand all the physical and psychological effects that a terror attack has on children. The intention of this chapter is to provide a framework from which local and national efforts can be developed. In the event of a chemical attack, prior preparation and planning will make the difference to whether lives are saved or further lives are lost. Efforts to improve upon current recommendations for managing pediatric chemical casualties must continue in order to better protect this vulnerable population.

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CHAPTER

69

Physiologically Based Pharmacokinetic/ Pharmacodynamic Modeling of Countermeasures to Nerve Agents

Elaine Merrill, Chris Ruark, Jeffery Gearhart and Peter Robinson

INTRODUCTION

Physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling has proven useful in many areas of toxicology and therapeutics. This quantitative, mechanism-based approach has allowed limited experimental *in vivo* and *in vitro* data to be quantitatively integrated with physiological data to facilitate predictions of the behavior of organisms under different exposure conditions. Specifically, it has been used for:

- Extrapolation across species (particularly experimental animals to humans)
- Extrapolation across dosing scenarios (such as high to low doses)
- Extrapolation across routes of exposure

Models have been developed for a number of chemical warfare (CW) agents (Langenberg et al., 1997; Worek et al., 2005; Aurbek et al., 2006; Sweeney et al., 2006; van der Merwe et al., 2006; see also Chapter 58) and surrogates, including diisopropyl fluorophosphate (DFP) (Gearhart et al., 1990; 1994), diazinon (Poet et al., 2004), and chlorpyrifos (Timchalk et al., 2002a,b). In the case of agent countermeasures, PBPK/PD modeling also allows the prediction of therapeutic efficacy under varying exposure scenarios. Specifically, it may allow therapeutic regimens to be optimized to particular exposure scenarios (routes, exposure times, concentrations). To do so, PBPK/PD models of individual agents and countermeasures must be combined to take into account their interactions, whether they are pharmacokinetic or pharmacodynamic. Such modeling of agent or (multiple) counteragent combinations is an example of mixtures modeling (Krishnan et al., 1994).

There is little in literature approaching a complete model of the kinetic and dynamic interaction of nerve agent (NA) and its current (multiagent) or potential countermeasures. There have been, however, a number of important steps in that direction, both on the level of model development and in the experimental generation of data useful for the development and validation of such models.

We restrict our discussion here to countermeasures of NAs and do not discuss other chemical agents such as mustards and cyanide and their antidotes. However, the modeling ideas outlined here can also apply to other agents.

BACKGROUND

Organophosphate compounds (OPs), such as the NAs, sarin (GB), soman (GD), tabun (GA), and VX, exert their toxicity via inhibiting acetylcholinesterase (AChE). As a result, Acetylcholine (ACh) accumulates in the synapses and neuromuscular junctions, resulting in hyperstimulation of muscarinic and nicotinic cholinergic receptors. Clinical symptoms include salivation, lacrimation, urination, defecation, and possibly convulsions (Myhrer et al., 2007). At moderate to high doses, seizures rapidly progress to *status epilepticus* (SE), which can lead to irreversible brain damage or death (Filliat et al., 1999; Carpentier et al., 2001). Survivors suffer long-lasting cognitive deficits and behavioral changes (McDonough et al., 1995) largely attributable to neuronal degeneration of hippocampal structures. Under acute high-level

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exposures, the focus of countermeasure treatment is the prevention of seizures, respiratory failure, and bradycardia due to convulsive spasms. For low-level exposures, and for the survivors of high exposures, the concern shifts to minimizing the cognitive impact of the agent that may or may not be related to the degree of seizure activity.

McDonough and Shih (1997) hypothesized that NA-induced seizures follow three main phases. Initially, approximately 5 min after exposure, hypercholinergic activity triggers the seizure. A transitional phase of cholinergic and glutamatergic hyperactivity follows as a self-sustaining glutamate–NMDA receptor (NMDAR)-mediated positive feedback loop begins to develop. After approximately 40 min, a predominantly glutamatergic phase sustains seizures, leading to neuronal damage predominantly in the hippocampus, amygdala, piriform cortex, thalamus, and entorhinal cortex across species (Olney et al., 1983; Wade et al., 1987; Carpentier et al., 1990; Fosbraey et al., 1990; Lallement et al., 1992; Solberg and Belkin, 1997).

High levels of glutamate are directly neurotoxic because overstimulation of glutamate ionotropic receptors allows excessive influxes of Na^{2+} and Ca^{2+}, causing prolonged depolarization of postsynaptic membranes (Bittigau and Ikonomidou, 1997). A cascade of events results from the delayed Ca^{2+} overload, leading to excitotoxic cell necrosis (McDonough and Shih, 1997; Solberg and Belkin, 1997). In addition, as the time lapse before anticonvulsant treatments increases, the Ca^{2+} influx induces transcription factors that mediate significant increases in damaging pro-inflammatory peptides, such as IL-1β, IL-6, and mRNA of TNF-α (Chapman et al., 2006).

The content of this chapter is not inclusive in the sense of taking one through the complete development and validation of a complex PBPK/PD model, which would describe the kinetics of each component, together with the pharmacodynamic interactions between selected NAs and countermeasures. Rather, this chapter briefly describes some examples of progress made in quantitative modeling and exploring how specific countermeasures interfere in this NA-induced cascade of events, and how such quantitative approaches could be used to develop improved treatment regimens.

CURRENT COUNTERMEASURES

Conventionally, NA poisoning is treated by a combination of prophylactic and post-exposure therapy, which target the three post-exposure phases of neurotransmitter systems described. Prophylactic treatments are designed to circumvent aging of the NA–AChE complex and consist of carbamate anticholinesterases (e.g., pyridostigmine) to bind AChE reversibly. Current carbamate pretreatment regimes bind 30% to 40% of available red blood cell (RBC) AChE, thereby protecting some of the enzyme from irreversible OP binding (McDonough and Shih, 1997). Carbamates, however, are not without side effects. Partial AChE blockage by pyridostigmine results in a transient overstimulation of the AChR, mimicking mild NA poisoning with nausea, diarrhea, shortness of breath, and dizziness (Abraham et al., 2002). Moreover, repeated prophylactic administration of carbamates is associated with detrimental and debilitating changes in nerve and muscles function (Hudson et al., 1986).

Standard post-exposure treatments include concurrent administration of anticholinergics, such as the muscarinic cholinergic blocker atropine sulfate, and AChE reactivators, such as obidoxime and pyridine-2-aldoxime methylchloride (also known as 2-PAM). Oximes cannot reactivate OP-inhibited AChE that has already "aged." Therefore, traditional oxime treatment is considered to be less effective for those agents such as soman, for which aging is rapid (Worek et al., 2005).

If seizures develop, then treatments that attempt to attenuate the hyperglutamatergic phase are required. Anticonvulsants, such as the benzodiazepine, diazepam, may be used. Diazepam is a lipophilic agonist of the γ-aminobutyric acid A ($GABA_A$) receptor, the most predominant inhibitory receptor of the CNS. The $GABA_A$ receptor is ubiquitously distributed. Therefore, its activation or inactivation affects virtually all brain regions, making dosage critical. Treatments with traditional benzodiazepines depend on administration within a very narrow time span to be effective because profound neuropathological changes are usually detected within 20 min of the onset of seizures (Lallement et al., 1994; Leadbeater et al., 1985; McDonough et al. 1995; Shih et al., 1999). By themselves, benzodiazepine anticonvulsants provide limited protection, and seizures recur with desensitization of $GABA_A$ receptors (Goodkin et al., 2005). In addition, diazepam has consistently failed to eliminate the neuropathological changes resulting from NA-induced seizures (McDonough and Shih, 1997). Furthermore, in many battlefield and civilian exposure scenarios for NAs, pretreatments are unrealistic and post-exposure anticonvulsants are likely to be delayed. Epidemiological studies have shown that time to seizure treatment in the United States varies broadly, with only approximately 41% of all patients receiving their first antiepileptic drug within 30 min (Pellock et al., 2004).

NOVEL COUNTERMEASURES

Because of the undesirable side effects of the standard treatment regimes, and because of limitations of traditional benzodiazepines to adequately control seizures, the

need for alternative therapeutics still exists. The focus of OP intoxication treatment is also expanding from immediate survival to include the prevention of long-term cognitive deficits. Other antidotes have been proposed that demonstrate promise in reducing excessive glutamatergic activity and/or prevent destabilization of Ca^{2+} homeostasis, because excitotoxic mechanisms of seizure activity ultimately result in Ca^{2+} overload and activate various enzymes that lead to necrosis of the cell. Attempts have been made by blocking the *N*-methyl-D-aspartate (NMDA) or alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, two subtypes of CNS glutamate receptors (Braitman, 1989; Lallement et al., 1994; McDonough and Shih, 1995; Raveh et al., 2003). NMDA-type glutamate receptors are glutamate-gated cation channels whose permeability to Ca^{2+} underlies aspects of normal synaptic plasticity. Excess Ca^{2+} influx through NMDARs mediates cell death in certain neurodegenerative pathologies. Therefore, neurons must precisely control NMDAR synaptic density, which is negatively regulated by global neuronal activity.

A number of glutamate receptor antagonists have demonstrated neuroprotective and anticonvulsive effects in animals (Urbanska et al., 1998). The high-affinity NMDAR antagonist, ketamine, has shown promise when administered intramuscularly with atropine sulfate up to 30 min after poisoning (Dorandeu et al., 2005). Other noncompetitive NMDA antagonists, like dizocilpine (MK-801) and N-(1-[2-thienyl]cyclohexyl)3,4-piperidine (TCP), display the ability to terminate seizures when treatment is delayed by 40 min but seriously decrease respiration (Carpentier et al., 1990; Shih, 1990; Shih et al., 1991; McDonough and Shih, 1997; Shih and McDonough, 1997).

An unusual NMDAR antagonist that recently demonstrated potent neuroprotection against GD-induced seizure-related brain damage in rats is dexanabinol. Dexanabinol is a nonpsychoactive synthetic cannabinoid that acts as a highly selective, low-affinity NMDAR antagonist (Feigenbaum et al., 1989; Eshhar et al., 1993). In GD-exposed rats ($1.6 \times LD_{50}$ subcutaneous), dexanabinol administered intraperitonealy at either 5 or 40 min after the onset of seizures reduced median brain lesion volume 86% and 81%, respectively (as assessed by microtubule-associated protein 2 [MAP2]-negative staining) (Filbert et al., 1999). When administered 5 min after seizure onset and repeated every 6 h up to 24 h, 98% reduction was seen. However, it is not known if the same degree of protection would be afforded if measurements were taken later than 24 h after seizure onset. Dexanabinol did not diminish seizure activity in any of the GD-treated rats, suggesting that its neuroprotective effects may be due to its properties as a potent antioxidant and free radical scavenger, as well as its ability to inhibit the production of tumor necrosis factor-alpha (TNF-α) (Shohami et al., 1997), thus also blocking the signal transduction pathway that activates nuclear factor B (NFB).

Data to date also indicate that enzyme-based bioscavengers show much promise for the next generation of pretreatments or antidotes, with potentially no observable side effects. Candidate bioscavenger proteins either bind and sequester toxic OPs (such as serum cholinesterases and carboxylesterases) or catalytically break down the OP into nontoxic products (such as human serum organophophorus acid anhydride hydrolases [OPAH] or paraoxonase [HuPON]). These scavengers, as well as carboxylesterase (CarbE), are each capable of providing protection against 2–16 × LD_{50}s of GD, GB, or VX, depending on the scavenger and the test species (rat, mouse, rabbit, guinea pig, or rhesus monkey), with little to no deficits in behavioral testing (Li et al., 2005). Human clinical trials of several bioscavengers are now underway that could lead to FDA licensure.

Human serum paraoxonase (HuPON1) is a Ca^{2+}-dependent enzyme that effectively hydrolyzes many toxic AChE-inhibiting organophosphates (Blum et al., 2008). HuPON1 is present in high-density lipoproteins of human plasma and plays an important role in protecting lipoproteins and cell membranes from oxidative damage. Because it is a human enzyme (Li et al., 2005), it could potentially be used as either a prophylaxis or medical post-treatment for NA exposure, without causing adverse immune reactions (Draganov and LaDu, 2004). Human PON1 has catalytic activity against the pesticide paraoxon and the NAs sarin (GB), soman (GD), and cyclosarin (GF), but shows only minimal activity against the nerve agent VX (Blum et al., 2008). The advantage of catalytic scavengers is the very small amount of the exogenous enzyme needed to hydrolyze and detoxify large amounts of NA.

PBPK/PD MODELING

For obvious ethical reasons, only animal experiments can be used to evaluate new NA antidotes. However, the extrapolation of data from animals to humans is hampered by marked species differences. Currently, quantitative analyses addressing the co-treatment of countermeasures and medicinal treatments simultaneous with or immediately preceding or after NA exposures have been very limited. The current paradigm thus relies on experimentation in various animal models to determine efficacy and then, following the classical model for pharmaceutics, scales the animal results to the human exposure scenario. In the case of NA countermeasures, short of controlled human experimental studies, this leaves a large uncertainty factor regarding the protection of military personnel from NA threats and may severely limit or compromise operational risk

management (ORM). One solution to this dilemma is to develop validated mathematical models to predict the biological impact of simultaneous and sequential low-level exposures to specific NAs, together with specific countermeasures, countermeasure combinations, and dosing regimens.

PBPK/PD models can be used to explore the interactions between agents, countermeasures, and the organism to integrate multiple animal (*in vivo* and *in vitro*) and human (typically *in vitro*) data sets and to develop tools for countermeasure design and dosing regimen selection to maximize countermeasure administration efficacy. The models make quantitative use of differences in physiological parameters between test animals and humans, such as organ blood flow. The result of this approach is the development of an interaction model quantifying competition for AChE binding sites between specific agents and countermeasures, as well as other relevant pharmacodynamic and pharmacokinetic modes of interaction and the development of methods to optimize countermeasure therapies for specific NA exposure scenarios in humans.

The process of applying mathematical constructs to describe experimental results often reveals patterns in the agent's pharmacokinetics or dynamics that might not otherwise be discernable. Failure of a model's simulations to predict experimental measurements sometimes prompts questioning of the data, such as the reliability of the quantitative methods, sample collection, or exposure techniques. More often, it may indicate that greater complexity in the structure of the model is required to capture the behavior of the data. This is another primary reason for developing models, that is, to create hypotheses (model structures) that are falsifiable, leading to improved models and improved predictions in an iterative process.

There are currently a number of critical toxicological data gaps related to NA exposure at both acute high levels and repeated low levels. Many of these gaps are due to the impossibility of conducting controlled human experiments involving NA exposure. Filling these data gaps for exposure to NAs is essential for predicting performance degradation of personnel, enhancing risk assessment modeling tools, and defining detection thresholds that are physiologically relevant. The purposes of filling such data gaps are to develop a valid method for predicting dose–response effects for exposures to low NA agent concentrations/doses over long durations, and to identify threshold exposure conditions at which toxicologically significant effects occur. PBPK/PD modeling provides a means to fill these gaps. Because of the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route in a particular species to develop and validate the PBPK model, and then that model can be used to simulate and predict the kinetics and pharmacodynamics in another species, such as the human exposed via another exposure route. The power of PBPK/PD models thus lies in aiding the ability of scientists and decision-makers to make reliable quantitative predictions concerning the potential health effects of real-life human exposures. In addition, this modeling approach can be useful for the development of meaningful therapeutic animal models. After all, therapeutics that have been successfully tested in animals can ultimately be tested in human clinical trials. However, their therapeutic efficacy against NAs can only be tested in animals.

DEVELOPMENT OF PBPK/PD MODELS

Critical components to PBPK models include species-specific physiological parameters and chemical-specific parameters. Species-specific physiological parameters include the organ weights and blood flow rates for

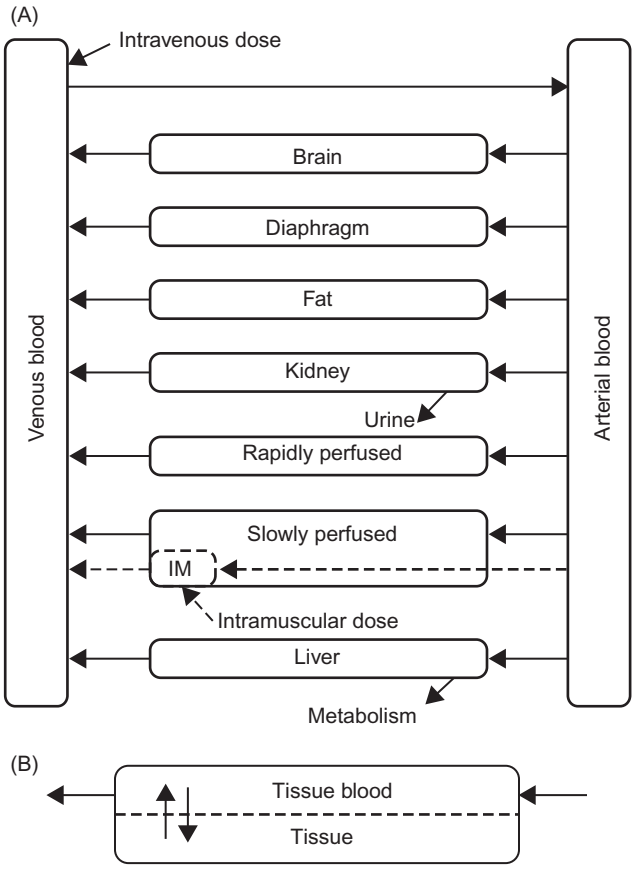

FIGURE 69.1 PBPK model schematic of the distribution of the oxime TMB-4 in rats and humans. Each tissue compartment depicted in schematic A includes diffusion limitation as shown in schematic B (Sterner et al., 2013).

the defined compartments in the PBPK model. These values are most often available in the published literature (Brown et al., 1997; Peeters et al., 1980) and, when lacking, can be derived by appropriate scaling factors from similar species. Chemical-specific parameters may include tissue to blood partition coefficients and metabolic rate constants, which may be measured experimentally or predicted, as described in the next section on quantitative structure–activity relationship (QSAR) methods. The basic structure of a PBPK model used to describe pharmacokinetics and pharmacodynamics of a compound is similar to the model presented in Figure 69.1, which predicts the absorption, distribution, metabolism, and excretion (ADME) of the oxime, TMB-4 (Sterner et al., 2013).

Although PBPK models can be used to predict ADME, they can be expanded to quantitatively describe the compounds pharmacological mechanism(s). Figure 69.2 shows a schematic representation of the pharmacodynamic processes involved in the interactions between NAs such as sarin with the organism (via AChE inhibition), together with interactions with pretreatments (pyridostigmine bromide [PB]) and countermeasures (oxime, atropine, and diazepam). When this PD submodel is linked to the PBPK model via the simulated concentration of a particular NA or therapeutic in a selected target tissue (i.e., plasma, RBCs, diaphragm, and brain), it provides a framework for describing these biochemical processes quantitatively. Linking it with the PBPK model also puts the biochemical processes into a physiological context. This allows extrapolation of results to multiple species, to description of relevant exposure scenarios to address potential health effects, and to optimization of pretreatment and countermeasure delivery.

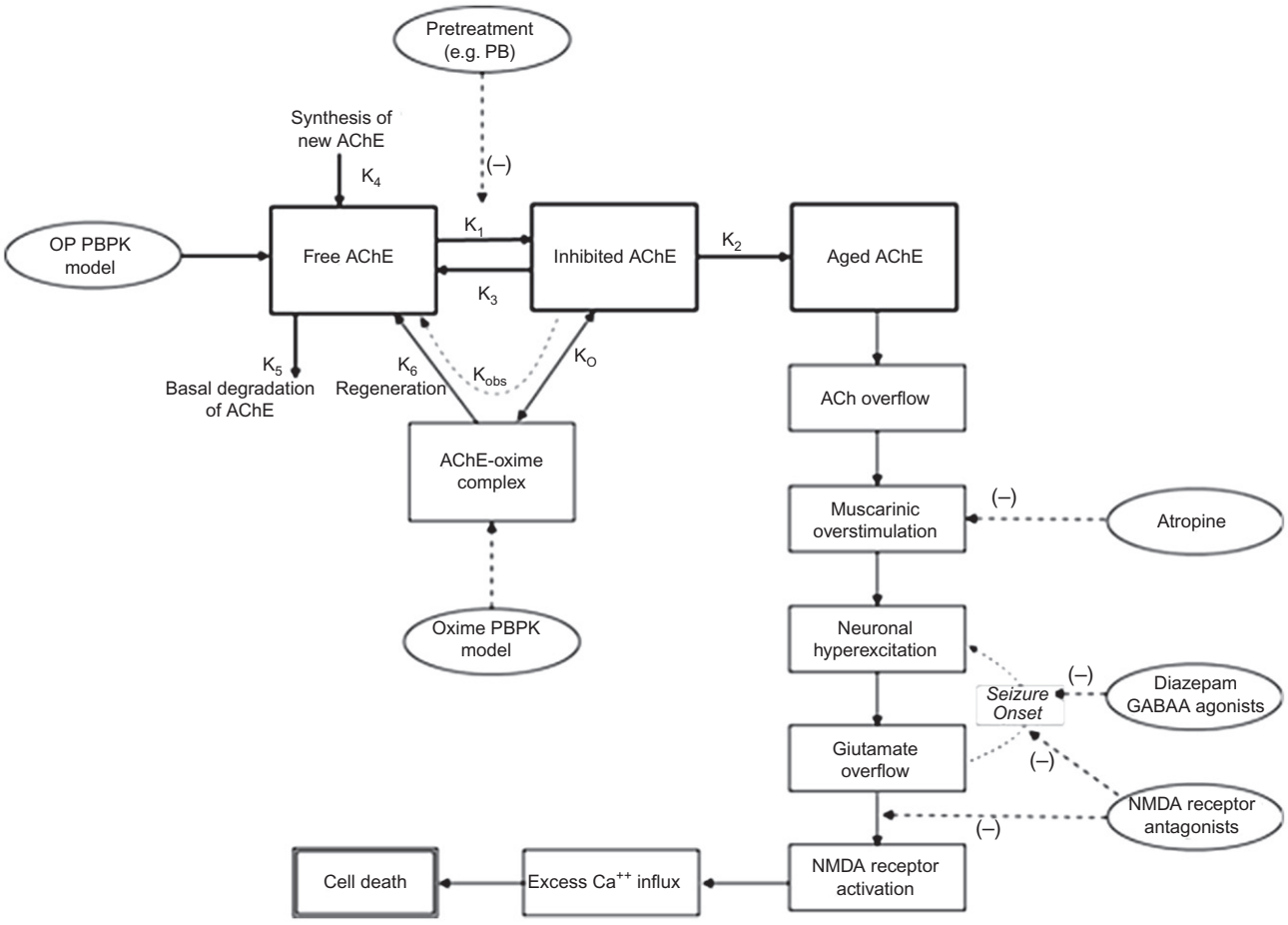

FIGURE 69.2 Schematic for a PD model of AChE inhibition and aging by OPs such as sarin, together with the interaction of pretreatment (pyridostigmine bromide [PB]), and countermeasure treatment (oxime). Pyridostigmine acts as a reversible cholinesterase inhibitor, oxime acts as an AChE regenerator. k_{obs} is an "effective" first-order rate that can be derived as: $k_{obs} = (k_r * [Ox])/(K_D + [Ox])$, when [Ox]≫[Inhibited AChE]. Potential links for the countermeasures atropine, a muscarinic receptor antagonist, and diazepam, a positive allosteric modulator of the $GABA_A$ receptor and NMDA antagonists, are shown without detailed kinetic interactions.

EXPERIMENTAL AND QSAR METHODOLOGIES TO PREDICT BLOOD AND TISSUE PARTITION COEFFICIENTS

As shown in Figure 69.1, PBPK modeling utilizes mathematical compartments that conceptually represent the physiology of an organism to depict the pharmacokinetics of chemicals exposed to the body. Traditional PBPK models depend on knowing the partitioning of the agent in key tissues of the organism. The distribution of a chemical within a tissue/compartment is most commonly represented by the following Eq. (69.1) (Krishnan and Andersen, 2001):

$$\frac{dA_t}{dt} = PA\left(CV_t - \frac{C_t}{P_t}\right) \tag{69.1}$$

where A = amount of chemical in the tissue, PA = permeation area cross product for the tissue, CV_t = concentration in venous blood emerging from the tissue, C_t = concentration in tissue, and P_t = tissue–blood partition coefficient. Note that $A = C_t \times V_t$, where V_t is the volume of the tissue.

PBPK modeling relies heavily on a proper definition of the partition coefficient because this parameter drives chemical uptake into the tissue. It is generally identified for PBPK modeling as the concentration ratio of the fraction in the tissue to the fraction in the blood at equilibrium (Schmitt, 2008), as shown in Eq. (69.2).

$$P_t = \frac{C_{\text{Tissue}}}{C_{\text{Venous}}} \tag{69.2}$$

The vial equilibration method is the most common *in vitro* method for determining partition coefficients for volatile or semivolatile materials and has been used most successfully for volatile organic solvents (Gargas et al., 1988). Tissues are harvested from the species of interest and incubated with the test compound until equilibrium is reached between the tissue and the headspace in the vial. The blood/air or tissue/air partition coefficients are given by the ratio of the concentrations of the chemical in the blood or tissue relative to its concentration in the headspace. Tissue–blood partition coefficients are calculated from the respective tissue/air and blood/air values. A number of operational equations have been derived to calculate these ratios under specific experimental conditions. Time to steady state is critical and should be optimized for the test compound. Metabolism of the compound in exposed tissue samples must be controlled. Analysis is performed by gas chromatography in a verified linear range. Human tissues can be obtained from tissue bank organizations to provide species specificity to models developed with human data. To estimate partitions for compounds of low volatility or nonvolatility, the *in vitro* filtration method of Jepson et al. (1994) can be used. Limitations of all such *in vitro* methods include issues of how representative the tissue sample is of the often quite heterogeneous tissue in the animal, and how closely the *in vitro* tissue preparation approximates the *in vivo* state. In addition, for highly lipophilic compounds, issues related to adsorption of the material onto glass and other surfaces need to be addressed.

Identifying the partition coefficient of a chemical for PBPK modeling is difficult because of the fact that most experimental methods fail to address this proper definition. *In vivo* methods most commonly track the kinetics of the chemical (radio-labeled or not). However, if enzymes are not properly inhibited, then metabolites can also be tracked instead of the parent compound, leading to contamination of the measured partition coefficient by the more water-soluble metabolite. Experiments are most commonly performed via a single dose at different routes of exposure, rarely leading to the steady state. To reach steady state, a critical point at which measurements should be made, a constant intravenous infusion of a chemical over an extended period of time until the concentration in the blood and tissue becomes constant must be made. Finally, protein binding is most commonly not accounted for, leading to a total concentration measured instead of the available free fraction. Compounds bound to proteins such as albumin, globulin, and lipoproteins reduce a chemical's tendency to partition into or out of tissues. This can be an extremely important issue depending on the chemical because some are upwards of 90–95% bound in the blood (Poulin and Krishnan, 1996a). These *in vivo* experimental issues are rarely addressed, which leads to utilization of *in vitro* measurements where there is greater control of the system.

Because experimental determinations of partition coefficients are slow and expensive, and are fraught with difficulties, particularly for NAs, an alternative is to predict tissue partitioning from more readily available chemical properties (particularly chemical structure), together with key biological properties of the tissue itself, such as its lipid composition, by means of QSAR (Ruark et al., 2008; Sterner et al., 2008). The pharmaceutical industry has utilized QSAR to obtain information of new drug candidates rapidly to help alleviate bottlenecks in the discovery process. A drawback to these models is that they have limited predictive power for scaling across species and to other compounds. However, by utilizing novel mechanistically based methods specifically suited to PBPK modeling, estimations of chemical warfare nerve agent partition coefficient properties can be developed.

Tissue distribution is predicted utilizing a variety of descriptors that range from both chemical-specific to tissue-specific, allowing for universal application of the methods across all species, tissues, and chemicals. A compound's

structure and their physiochemical properties, such as the log of the octanol:water distribution (LogP) and the octanol:water distribution mixture of different ionic forms (LogD), are commonly utilized in partition coefficient QSAR, or quantitative structure–property relationships (QSPR) models because they describe the relative affinity for a chemical between hydrophilic and hydrophobic phases. Octanol, being amphiphilic, represents the phospholipid bilayer, which provides a barrier for the cell, separating the extracellular from its intracellular components. However, many studies have shown that octanol is not a perfect surrogate for all lipids within organisms and many are beginning to pursue other surrogates such as vegetable oil and specific phospholipid membranes (Poulin et al., 1999; Schmitt, 2008). Other relevant properties include a wide range of descriptors predicted from *ab initio* calculations such as H-bonding acidity and basicity parameters, polarity, polarizability, molar refraction, McGowan volume, energies of the lowest unoccupied molecular orbital (E_{lumo}) and the highest occupied molecular orbit (E_{homo}), the maximum positive and negative atomic charge ($\sum Q$), the solvation of free energy (ΔG), as well as many others (Zhang and Zhang, 2006).

As far as tissue characterization is concerned, the fractions of lipid and water tend to be the dominating factors that influence partitioning into or out of the tissue. Some QSPR studies have included proteins that account for an electrostatic interaction with the amino or carboxyl terminal ends of an ionized chemical at physiological pH, as well as some nonspecific binding for neutral compounds. However, a recent QSPR model developed by Schmitt (2008) identified that nonspecific binding to proteins is approximately 40-fold weaker than to lipids. Therefore, proteins may only become of importance for tissues such as muscle and lung that contain a greater fraction of proteins than that of lipids. There have also been attempts at differentiating between phospholipids and neutral lipids (Poulin and Krishnan, 1995a,b; 1996a–c; Poulin and Theil, 2000, 2002; Poulin et al., 2001). Rodgers and Rowland (2006, 2007) later differentiated between acidic and neutral phospholipids to account for another electrostatic interaction with ionized chemicals within the tissue. There is also a need to distinguish between tissue and interstitial space, because the interstitial space is highly representative of blood plasma. Other tissue components that may be of interest include DNA, RNA, and intracellular organelles. Binding to DNA appears to be highly correlated with β-blockers; however, the fraction of DNA in tissue is too low to make a substantial difference in the current predictions (Rodgers et al., 2005a,b). Some authors have also suggested differences in intracellular organelle pH as another means of accounting for differences in tissue partitioning for ionized chemicals (MacIntyre and Cutler, 1988; Ishizaki et al.,1996; Daniel and Wojcikowski, 1999; Siebert et al., 2004); however, the overall volume of these organelles in relation to the cytoplasm is very low, most likely not resulting in substantial differences.

Optimization of predictions can be made utilizing linear as well as nonlinear relationships by means of statistical methods to correlate chemical and physiological descriptors to experimental data sets. These statistical methods include multilinear partial least-square analysis, principal component analysis, and neural networking. Many of these tools are included in QSPR/QSAR packages through companies such as Advanced Chemistry Development, SemiChem, EduSoft, BioByte, TOPKAT, MDL, ChemSilico, Pallas, Pharma Algorithms, and others.

A review of the literature will reveal that several QSPR equations have been developed to predict PCs of drugs for use in PBPK models and many are suitable for a wide variety of chemicals. It appears that the greatest deviations in predicted versus experimental measurements of PCs are largely due to an experimental uncertainty or misinterpretations of the data rather than incorrectness of the models. Strategies still need to be developed for other macromolecular binding (ά-acid glycoprotein) and processes such as blood–brain barrier (BBB) permeation and active transport, but it appears that the methods thus developed are suitable for preliminary PBPK modeling.

INTERACTION PBPK/PD MODEL FOR NAS AND COUNTERMEASURES

As stated, a number of PBPK/PD models have been developed for individual nerve agents (sarin, VX, soman, and cyclosarin) in multiple species. Chapter 58 in the current volume discusses the development of such models. Standalone PBPK or compartmental models have also been developed that describe the pharmacokinetics of certain countermeasures, such as diazepam (Igari et al., 1983; Gueorguieva et al., 2004) and oximes (Stemler et al., 1990; Sterner et al., 2013). However, to date, few models for specific countermeasures have been harmonized and linked to NA PBPK/PD models to be able to quantitatively describe their pharmacokinetic and pharmacodynamic interactions. This is partly due to the fact that most PBPK/PD models developed for NAs and other OPs focus on the inhibition of ChEs as the critical endpoint. The lack of a mathematical description of the disruption of other complex biochemical pathways presents a problem for linking these NA models to those of many countermeasures. For example, the conventional NA countermeasures, atropine and diazepam, as well as many novel countermeasures, do not directly impact ChE kinetics because they act at sites distinct from the active site of the esterases, such as muscarinic, $GABA_A$, or NMDARs (Figure 69.2).

Interactive models that have been developed and validated exist for countermeasures that compete with

TABLE 69.1 Rate of Activation of Inhibited Erythrocyte ChE by Various Oximes and Hydroxmic Acids

	Inhibitors		
Compound	**TEPP**	**DFP**	**Sarin**
Diisonitrosoacetone	8.4	0.8	24.3
Monoisonitrosoacetone	6.8	0.7	22.1
*iso*Nitrosoacetophenone	10.7	5.1	4.1
*iso*Nitrosoacetylacetone	0.7	NA	1.1
Picolinohydroxamic acid	2.9	0.6	0.2
Micotinohydroxamic acid metabolide	0.3	0.05	0.3

Source: Davies and Green (1956).
Rates of reactivation are given in L/mol/min.: temp. = 25°C, pH = 7.4.
NA, not available.

TABLE 69.2 Rate Constants for Interactions of AChE, OPs and Oximes

		Reactivation Constant		
Inhibitor	**Oxime**	k_r **(min^{-1})**	K_D **(μM)**	**[Oxime] (mM)**
MFPCh	Obidoxime	0.020	1133	0.5–4
	2-PAM	0.004	2949	1–5
	HI 6	0.076	1233	0.3–2
	HLö 7	0.051	606	0.1–4
MFPβCh	Obidoxime	0.015	1658	0.5–4
	2-PAM	0.002	3131	0.5–10
	HI 6	0.090	859	0.5–4
	HLö 7	0.078	458	0.5–4
MFPhCh	Obidoxime	0.009	3547	1–5
	2-PAM	0.003	3837	1–5
	HI 6	0.028	1037	0.5–4
	HLö 7	0.011	599	0.3–3

Source: Worek et al. (2004).

NA inhibition of ChE or the regeneration of the free enzyme from its inhibited state, such as pyridostigmine and oximes, respectively. Numerous data in the literature describe the kinetics of interaction between nerve agents (and other OPs) and specific countermeasures at active enzyme sites of concern. For example, Davies and Green (1956) have measured the rate of reactivation of inhibited erythrocyte ChE by various oximes (Table 69.1). More recently, Worek et al. (2004) determined kinetic rate constants of inhibition, reactivation, and aging for different NAs, pesticides, and oximes with human erythrocyte AChE (see Figure 69.2 and Table 69.2), as described by the reactions listed here:

$$[E] + [OP] \xrightarrow{k1} [EP]$$
$$[EP] + H_2O \xrightarrow{k2} [EA]$$
$$[EP] + H_2O \xrightarrow{k3} [E] + [OP]$$
$$[EP] + [OX] \xleftrightarrow{k_D} [EPOX] \xrightarrow{k_r} [E] + [POX]$$

where [E] is the active enzyme (i.e., AChE), [OP] is the organophosphorus compound, [EP] is the reversibly phosphylated AChE, [EPOX] is the Michaelis–Menten-type phosphyl-AChE-oxime complex [EPOX], [OX] is the oxime, [POX] is the phosphylated oxime, and [EA] is the dealkylated, "aged" AChE. The reaction rates used describe the bimolecular inhibition (k_1), aging (k_2), spontaneous (k_3), and oxime-induced reactivation. The dissociation rate, K_D, is equal to the ratio [EP] × [OX]/[EPOX] and (k_r) represents a first-order rate constant for the displacement of the phosphyl residue from [EPOX], resulting in regenerated enzyme and a phosphorylated oxime.

Hence, both a high affinity of the oxime to the "inhibited" or phosphylated AChE and a high regeneration rate (k_r) are critical for oxime efficacy. Using *in vitro* enzyme kinetic constants and *in vivo* inhibitor and oxime concentrations, Worek et al. (2005) were able to extrapolate the percent of AChE inhibition in humans after sarin or cyclosarin (intravenous) and a simultaneous intramuscular injection of various oximes. Similarly, kinetic models have been developed to predict a dose range of human butyrylcholinesterase (HuBChE) required to maintain an adequate residual RBC AChE level after exposure to sarin, soman, or VX (Ashani and Pistinner, 2004). These and other data sets can be readily integrated into the full PBPK/PD modeling framework, as shown in Figures 69.1 and 69.2. For example, Figure 69.3 shows a simulation of RBC AChE inhibition and regeneration in the Rhesus monkey after exposure to sarin, followed by 2-PAM administered at 9 min after sarin and produced using the PBPK/PD model for sarin presented in Chapter 58. The lower simulation represents AChE inhibition after sarin exposure alone, whereas the upper simulation was generated by fitting an effective first-order rate constant (k_3^{eff}), which can be derived as shown in Eqs. (69.3) and (69.4). Improved simulation of the early time points of AChE regeneration may be achieved through linking the sarin model with a PBPK for 2-PAM, which would capture the distribution phase of the countermeasure as well taking into account its affinity.

$$k_{\text{obs}} = \frac{k_r^*[Ox]}{K_D + [Ox]} \tag{69.3}$$

$$k_3^{\text{eff}} = k_3 + k_{\text{obs}} \tag{69.4}$$

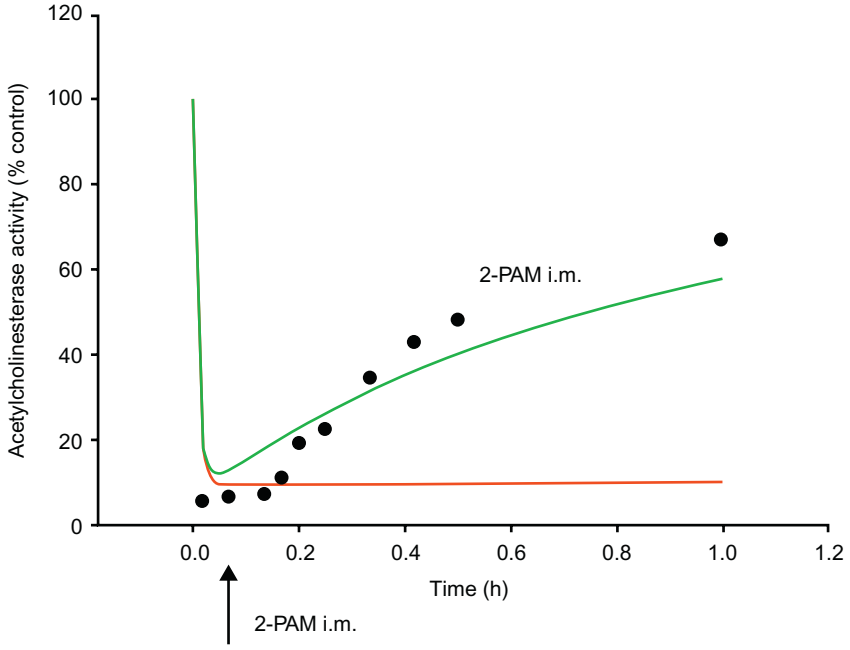

FIGURE 69.3 RBC AChE inhibition in Rhesus monkey after administration of sarin 0.75 LD_{50} (15 µg/kg) intravenous, and with 2-PAM (25.8 mg/kg) administered intramuscularly at 9 min after sarin intravenous administration. Atropine was administered (0.4 mg/kg) intramuscularly 15 min before sarin administration. The filled circles indicate experimental data (Woodard et al., 1994); the curves show our PBPK model simulations of AChE activity after sarin, both with (upper curve) and without (lower curve) 2-PAM administration.

Specific model features relevant to the agent's route of exposure and its biological effect can be incorporated when appropriate, for example, quantitative models for ciliary removal of NAs in the upper respiratory tract during inhalation exposure. In addition, it is important to take into account (and incorporate into the model) the limitations of the analytical methods used to quantify circulating and tissue levels of NA, such as the regeneration of NA from its binding sites. For example, it is desirable to model competition for AChE binding sites at erythrocytes and at target sites in the brain; the former because this regenerated NA from RBCs can be interpreted to calculate a measure NA systemic exposure and the latter, of course, because of the health impact. In general, the PBPK models can be linked via interaction processes at target sites.

Pharmacokinetic/PD models have been developed and used to predict the pharmacological effect of seizure activity for several anticonvulsants for which drug receptor kinetics were available from either *in vitro* or *ex vivo* binding studies. Diazepam, an agonist of the $GABA_A$ receptor, is among the NA antidotes, for which pharmacodynamic impact on electroencephalogram (EEG) activity has been modeled. Dunhof et al. (2007) have developed compartmental kinetic models for various anticonvulsants and modeled their ligand receptor pharmacodynamics. EEG parameters have been used as pharmacodynamic endpoints (Tuk et al., 1999; van der Graaf et al., 1999; Bueters et al., 2003; Dunhof et al., 2007). These receptor binding models predict changes in receptor binding and pharmacodynamics as a function of changes in the pharmacokinetics of the ligand as described in Eq. (69.5):

$$E(C) = E_{maxA} \cdot \left(\frac{C_A^N}{K_A + C_A} \right) \tag{69.5}$$

When E(C) is the observed effect at concentration C, E_{max} is the maximal effect (based on EEG components), K is the concentration at half-maximal effect, and N is the Hill factor, a constant expressing the sigmoidicity of the concentration–effect relationship. Subscripts A refer to the specific ligand of a ligand-gated ionotropic receptor.

Unfortunately, the impact of anticonvulsants on NA-induced seizures has not been modeled in this way. Given that many countermeasures have different ligand receptor interactions, such an approach would need to be expanded to the other excitatory and inhibitory neurotransmitters and their respective receptors, which are involved in seizure induction and sustainment. However, validation of such an approach, using components of gross EEG recordings, may not be plausible because of the large number of ionotropic channels represented. An endpoint such as sustained elevated glutamate may be more appropriate.

In addition, many target receptors undergo rapid changes in their level of expression during status

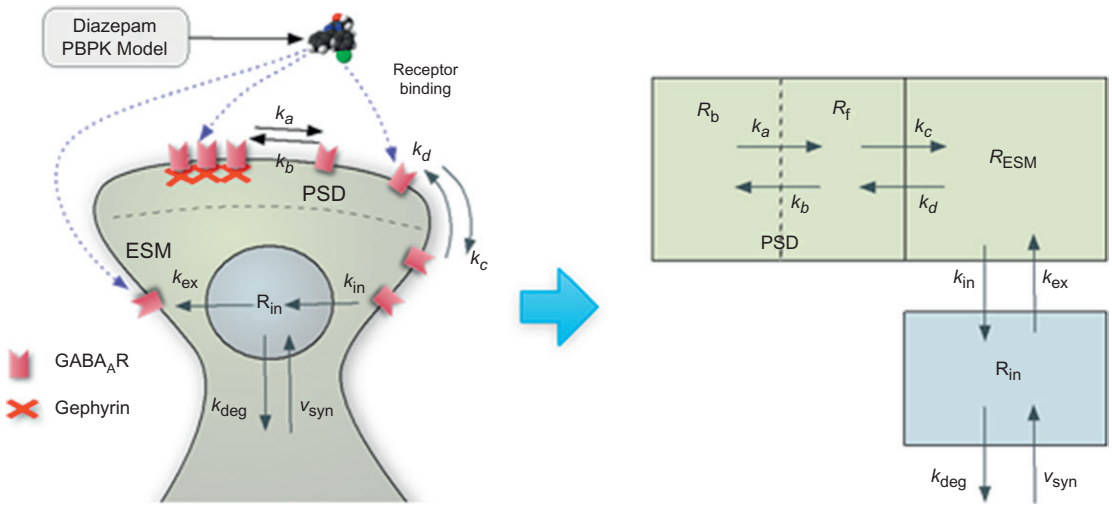

FIGURE 69.4 Simplified model of GABA$_A$R trafficking. *Rb* and R_f represent surface GABA$_A$ *Rs* within the PSD that are either bound to scaffolding proteins or free, respectively. R_{ESM} represents extrasynaptic GABA$_A$Rs and R_{in} represent internalized receptors. First-order rates are used to describe binding (k_b) and unbinding (k_a) of receptors to scaffolding proteins within the PSD, lateral movement between the synaptic and extrasynaptic membrane (k_c and k_d), internalization (k_{in}), recycling and membrane insertion (k_{ex}), and degradation (k_{deg}). Synthesis of nascent receptors is described with a zero-order rate, V_{syn}. *Adapted from Merrill (2012).*

epilepticus. GABA$_A$ receptors are undergoing rapid internalization, whereas AMPA and NMDARs are increasing at excitatory synapses, aggravating the loss of inhibitory tone (Naylor et al., 2005; Naylor, 2010). Figure 69.4 illustrates a basic model of GABA$_A$ receptor trafficking that can be developed into a full PBPK/PD model to include time-critical changes in target receptor activity (Merrill, 2012), leading to benzodiazepine pharmacoresistance. Such a model could be expanded to include other therapeutic targets, such as the NMDAR; when linked with PBPK models for the corresponding therapeutics, they can be used to optimize dosing based on the time from onset of status epilepticus.

Although the receptor targets of various NA countermeasures vary distinctly, be they ion channel-linked, G-protein-linked or enzyme-linked, their pharmacodynamic effects often converge on the final steps leading to the disruption of intracellular Ca^{2+}, beyond which cell death is inevitable. Hence, ultimately, a systems biology approach may provide the best predictions of countermeasure efficacy, especially for modeling long-term low-level effects. The Systems Biology Markup Language (SBML) project is an effort to create a machine-readable format for representing computational models in biology. SBML provides an input and output format, so that different software tools can operate on the same representation of a model, removing chances for errors in translation. SBML also provides convenient model databases, such as Biomodels (www.biomodels.net) for sharing these models. Neural Open Markup Language (NeuroML) similarly facilitates building, simulating, testing, and publishing of models describing channels, neurons, and networks of neurons. Future integration, via a common computational language, of these molecular and cellular level models with PBPK models will permit the development of multiscale models that will link external exposure and tissue dosimetry with NA-induced neurotoxicity. This multiscale approach is necessary for quantitative assessments of the health risks associated with NA exposure.

HEALTH EFFECTS ASSESSMENT AND COUNTERMEASURE OPTIMIZATION

Once validated, PBPK/PD models can be used to predict efficacy of specific countermeasures, countermeasure combinations, and dosing regimens for specific NAs and combinations thereof. To do so, appropriate measures of efficacy need to be defined. Perhaps the simplest way to estimate the effectiveness of a particular countermeasure is to determine the degree of a receptor activation *at the target site of concern* (e.g., brain or brain region) at a certain time or period of time after the administration of the countermeasure(s), compared with the situation in which no countermeasure is administered. Expressed as a simple ratio, countermeasure effectiveness (CE) may be written in terms of the time-weighted average concentration (from time $t = T_1$ to $t = T_2$) of receptor occupancy (obtained from the PBPK/PD model):

$$CE = \frac{\int_{T_1}^{T_2} [E]_c \, dt}{\int_{T_1}^{T_2} [E] dt}$$

Here, the numerator is calculated by running the model in the presence of the countermeasure, whereas the numerator is obtained in the absence of countermeasures. The ratio CE can be calculated under a variety of countermeasure dosing regimens to determine an optimum for each specific NA exposure scenario. Clearly, if such an exploration of countermeasure effectiveness were to be conducted experimentally, without the benefit of modeling, then it would be prohibitively expensive and time-consuming, thus demonstrating the usefulness of the modeling. The information obtained in this way would provide a rational basis for designing countermeasure delivery systems for optimized effectiveness. Ultimately, such an approach would also aid in the design of novel countermeasures, either to be used alone or to be integrated into an optimized countermeasure delivery "package."

A separate, although related, application of the proposed modeling approach is the development of meaningful therapeutic animal models based on rational animal to human scale-up. In such an application, dosing regimens for existing countermeasures and countermeasure combinations, and particularly novel countermeasures, can be tested in experimental animals with the knowledge that the results obtained can be extrapolated to humans by taking into account the appropriate species differences incorporated into the models.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

It can be expected that, in the near future, PBPK/PD models will be developed that can predict the interaction between mixtures of NAs and countermeasures and their opposing pharmacodynamic effects. Validation of such complex modeling efforts is data-intensive and will rely on compilation of existing *in vitro*, *in vivo*, and *ex vivo* data. Unfortunately, the majority of toxicological studies of NAs measure lethality, with little or no additional collection of time-course data of pharmacodynamic endpoints. Similarly, interaction studies between NAs and antidotes often measure only efficacy in terms of a reduction in cell death. This is understandable because of the difficulty and cost in measuring these NAs in biological matrixes and the large number of animals involved in collecting time-course data. Measurements of neurotransmitter changes after OP and countermeasure exposures are very difficult, and often there is uncertainty in deciding which biomarker is most predictive of an effect that is not easily quantifiable, such as cognitive deficits. PK/PD analyses may not always be possible if the relationships between the input (the concentrations of the NA and countermeasure) and the output become too indirect. However, it should always be remembered that even without formal concentration–effect analyses, there is always much to be gained if a pharmacological countermeasure test is performed with intensive sampling of PK and PD parameters. Differences in PK/PD parameters give quantitative information about different neurotransmitter systems in the central and peripheral nervous systems, which cannot be distinguished by pure statistical group comparisons. PK/PD analyses largely eliminate pharmacokinetic variability and provide detailed and functional characteristics of pharmacological systems in the brain or other target organs.

With specific regard to finding novel therapeutics for preventing or ameliorating NA-induced brain damage, PBPK/PD modeling provides a means to extrapolate experimental findings in animals to humans. Differences seen in the efficacy of different therapeutics may sometimes be explained by pharmacokinetics. Perhaps the therapeutic dose reaching a critical tissue, such as the diaphragm, heart, or brainstem is too little, too late, or not long enough. In addition, many of the animal toxicity studies performed to assess the efficacy of a therapeutic against NA measure a biomarker, such as cell death within 24 h from NA exposure. Biomarkers measured at 24 h may not always be indicative of outcome. Whether any protection seen at that point would still remain if the biomarker (e.g., cell death) were measured days, weeks, or months later then remains unknown. For example, damage seen in thalamus at 24 h might later extend to areas with which it has strong reciprocal connections, such as the cerebral cortex. Finally, in the quest for antidotes that may rescue one from cognitive deficits later, when realistically administered beyond the time when the glutamatergic phase takes control of seizure activity (i.e., > 40 min after NA exposure), predicting biomarkers that indicate the promotion of physiological balance in the brain, rather than the attenuation of seizure activity, may become the most important endpoints for predicting the efficacy of novel countermeasures.

Given the unsustainable cost and time involved in bringing novel therapeutics to market, the use of quantitative techniques to improve countermeasure development are being used increasingly more. Several opportunities exist in drug development, which could lead us on a more direct path toward finding suitable countermeasures, for example, more complete integration of the available knowledge of therapeutic candidates early in their development using PBPK/PD model(s) for NAs and countermeasures together. This should be started early, in the preclinical phases of drug development. Extrapolation of animal PBPK/PD models to humans for simulating initial clinical trials should be preformed to evaluate the design of proposed clinical trials of a drug development plan, and to provide proof of concept. This ability to mathematically explore various exposure–response relationships of NA(s) and

countermeasure(s) is useful for designing optimal countermeasure regimens and identifying the limits of the therapeutic window after NA exposure. Uncertainty analysis of the parameters of the model may identify biomarkers that may be critical in describing the data. Ways of defining and decreasing the uncertainty in efficacy and safety (risk assessment) in the drug and disease model are needed.

Finally, the mechanisms (e.g., diffusion-limited transport, receptor binding, protein upregulation or desensitization) described in a particular NA countermeasure model should be updated as new information is generated, thereby using the models as knowledge management tools. Thus, modeling the PK/PD of neuroprotectants in the presence of NA may provide direction and guidance early during clinical development, when there is opportunity to change direction and plans.

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CHAPTER

70

Strategies to Enhance Medical Countermeasures After the Use of Chemical Warfare Agents on Civilians

David A. Jett and David T. Yeung

INTRODUCTION

Chemical warfare agents (CWAs) are highly toxic chemicals that have been used in military conflicts, beginning in World War I and continuing in more recent conflicts in the Middle East, specifically during the Iran–Iraq war in the 1980s. However, ever since the 2001 terrorist attacks in the United States, there has been a heightened awareness of the potential deployment of biological, radiation/nuclear, and chemical threats against the civilian population as well. Underscoring these concerns are events surrounding the use of the CWAs sarin and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX) by the terrorist organization Aum Shinrikyo against Japanese civilians in the 1990s (Vale, 2005; Yanagisawa, 2006). These concerns were highlighted again when sarin was deployed against civilians in the suburbs of Damascus, Syria, in 2013, during the Syrian civil war (Dolgin, 2013). Divisions within the US Department of Health and Human Services (HHS) and other federal agencies have sustained a highly focused effort to assess, and if necessary, improve current emergency response capabilities in the event of a terrorist event. These efforts include both nonmedical countermeasures, such as personal protective equipment (PPE) for first-responders who must enter a contaminated site, and medical capabilities, such as safe and effective antidotes and diagnostic tools to reduce mortality and morbidity after a chemical attack. These countermeasures are also important for large scale industrial accidents involving toxic chemicals.

OVERALL STRATEGIC APPROACH

The strategic research plan and research agenda for civilians aims to improve the nation's ability to diagnose, prevent, and treat injuries resulting from chemical attacks or accidents. Medical interventions must be appropriate for a diverse civilian population, and treatment strategies must take into account how a toxic chemical enters the body and the time window for possible medical intervention, which is often very short. Thus, treatments must be administered easily and rapidly in a situation involving mass casualties. Both immediate and long-term effects of exposure to chemicals must be understood to ascertain whether treatments are needed and whether they can be developed. Drugs should be chemically and physically stable so that they are amenable to prepositioning and stockpiling, and pretreatments for first-responders to a contaminated site should be considered, especially when decontamination is impossible.

In 2006, the US Congress appropriated funds to the National Institutes of Health (NIH) to implement the National Strategic Plan and Research Agenda for Medical Chemical Countermeasures (http://www.niaid.nih.gov/topics/BiodefenseRelated/ChemicalCountermeasures/Documents/nihstrategicplanchem.pdf). This program, led by the National Institute of Neurological Disorders and Stroke (NINDS) and coordinated by the National Institute of Allergy and Infectious Diseases (NIAID) with participation from several other NIH institutes and centers, is called Countermeasures

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Against Chemical Threats (CounterACT; http://www.ninds.nih.gov/counteract). This program has two important goals. First, it supports basic research to learn information about the toxicology of chemical threat agents so that novel therapeutic targets and approaches can be identified. In this regard, the program serves as a science and technology base for the larger federal research enterprise. Second, CounterACT supports more advanced translational research with promising lead compounds that would be eligible for transfer to the HHS Biomedical Advanced Research and Development Authority (BARDA; http://www.phe.gov/ABOUT/BARDA/) for more advanced studies that are required to obtain approval from the US Food and Drug Administration (FDA). The CounterACT and BARDA programs operate within a highly collaborative environment that includes similar research agencies within the US Department of Defense (DOD).

COUNTERACT PROGRAM STRUCTURE

From its inception, the CounterACT program has employed a strategy of building partnerships among laboratories within industry, academic, and federal sectors. CounterACT has specifically relied on the vast experience and resources within the DOD, as it had been the primary federal agency responsible for CWA research, including countermeasure development. The program has used the expertise of researchers in chemical warfare and engaged a vast pool of NIH-funded experts in scientific areas that are very relevant to some of the central questions being posed in this field. For example, if untreated, chemical nerve agents can cause seizures and possibly neuropathological sequelae, conditions that share molecular mechanisms and phenotypes with many neurological illnesses such as epilepsy and stroke. These disorders are research areas that the NIH has supported for decades, and this has provided an unprecedented opportunity for CounterACT to engage researchers and neurologists from these fields. Similarly, the partnership between HHS and DOD facilitated by CounterACT has also led to excellent collaborations among scientists who otherwise would rarely interact. These efforts have already produced promising, novel approaches in the development of medical chemical countermeasures. Research within CounterACT has already resulted in over 600 publications in some of the most prestigious scientific journals (see www.ninds.nih.gov/counteract for more information).

The CounterACT program has supported over 60 individual research projects, multiple contracts, and at least eight different Research Centers of Excellence located at many of the top laboratories across the United States thus far. Most, if not all, of these programs have collaborations with other domestic and foreign laboratories, resulting in a large interdisciplinary and vibrant collaborative research network. This network of researchers maintains collaborative interaction by sharing new and exciting results at scientific meetings sponsored by both the NIH and international research societies, such as the Society of Toxicology, American Thoracic Society, and Society for Neuroscience. As a result, new collaborations among scientists in disciplines such as neuroscience, dermatology, and pulmonary research are formed at these meetings each year.

SCOPE OF RESEARCH

The CounterACT program focuses on basic and translational research on the development of novel therapeutics that will enhance medical response capabilities against chemical threats during an emergency. *Chemical threats* are defined as highly toxic chemicals that could be used intentionally in a terrorist attack or could be released from transportation and storage facilities after an accident or a natural disaster, causing mass casualties. The civilian threat spectrum includes not only traditional CWAs, but also thousands of other highly toxic industrial compounds and materials (TICs/TIMs). However, based on availability and the feasibility of mass exposure, the number of chemicals posing a serious threat is smaller (Figure 70.1). Some TICs/TIMs are stored at large industrial facilities and transported by rail and other means across the United States. Accidents and resulting fatalities involving human exposures to these chemicals are not uncommon (Figure 70.2). In fact, the number of annual incidents involving exposure to hazardous substances is in the thousands within the United States alone (Mannassaram et al., 2003; Broughton, 2005; Buckley et al., 2007). While CWAs are rarely implicated outside military facilities or off the battlefield, there are major exceptions, such as the terrorist attacks in Japan and the recent use of sarin in Syria.

The scope of research supported by the CounterACT program includes mechanistic research to identify targets for therapeutic development. If targets are known, the program supports the creation and development of screening assays that must be validated with appropriate pharmacology, biological activity, and other factors that increase the likelihood that candidate therapeutics emerging from the *in vitro* screening activity will be effective *in vivo*. As such, creation and validation of animal models of chemical effects on humans is a critical component of the program. The primary goal of this effort is to develop models of lethality and serious morbidity that are based on the best available data in humans. Using these models, the program supports preliminary proof-of-principle data on the efficacy of candidate

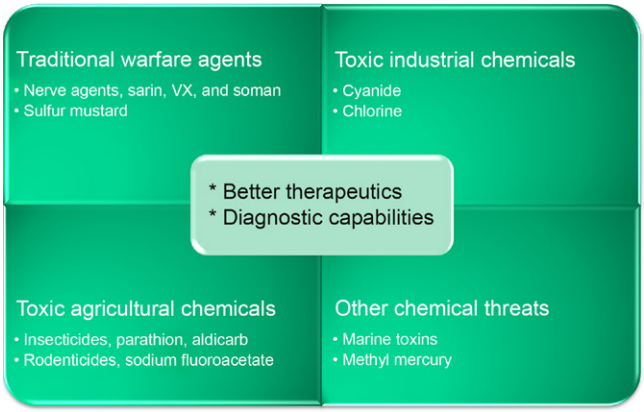

FIGURE 70.1 The civilian chemical threat spectrum, including general categories of chemical threats and some examples of chemicals within each category.

FIGURE 70.2 Train wreck and chlorine spill in Graniteville, SC, in 2005; *Source: accessed from www.cen.online.org, June 23, 2008.*

therapeutics, as well as more advanced efficacy studies and preclinical research, including safety and pharmacokinetic/pharmacodynamic studies that are necessary to obtain regulatory approval and licensure.

CounterACT not only supports the discovery of novel compounds with requisite therapeutic activity and acceptable safety profiles, but also alternate routes of administration for FDA-approved therapeutics that would be safe and effective and may be easy to administer during a mass casualty scenario [e.g., intramuscular injection (Rebmann et al., 2009)]. Although antidotes that are specific to a chemical agent are supported, the program is also very interested in developing countermeasures that can be effective against the acute effects and pathologies common to multiple chemical threat agents. This latter approach would allow the development of a therapy that can be used to treat injuries caused by a spectrum of chemical threats with common mechanisms and pathologies. Special consideration is given to research that is relevant to populations who are particularly vulnerable, including the young, the elderly, pregnant women, and individuals with preexisting medical conditions. Children and pregnant women, for example, have been shown to be much more sensitive to the toxicity of some CWAs and thus may require specialized medical management after exposure (Baker, 2007). Consequently, animal models and studies that address these vulnerabilities, as well as long-term effects after an acute exposure, are of keen interest.

THE CIVILIAN VERSUS MILITARY THREAT SPECTRUM

CWAs can generally be divided into four broad categories: traditional nerve agents, such as sarin and VX; vesicating agents, such as sulfur mustard; pulmonary agents, such as chlorine and phosgene; and metabolic poisons, such as cyanide and hydrogen sulfide. Detailed descriptions of medical interventions for each of these CWAs can be found elsewhere in this book, so they will be discussed only briefly here to illustrate the different approaches undertaken by the NIH research community to develop medical countermeasures for treating civilians.

Military research on the effects of the organophosphorus (OP) nerve agents and mustard gases has been ongoing for several decades since their discovery and use in past wars. This work, along with efforts in the nonmilitary research community, has led to the development of useful therapeutics and diagnostic tools primarily for use to protect the soldiers on the battlefield. Of particular note are the development of more effective PPE, prophylactic drugs such as pyridostigmine bromide (PB) that reversibly binds to acetylcholinesterase (AChE; Gordon et al., 2005), and portable antidote kits like the MARK-1, Antidote Treatment Nerve Agent Autoinjector (ATNAA), and Duodote autoinjectors (Henretig et al., 2002). Key differences between the civilian and military populations pose some unique challenges to researchers in this field (Figure 70.3). First, the typical soldier is fit and healthy and between the ages of 18–45, whereas the civilian demographic is much broader, including pediatric and elderly people, pregnant women, and individuals with preexisting medical conditions such as asthma, heart disease, and diabetes. These factors will be extremely important when deciding the specific type and dosage of medical countermeasures that can be administered during a civilian chemical emergency. Fortunately, some military countermeasures can be adapted to special populations such as children (Rotenberg and Newmark, 2003; Baker, 2007). However, the development and validation of good animal models

Military focus:
- Warfighter: 18–45 and healthy
- Open air environment
- High number of casualties is the goal
- Prophylactic measures is focus

Civilian focus:
- Pediatric - elderly
- Preexisting medical conditions
- Could happen in closed environment
- No need for high casualties
- Post-exposure therapies is focus
- First responders and DeCon personnel

FIGURE 70.3 Civilian and military challenges in countermeasure development.

to simulate these special segments of the population are still urgently needed.

Another important difference between the military and civilian sectors is that military officials may be able to predict with some degree of certainty when CWAs could be used, and thus equip the warfighter with pre- and post-exposure countermeasures, such as PB and Duodote kits. However, since it is nearly impossible to predict when a civilian attack may occur, the NIH has been primarily focused on treating victims after exposure to CWAs. The exception to this approach is that there is some current research into prophylactic treatments that could be administered to first-responders who must enter a contaminated site or those who must treat victims that may still be contaminated, such as healthcare providers in the emergency department.

BASIC/MECHANISTIC RESEARCH AND TARGET IDENTIFICATION

Support of basic or fundamental research efforts is an important component of the NIH mission statement (http://www.nih.gov/about/mission.htm). To that end, the CounterACT program supports basic research efforts to identify molecular mechanisms of toxicity exerted by chemical threat agents for the purpose of identifying novel therapeutic targets. For example, the program supports research into the mechanisms of nerve agent-induced seizures with respect to temporal and regional changes, roles of cardiac versus neuronal mitochondria in cyanide toxicity, or the cellular and molecular basis for agent-induced pulmonary edema. Similarly, understanding the toxicokinetics related to the different routes of exposure to chemical agents is also critical to the development of novel medical countermeasures.

Some of the most toxic chemical threat agents selectively target molecular sites and processes within the nervous system. They may bind directly to receptors or ion channel proteins, while others target metabolic enzymes. Some chemical threat agents can inhibit neuronal respiration, leading to anoxia, whereas others may produce oxidative stress, leading to cell death and neuropathological lesions. These molecular effects and the resulting signs and symptoms of toxicity depend on the level of and duration of exposure to the dose which, in the case of a terrorist attack or industrial accident, will likely be a high dose during a short period of time. Lethal exposures to the neurotoxic chemicals usually result in disruptions of critical neurological functions, such as central and peripheral control of cardiac and respiratory functions. Other serious but nonlethal effects can include seizure activity, including *status epilepticus*, paralysis, and loss of consciousness. Neurologic sequelae, sometimes occurring years after an exposure incident, may include emotional and cognitive abnormalities and weakness in the limbs (Yamasue et al., 2007).

IN VITRO AND *IN VIVO* MODELS FOR EFFICACY SCREENING

Development and validation of *in vitro* and/or animal models for rapid screening of molecular libraries to identify potential medical countermeasures is another priority of the CounterACT program. These models include nerve agent-induced seizures in small mammals, models of direct pulmonary injury from an inhaled chemical source such as sulfur mustard, animal models of cellular intoxication in the case of cyanide and sodium azide, and medium-throughput models of dermal or ocular injuries caused by vesicants like lewisite. To accelerate scientific progress and the development of new therapies, these models must also be rigorously designed (Landis et al., 2012) and be amenable to use under Good Laboratory Practice (GLP) methodology to allow regulatory review by the FDA. Since strict adherence to GLP standards may be expensive, earlier efficacy screens to identify potential hits are usually performed under non-GLP conditions.

For specialized efficacy testing, CounterACT investigators are encouraged to utilize the centralized testing facilities that the NIH has provided. One such facility is the NINDS Anticonvulsant Screening Program (ASP) at the University of Utah (http://www.ninds.nih.gov/funding/research/asp/index.htm). The NINDS ASP has screened compounds for potential therapeutic activity in the treatment of epilepsy for decades. Capitalizing on this considerable experience and the medium-throughput screening models that are already in place within the ASP, the CounterACT program has developed an additional testing arm at the ASP devoted to seizures induced by OP chemicals. This CounterACT arm aims to identify compounds for efficacy in alleviating OP-induced

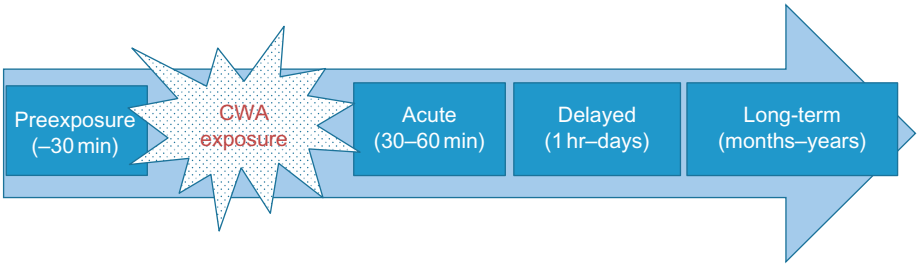

FIGURE 70.4 Timeline of morbidity progression after CWA exposure and opportunities to intervene.

seizures, as well as those that may be capable of preventing the neuropathology that could occur after nerve agent exposure.

One of the major challenges of developing an NIH program on medical chemical countermeasures is that many of the highest-priority chemical threat agents (such as OP nerve agents) are highly toxic and can be used only in approved, usually military, research facilities. Consequently, the NIH has partnered with the US Army Medical Research Institute of Chemical Defense (USAMRICD; http://chemdef.apgea.army.mil/) to conduct chemical countermeasure research against CWAs that cannot be done outside DOD laboratories and that takes advantage of the CWA research expertise within DOD.

One particular challenge for the program is to define the effects of chemical agents that are nonlethal but still represent serious morbidity, both in the short term, immediately in the aftermath of CWA exposure, and in the long term. These long-term effects could manifest immediately after the incident and last chronically for months to years afterward, or they may not develop until much later (see Figure 70.4). It is important to define these endpoints so that potential therapeutics can be tested to determine if these often-debilitating illnesses and disabilities can be prevented. As such, animal models that are predictive of these long-term effects in humans are needed to assess the efficacy of potential therapies. These models must also clearly define treatment modalities that are consistent with the context of intended use in humans, and thus should be based on the best available human data. Data from terrorist attacks is rare, fortunately, but much can still be learned from exposures that occur from industrial accidents and occupational situations.

ADVANCED PRECLINICAL DEVELOPMENT AND CLINICAL STUDIES

Once a lead compound is identified, optimization of that compound into a viable drug candidate is crucial. Using medicinal chemistry, it may be possible to optimize a compound to exhibit the best possible pharmacokinetic profile for a desired formulation and preferred route of administration. This is an iterative process that is coupled to biological assays of efficacy, safety, and pharmacokinetics. General chemistry and manufacturing support is critical once a lead compound is identified. These support activities include current Good Manufacturing Principle (cGMP)–compliant stability studies to support regulatory submission. Manufacturing processes and procedures under cGMP conditions must be developed, and reagent and clinical grade drugs must be synthesized in amounts sufficient for preclinical evaluation and Phase I and II clinical trials. The CounterACT program uses several core NIH resources, including the CounterACT Preclinical Development Facility (CPDF) discussed next, to support these preclinical activities.

The CPDF provides researchers with access to state-of-the-art resources to conduct pharmacokinetic, safety, chemistry, and formulation studies under both GLP and cGMP conditions when a lead compound is ready for Investigational New Drug (IND)–enabling studies. Alternatively, the CPDF may conduct smaller pilot studies for promising compounds that require additional characterization before further efficacy or preclinical testing. This facility and other programs under development may also support regulatory affairs on an individual basis for CounterACT investigators. For clinical trials, the CounterACT program takes advantage of other established resources at the NIH, such as the NINDS Neurological Emergency Treatment Trials (NETT) Network (http://www.nett.umich.edu/nett/welcome). The NETT conducted a Phase II/III double-blind, randomized, noninferiority clinical trial to compare the efficacy of intramuscular midazolam with that of intravenous (IV) lorazepam for children and adults in *status epilepticus* treated by paramedics (Silbergleit et al., 2011, 2012). Results of this study are of interest because they suggest that this drug may also be effective in treating seizures caused by nerve agents in adults and children.

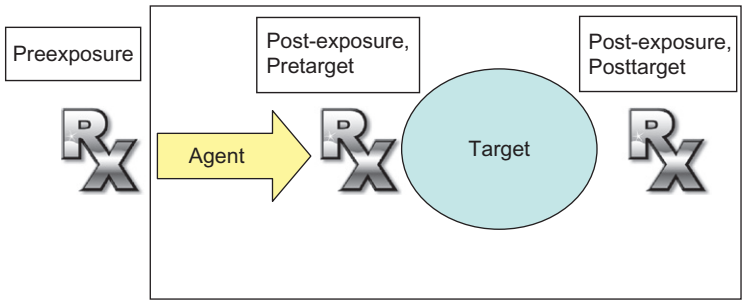

FIGURE 70.5 Different opportunities for medical intervention to reduce mortality and morbidity caused by CWAs.

PRETREATMENTS

For the purpose of this chapter, we define *pretreatments* as those medical countermeasures to be administered before CWA exposure (see Figure 70.5). Personnel who would be pretreated with medical countermeasures include first-responders, such as emergency medical technicians (EMTs) and persons responsible for site decontamination. Emergency department medical personnel, who are likely to treat individuals shortly after a chemical incident, may also require pretreatment to prevent incapacitation from exposure to residue and off-gassing of CWAs from patients. These secondary exposures could be deadly and lead to more loss of life, both during and after an event. In theory, prophylactic drugs would be administered in conjunction with proper PPE to first-responders before they enter a CWA "hot zone." However, depending on the possible adverse effects and pharmacokinetics of the proposed drugs, there may be some operational difficulties in the implementation of a prophylaxis program. For example, compliance by otherwise healthy individuals may present an issue if there are potential side effects. These potential side effects that may occur when a countermeasure is administered to otherwise normal and unexposed individuals illustrates the importance of favorable safety profiles, especially if the intended indication is for prophylaxis use. Side effects in first-responders and care providers could compromise their ability to respond effectively to chemical events involving civilians.

Another challenge for the prophylaxis approach is the time needed for the proposed drugs to achieve or maintain maximal therapeutic levels when a first-responder arrives at the contaminated site. Similarly, the half-life of the drugs must ensure lasting therapeutic efficacy to protect first-responders during the window of potential exposure. However, if these challenges can be overcome, pretreatments against the toxic effects of chemical agents for emergency first-responders would represent a significant advancement. In addition to prophylactic drugs, better dermal and ocular medical protectants should be considered for inclusion as preexposure treatments, especially for medical personnel who may have to work in areas where off-gassing may occur by individuals exposed to CWAs.

The only pretreatment currently approved by the FDA for nerve agents is PB, which was first approved for treating myasthenia gravis and is now approved for treating intoxication by soman only. It is to be used by military troops at high risk of exposure to soman on the battlefield. PB is a reversible inhibitor of AChE and protects it from permanent inhibition by soman. Since it is itself an inhibitor of the target enzyme in nerve agent toxicity, it is contraindicated after exposure, and AChE inhibition has already occurred. The US military has led the efforts to explore other potential pretreatments. One effort involves the use of serum-derived or recombinant butyrylcholinesterase (BChE) as a stoichiometric scavenger of nerve agents that could be administered as a prophylactic treatment (Lenz et al., 2007). Previous studies have shown that administering the exogenous human BChE enzyme can protect guinea pigs and cynomolgus macaques from death against extremely high doses of soman or VX (Saxena et al., 2011). The challenge with this stoichiometric approach is rapidly reaching and maintaining an effective dose when each protein molecule binds only 1 molecule of nerve agent, so another approach is being explored that adds a catalytic function to scavenger molecules (Gupta et al., 2011; Cherny et al., 2013).

POST-EXPOSURE (PRETARGET) PROPHYLACTIC THERAPIES

Most CWAs reach their physiological targets at toxic levels to cause serious illness and even fatality in only minutes after exposure. The medical intervention in this case is to reverse the toxic action at the target site immediately or treat the physiological response (symptoms) as soon as possible. Another possibility is that some CWAs may exert their toxic effects more slowly and not

reach the target site at critical levels until after a period of time. This may be due to the route of exposure, such as the relatively slower dermal route versus more rapid inhalation, or due to slower toxicokinetics and toxicodynamics. In this instance, the opportunity for medical intervention may be greater, and a post-exposure, pretarget or post-exposure prophylaxis approach would be beneficial (Figure 70.5). Under this paradigm for treating CWA intoxication, reversible cholinesterase inhibitors and scavengers may be utilized to protect peripheral AChE before the systemic concentration of irreversibly inhibited OP nerve agent–AChE reaches a critical and toxic level. Thus, higher success rates with potential therapeutics may be expected after dermal exposures to lower-volatility CWAs such as VX since there would be time to protect AChE before all the VX enters the systemic circulation (Mumford et al., 2011).

Consequently, therapeutics that detoxify or remove chemical agents from systemic circulation, such as protein scavengers of nerve agents or newer cobalamine-based molecules that bind and scavenge cyanide, show promise as well. For cyanide intoxication, the Cyanide Antidote Kit has been used for several years to remove cyanide from circulation by forming cyanomethemoglobin (using nitrites) or thiocyanate (using sulfur donors) complexes that are less toxic and more readily excreted. Hydroxocobalamin marketed as the Cyanokit is a relatively new FDA-approved product for IV treatment of cyanide poisoning. An even more effective scavenger is cobinamide, a related compound under development (Chan et al., 2010). This drug binds cyanide with very high affinity and can be administered by intramuscular injection, which would be very suitable for a chemical attack or an emergency scenario with mass casualties (Brenner et al., 2010). These drugs can be effective if they can remove cyanide before it reaches critical targets that regulate cellular respiration. Next-generation cyanide antidotes may include drugs that could preserve mitochondrial respiration via direct interaction with the targets, sulfur donors with longer half-lives, or novel nitric oxide-based approaches.

POST-EXPOSURE POSTTARGET TREATMENT

For many CWAs, there is little opportunity to medically intervene when the dose is high and rapidly delivered to the target site. In these situations, high doses of CWAs are almost always fatal. If the action at the target site does not cause immediate death, there are several strategies that can be employed to reduce the impending morbidity and lethal effects. At the molecular level, AChE reactivators such as oximes can be administered during a nerve agent exposure incident to remove the agent from the active site of AChE. Administration of oxime therapy will succeed only if irreversible covalent modification of the nerve agent–AChE complex has not occurred (a process called *aging*). Un-aged AChE enzyme is thus restored to the active state and can continue the hydrolysis of the neurotransmitter acetylcholine (ACh). This intervention was developed through an intimate knowledge of the molecular mechanism of nerve agent toxicity. CounterACT researchers are using this knowledge to develop AChE reactivators that are more effective in removing nerve agents from AChE (Topczewski et al., 2013), and also possess chemical characteristics that allow them to cross the blood–brain barrier (BBB) and enter the CNS (Kovarik et al., 2013). For further details on CWAs, AChE reactivators, and the BBB, see Chapter 49.

In cases where intricate knowledge at the molecular level is not yet available to allow reversal of the toxic action on the target, the intervention is to treat the effects of target site activation (or inactivation). For example, at sublethal doses of nerve agents, medical intervention primarily involves combating the effects of nervous system activation and cholinergic crisis. As discussed in other chapters, atropine is used to block the overstimulation of cholinergic receptors, and anticonvulsants such as diazepam are used to prevent or treat seizures. Similarly, victims exposed to a sublethal concentration of the fast-acting poison cyanide are treated with ventilation and supportive care to delay mortality and morbidity until they can be taken to medical facilities. Another important area of research is to identify new approaches to mitigate the pulmonary edema that typically develops after lung injury caused by agents such as chlorine and phosgene.

Whether there is sufficient time after an exposure to the CWA for a therapy to be beneficial depends on the initial effective dose. This determination is based on several factors, including the initial level of the agent released, proximity to the point of release, route of exposure, and duration of exposure. If the effective cumulative dose is low, the risk of an acute, life-threatening intoxication will likely not be an issue, but there is evidence that acute sublethal exposures to chemical nerve agents may be associated with long-term neurological sequelae (Yanagisawa, 2006 and chapter 4). Some agents, such as chlorine and sulfur mustard, may also cause delayed toxicity after initial exposure or long-term pulmonary effects (such as fibrosis) years later (Chen et al., 2013; Mo et al., 2013; Veress et al., 2013 and chapter 5). Therefore, medical interventions aimed at improving the long-term health outcomes of nonlethal exposures are critical. Since the immune responses observed in the pulmonary system after sulfur mustard or TIC exposure share similar characteristics, it is likely that therapeutic drugs that prevent, slow, or halt the processes of

inflammation and subsequent cellular degeneration will probably have great utility across many different types of chemical agents. Drugs such as anti-inflammatory, antioxidant, anti-necrotic/apoptotic compounds, or those that promote cell survival such as growth factors may be effective against various chemical threats that cause these pathologies.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

With the increasing threat of terrorism, the development of medical products for use in the event of a chemical attack on civilians has become a high priority. Civilian-focused research resides within the HHS, which established a program in 2006 to develop and stockpile medical products for chemical threats. This NIH strategic plan takes into account the ongoing efforts with the DOD, and it partners with laboratories already working in this area of study. Strategies for enhancing medical preparedness in civilian locations are similar to those of the military. At NIH, a broad research community that otherwise would not be involved in CWA research is being engaged to take advantage of its critical expertise in areas of scientific endeavor relevant to the civilian and military CWA research programs. The scientific approach seeks opportunities to medically intervene during the course of exposure through acute and long-term responses. Understanding long-term chronic effects after a chemical exposure is critical, especially if better acute treatments can increase survivability from an otherwise lethal exposure. With regard to these nonlethal effects, it will be critical to study the human condition of survivors of chemical exposures in order to develop animal models and other techniques for predicting the efficacy of candidate therapeutics in humans. Finally, a major focus of future research strategies will be to address the large number of chemical threats that pose a threat to national security, especially for those where little is known about their toxicity and potential therapeutic interventions.

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CHAPTER

71

Pyridinium Oximes in the Treatment of Poisoning with Organophosphorus Compounds

Milan Jokanović

INTRODUCTION

Organophosphorus (OP) compounds have long been used as pesticides and have been developed into warfare nerve agents such as tabun, soman, sarin, VX, and others. Exposure to even small amounts of an OP compound can be fatal. Death is usually caused by respiratory failure resulting from the paralysis of the diaphragm and intercostal muscles, depression of the brain respiratory center, bronchospasm, and excessive bronchial secretions. The mechanism of OP poisoning involves phosphorylation of the serine hydroxyl group in the active site of acetylcholinesterase (AChE), which leads to the inactivation of this essential enzyme. As the result of AChE inhibition, the accumulation of acetylcholine (ACh) at cholinergic receptor sites produces continuous stimulation of cholinergic nerves throughout the central and peripheral nervous systems, motor convulsions, and epileptic seizures. Accumulating evidence indicates that seizure events are linked to irreversible long-term compromise of cognitive functions and alteration of CNS electrical excitability (Terry et al., 2014). Presently, therapy of acute OP poisoning includes administration of atropine as a muscarinic ACh receptor antagonist, together with an oxime, pralidoxime (2-PAM), HI-6, or congeneric bisquaternary pyridinium compounds such as TMB-4 or LüH-6 and CNS depressants such as diazepam. When administered *in vivo*, quaternary oxime reactivators largely remain in blood and peripheral tissue capable of crossing the blood–brain barrier (BBB) in small amounts and reactivating OP-inhibited brain AChE. This chapter describes the mechanisms of OP actions and the role of pyridinium oximes as AChE reactivators in the treatment of OP poisonings.

INTERACTION OF CHOLINESTERASES WITH OP INHIBITORS

The different types of cholinesterases (ChEs) in the human body are characterized by their location in tissues, substrate affinity, and physiological function. The principal ones are AChE (EC 3.1.1.7), predominantly found in the nervous system and also present in the outer membrane of red blood cells (RBC). Plasma ChEs (EC 3.1.1.8, ChE) are another group of enzymes present in plasma, liver, cerebrospinal fluid, and glial cells. Under normal physiological conditions, AChE breaksdown ACh, which is the chemical mediator responsible for conduction of nerve impulses at the sites of cholinergic transmission. Its physiological role in blood is not understood. However, ChE is also an esterase that can react in a similar way with most of the compounds that react with AChE. Although the effects of inhibition of AChE can be fatal, depending on the dose used, inhibition of ChE does not have any known consequences on normal body functions (Jokanović and Maksimović, 1997). ChE is a circulating plasma glycoprotein synthesized in the liver and does not serve any known physiological function. Recent evidence indicates that ChE may have roles in cholinergic neurotransmission, other nervous system functions such as cellular proliferation and neurite growth during the development of the nervous

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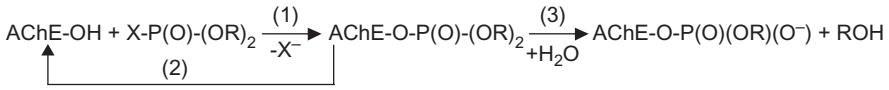

FIGURE 71.1 Interaction of acetylcholinesterase (AChE-OH) with OP compounds. Reaction 1 shows interaction of organophosphate molecule with the serine hydroxyl group at the active site of AChE. Inhibited AChE cannot further serve its physiological function, which causes the accumulation of ACh at the nerve endings. Reaction 2 is spontaneous reactivation of inhibited AChE, which occurs very slowly for most OP compounds. Reaction 3, called "aging," represents nonenzymatic time-dependent loss of one alkyl group (R) bound to the phosphorus. The aging reaction depends on the chemical structure of the inhibitor and leads to a stable nonreactivatable form of phosphorylated AChE. X indicates acyl radical (i.e., Cl^-, F^-, CN^-, *p*-nitrophenol).

system, and processes in neurodegenerative disorders (Darvesh et al., 2003).

AChE terminates the action of ACh at the junctions of the various cholinergic nerve endings with their effector organs or postsynaptic sites. In the presence of OP inhibitors, AChE becomes progressively inhibited and is not further capable of hydrolyzing ACh to choline and acetic acid (Jokanović and Maksimović, 1997). Consequently, ACh accumulates at cholinergic receptor sites and produces effects equivalent to excessive stimulation of cholinergic receptors throughout the central and peripheral nervous systems.

Both substrate and inhibitors react covalently with the esterases in essentially the same manner because of the fact that acetylation of the serine residue at AChE catalytic site is analogous to phosphorylation. In contrast to the acetylated enzyme that rapidly separates acetic acid and restores the catalytic site, the phosphorylated enzyme is stable (Figure 71.1). Inhibited enzymes can be spontaneously reactivated at different rates depending on the inhibitor; for organophosphates with branched alkyl groups such as warfare nerve agents, it may occur at a very slow rate.

The variations in the acute toxicity of OPs are the result of their different chemical structures, rates of spontaneous reactivation, and aging. An aged form of phosphorylated AChE is resistant to both spontaneous and oxime-induced reactivation. For example, when inhibited with VX, AChE in red blood cells spontaneously reactivates at a rate of approximately 1% per hour for approximately the first 48 h, whereas the VX–AChE complex ages very little during this period. The soman–AChE complex does not spontaneously reactivate because the half-time for aging is approximately 2–4 min. The half-time for aging of the sarin–AChE complex is between 5 and 12 h, depending on experimental conditions, and only approximately 5% of the enzyme undergoes spontaneous reactivation. The half-time for aging of the tabun–AChE complex is approximately 46 h (Karczmar, 1984; Sidell, 1997; Stojiljković et al., 2001; Milatović and Jokanović, 2009). For OP pesticides containing dimethyl phosphate groups, the half-time of spontaneous reactivation of phosphorylated AChE *in vitro* is approximately 1 h and the half-time of aging is 3.7 h, whereas these for diethyl phosphates are slower—approximately 31–57 h and 31 h, respectively (Worek et al., 1999; Mason et al., 2000; Eyer, 2003).

Although it has many phosphorylated AChE complexes, the aging reaction has major clinical importance and is an imperative problem, particularly in the treatment of soman poisoning. Aging with soman occurs so fast that no clinically relevant spontaneous reactivation of AChE can occur before aging has taken place. Hence, recovery of function depends on the resynthesis of AChE, which is a relatively slow process. After soman exposure, it is important to immediately administer atropine and oximes so that some extent of AChE reactivation occurs before the entire AChE has aged. Even though aging occurs slowly in the case of other warfare nerve agents and OPs, early oxime administration is of high clinical importance for treatment efficiency.

CLINICAL ASPECTS OF ACUTE OP POISONING

Signs and symptoms of acute poisoning with OPs are predictable from their biochemical mechanism of action and are directly related to the levels of AChE activity (Table 71.1). The types of effects manifested in these poisonings are muscarinic, nicotinic, and central signs and symptoms. The duration of effects is determined mainly by the properties of the compound: its liposolubility, the stability of the OP–AChE complex, and whether it is reactivatable after the use of ChE reactivators, such as oximes. It is important to note that only OPs containing the P═O bond, known as direct inhibitors, are potent AChE inhibitors, whereas those with a P═S group, indirect inhibitors, must be metabolically activated to the P═O group (Jokanović, 2001). The signs and symptoms of poisoning with direct inhibitors appear quickly during or after exposure, whereas those with indirect inhibitors appear slower and last longer, even up to several days after cessation of exposure.

TABLE 71.1 Signs and Symptoms of Poisoning with OP Compounds (Milatović and Jokanović, 2009)

Signs and Symptoms			Severity of Poisoning	Red Blood Cell AChE (% of Control)
Muscarinic	**Nicotinic**	**Central**		
Nausea, vomiting, diarrhea, salivation, lacrimation, bradycardia and arrhythmia, bronchoconstriction, bronchosecretion		Headache, dizziness, drowsiness, anxiety	Mild	>40
As above plus miotic pupils (unreactive to light), involuntary defecation and urination	Twitching of fine muscles, hyperreflexia, fasciculations	As above plus ataxia, psychosis, tremor, dysarthria, slurred speech	Moderate	20–40
	As above plus muscle weakness, reduced tendinous reflexes, paralysis affecting diaphragm and respiratory muscles	As above plus coma, convulsions, respiratory depression	Severe	<20

Clinical diagnosis is relatively simple and is based on medical history, circumstances of exposure, and the presence of clinical symptoms of poisoning. Confirmation of diagnosis can be made by measurement of red blood cell AChE or plasma ChE. Activities of these enzymes are accepted as biomarkers of exposure and/or toxicity of OPs. Red blood cell AChE is identical to the enzyme present in the target synapses and its levels are assumed to reflect the effects in target organs. Thus, RBC AChE is regarded as a biomarker of toxicity of these compounds. AChE in the brain is restored by *de novo* synthesis more rapidly than in erythrocytes, where AChE activity is recovered via erythropoiesis. The level of activity of ChE should be carefully interpreted because the normal range in healthy subjects is relatively wide, rendering interpretation in individual patients difficult unless the results of previous estimations in the patient are available. Inhibition of ChE does not provide accurate information related to the clinical severity of the poisoning. Many OP insecticides, for example, chlorpyrifos, demeton, and malathion, are apparently more potent inhibitors of ChE than they are of erythrocyte AChE and, consequently, ChE inhibition might occur to a greater extent than AChE inhibition (Jokanović, 2009).

The first 4–6 h are the most critical in acute poisoning with OP pesticides. If there is improvement in symptoms after initial treatment, then the patient is very likely to survive if adequate treatment is continued (IPCS, 1998; Jokanović, 2009). The duration of effects is determined mainly by the properties of the compound: its liposolubility, the stability of the OP–AChE complex, and whether it is reactivatable after the use of ChE reactivators such as pyridinium oximes.

The rate of spontaneous reactivation (Figure 71.1, Reaction 2) can be accelerated by pyridinium oximes that have a chemical structure that "fits" the structure of the inhibited AChE. The oximes can only be of benefit as long as inhibited AChE is not completely converted to the aged form.

ANTIDOTES IN THE TREATMENT OF OP POISONING

Atropine

Atropine acts by blocking the effects of excess concentrations of ACh at muscarinic cholinergic synapses after OP inhibition of AChE. Atropine is the initial drug of choice in acute OP poisoning that can relieve the following symptoms of OP poisoning: sweating; salivation; rhinorrhea; lacrimation; nausea; vomiting; and diarrhea. It can also help control bradycardia and circulatory depressions and dilating of the bronchi, and it can abolish bronchorrhea. Atropine does not bind to nicotinic receptors and cannot relieve nicotinic effects of OP compounds. In addition, there is evidence regarding the anticonvulsant properties of atropine in poisoning with soman and VX (McDonough et al., 1987; Zilker, 2005).

The standard dosing of atropine depends on the severity of OP poisoning. The initial dose is usually 2 mg in an adult (0.02 mg/kg in a child) administered every 5–10 min until hyperatropinization (flushing, dryness of the mouth, nose, lungs, and the skin, heart rate 80–100/min, normal blood pressure, mydriasis). The dose may be increased as required. Patients poisoned with OPs appear to be resistant to the toxic effects of atropine and may require relatively large doses of atropine administered during prolonged period of time. According to IPCS (2002) in severe OP poisoning, the total dose of atropine given during 5 weeks of treatment can be as high as 30,000 mg.

In an open-label, randomized clinical trial, Abedin et al. (2012) compared the efficacy and safety of conventional

bolus doses of atropine with individual incremental doses for atropinization followed by atropine infusion for management of OP poisoning. It was concluded that rapid incremental dose atropinization, followed by atropine infusion reduced mortality and morbidity from OP poisoning and shortened the length of the total hospital stay and recovery. They recommended that incremental atropine and infusion should become the treatment of choice for OP poisoning.

Diazepam

Benzodiazepines are CNS depressants, anxiolytics, and muscle relaxants. Their main site of action is at the gamma-aminobutyric acid (GABA) receptor. The $GABA_A$ receptor is a ligand-gated chloride ion channel and is part of a superfamily of receptors that also includes the nicotinic ACh receptor and the glycine receptor. GABA is the major inhibitory neurotransmitter in the mammalian CNS. Benzodiazepines, including diazepam, alter GABA binding at the $GABA_A$ receptor site in an allosteric fashion, but these drugs do not directly activate the receptors (Sellström, 1992; Marrs, 2004).

Currently, the most important anticonvulsant is diazepam. The combination of atropine and diazepam is more effective in reducing mortality than atropine or oxime alone. It was also shown that diazepam enhanced the efficacy of low doses of atropine. In the cholinergic nervous system, diazepam appears to decrease the synaptic release of ACh. The main consequence of the action of benzodiazepines in CNS is the hyperpolarization of neurons. This makes neurons significantly less susceptible to cholinergically induced depolarization. The ultimate result is cessation of convulsions propagation (Sellström, 1992; Marrs, 2004; Jokanović, 2009).

In patients poisoned with OPs, benzodiazepines may have a beneficial effect in reducing anxiety and restlessness, reducing muscle fasciculation, arresting seizures, convulsions, controlling apprehension and agitation, and possibly reducing morbidity and mortality when used in conjunction with atropine and an oxime. Diazepam should be given to patients poisoned with OPs whenever convulsions or pronounced muscle fasciculation are present. In severe poisoning, diazepam administration should be considered even before these complications develop. The recommended dose of diazepam in cases of OP poisoning is 5–10 mg intravenous in the absence of convulsions and 10–20 mg intravenous in cases with convulsions, which may be repeated as required (Johnson and Vale, 1992).

It appears that other anticonvulsant benzodiazepines, such as midazolam, avizafone, and lorazepam, are just as effective in stopping nerve agent–induced seizure as diazepam in a hospital setting. In animal studies, midazolam was even more effective than diazepam (Bokonjić and Rosić, 1991). FDA and the French army are considering approval of midazolam as treatment of nerve agent–induced seizures (Sidell et al., 2009; Masson, 2011; Jokanović, 2012). In addition to diazepam, Delacour and Dorandeu (2014) recommended administration of clonazepam in the management of severe OP poisoning.

Detailed therapeutic protocols used in the treatment of poisoning with OPs are presented in several excellent reviews (World Health Organization, 1986; Lotti, 1991; Bismuth et al., 1992; Johnson and Vale, 1992; Johnson et al., 2000; Marrs and Vale, 2006; Jokanović, 2009; Jokanović and Prostran, 2009; Jokanović et al., 2010; Jokanović, 2012; Balali-Mood and Saber, 2012).

Oximes

Extensive studies over the past decades have investigated the mechanism of oxime action. There is convincing evidence that the antidotal potency of oximes is primarily attributed to their abilities to reactivate the phosphorylated ChEs. Oximes reactivate phosphorylated ChEs by displacing the phosphoryl moiety from the enzyme because of their high affinity for the enzyme and powerful nucleophilicity. The rate of reactivation depends on the structure of the phosphoryl moiety bound to the enzyme, the source of the enzyme, the structure and concentration of oxime that is present at the active site, the rate of postinhibitory dealkylation known as aging, and the steric hindrance effects between oxime molecule and phosphoryl moiety attached to the active site of AChE (Jokanović and Stojiljković, 2006; Maxwell et al., 2008). Phosphorylated oximes are formed during reactivation reaction and some of them appear to be potent inhibitors of AChE (Luo et al., 1999; Ashani et al., 2003; Worek et al., 2007). It was shown that phosphorylated oximes of 2-substituted pyridinium compounds (e.g., 2-PAM, HI-6) are unstable, whereas those of 4-pyridinium aldoximes are markedly stable (Worek et al., 2000; Ashani et al., 2003; Kiderlen et al., 2005) (Figure 71.2).

Oximes bind to AChE as reversible inhibitors and form complexes with AChE at the acylation (catalytic) site, at the allosteric site, or at both sites of the enzyme and protect AChE from phosphorylation. When the reversible inhibitor binds to the catalytic site, the protection is due to direct competition between OP and reversible inhibitor. Binding of a reversible inhibitor to the allosteric site induces indirect protection of the active site. Differences in the mechanisms of enzyme reactivation and protection demonstrate how stereochemical arrangements of oximes can play a role in the potency of their therapeutic efficacy. Direct pharmacological effects, such as direct reaction with OPs (van Helden et al., 1996; Jokanović and Prostran, 2009; Jokanović, 2012) and anticholinergic and sympathomimetic effects may also be relevant for the interpretation of antidotal potency of oximes.

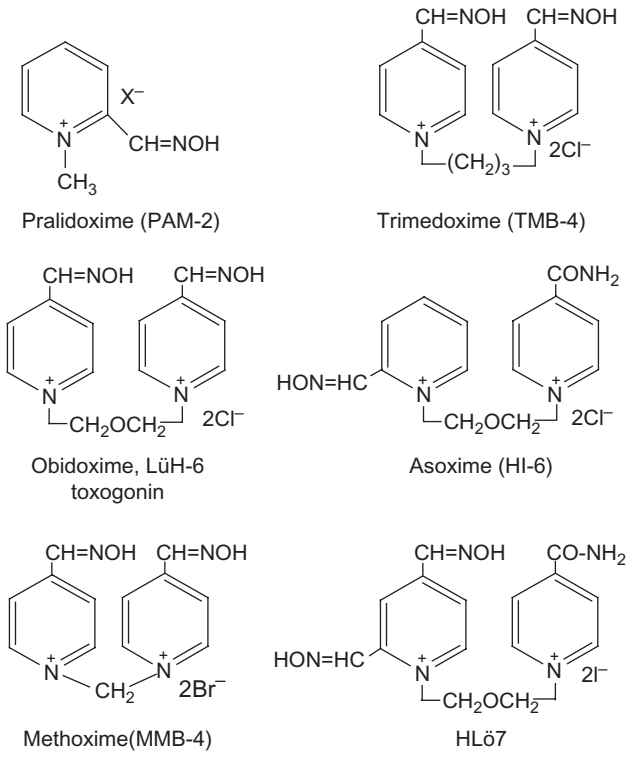

FIGURE 71.2 Chemical structure of pyridinium oximes used in treatment of OP compound poisoning. X indicates an anion (in PAM-2 it can be Cl^-, I^-, or methanesulfonate).

Mono-pyridinium and bis-pyridinium oximes are effective against OP-inhibited AChE in the peripheral nervous system (PNS) but have a limited penetration across the BBB. Limited penetration of HI-6 is due to its pharmacokinetic profile and the two quaternary nitrogen atoms in its structure. However, it appears that oxime penetration through the BBB is underestimated because soman can cause a seizure-related opening of the BBB (Carpentier et al., 1990; Grange-Messent et al., 1999) and thus enable the passage of higher oxime concentrations into the brain. Abdel-Rahman et al. (2002) have shown that 0.5–1.0x LD_{50} of sarin caused a dose-dependent increase in permeability of BBB in midbrain, brainstem, cerebrum, and cerebellum in rats 24h after poisoning. Sakurada et al. (2003) have determined the amount of 2-PAM passing across the BBB at approximately 10% of the given dose, which may be effective in reactivation of OP-inhibited AChE in brain. Obidoxime concentrations in brain were estimated to be 3–5% of plasma levels in rodents (Falb and Erdmann, 1969), and those of HI-6 were estimated to be up to 10% of the serum highest concentration (Cassel et al., 1997). Several studies (Kassa, 2002; Shrot et al., 2009) suggested that such oxime concentrations in the brain are apparently sufficient to produce biochemical and physiological effects in soman poisoning, particularly in specific brain regions, such as the medulla oblongata. These effects can contribute to survival after OP intoxication. Others claim that it is uncertain whether this amount is sufficient to reactivate nerve agent–inhibited AChE (Shih et al., 2011). When AChE reactivation was achieved in the pontomedullar region, good therapeutic effects of oximes were observed and survival of poisoned animals correlated with the AChE activity in this region (Bajgar et al., 2007). In addition, it was suggested that in pralidoxime and obidoxime penetration through the BBB, a carrier-mediated transport mechanism might be involved (Lorke et al., 2008). For further details on oximes crossing the BBB, see chapter 49.

PYRIDINIUM OXIMES IN MANAGEMENT OF POISONING WITH WARFARE NERVE AGENTS

Pralidoxime (2-PAM)

Pralidoxime was synthesized in the United States in 1955 (Wilson and Ginsburg, 1955). Its four salts, chloride (2-PAM Cl), methiodide, methylsulfate, and mesylate (P2S), were investigated and introduced into practice. 2-PAM is very effective in reactivating AChE inhibited with sarin or VX (Johnson and Stewart, 1970; Sidell and Groff, 1974; Harris and Stitcher, 1983; Mesić et al., 1991; Masuda et al., 1995; Nozaki and Aikawa, 1995), but it was not successful in the reactivation of the tabun-inhibited or soman-inhibited enzyme (Inns and Leadbeater, 1983; Koplovitz and Stewart, 1994).

Because 2-PAM, as quaternary pyridinium salt, penetrates the BBB only to approximately 10% of its blood concentration, its pro-drug pro-2-PAM (*N*-methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride), which apparently rapidly oxidizes in brain to 2-PAM, was synthesized and tested in experimental animals. Pro-2-PAM provided some reactivation of sarin-inhibited and VX-inhibited AChE in the brain, blood, and peripheral tissues of the guinea pigs, which was reflected by a limited ability to block or terminate seizures elicited by these agents. Pro-2-PAM provided marginal reactivation of blood, heart, and spinal cord AChE inhibited by cyclosarin, but it was not effective against cyclosarin-induced seizures (Shih et al., 2011). Pro-2-PAM was effective in suppression and elimination of seizures/status epilepticus in DFP-treated guinea pigs. In addition, pro-2-PAM provided a significant reduction of neurological damage in DFP-poisoned guinea pigs. In both cases, 2-PAM was ineffective (DeMar et al., 2010). Clement (1979) reported that pro-2-PAM was effective in protecting mice poisoned with DFP and sarin, but only very slightly effective against soman in guinea pigs. In summary, pro-2-PAM was not a significant improvement

over 2-PAM with regard to prophylaxis against noted OP compound. However, pro-2-PAM was less effective than 2-PAM in experimental poisoning with paraoxon in mice (Bošković et al., 1980).

When given together, atropine and 2-PAM act synergistically, involving different mechanisms; the net effect is protection/treatment of animals poisoned with very high doses of certain OPs. Studies with nonhuman primates proved that the combination of atropine and 2-PAM provided protection up to five-times the LD_{50} of all known nerve agents except soman (Stojiljković et al., 2001) and against 128x LD_{50} of paraoxon (Murphy, 1986).

2-PAM may be given by slow intravenous injection over 5–10 min, by intravenous infusion over 15–30 min, or by subcutaneous or intramuscular injection; it has also been given orally (Pralidoxime, 2009). The minimum therapeutic concentration of 2-PAM in plasma is 4 μg/mL, and this level is reached in approximately 16 min after a single injection of 600 mg 2-PAM Cl (Pralidoxime, 2007).

In the treatment of OP poisoning, atropine and 2-PAM should be given as soon as possible and maintained as long as needed. When the effects of atropine become apparent, 1–2 g of 2-PAM chloride, iodide, or mesylate should be given intramuscularly or intravenously and repeated after 1 h and then every 8–12 h if necessary. Alternatively, 2-PAM Cl may be given in an initial dose of 30 mg/kg by intravenous infusion over 20 min, or by intravenous injection over at least 5 min if pulmonary edema is present or if infusion cannot be given; the initial dose is then followed by intravenous infusion at a rate of 8 mg/kg/h (IPCS, 1998; Milatović and Jokanović, 2009; Pralidoxime, 2009). 2-PAM is relatively short-acting (the apparent half-life of 2-PAM Cl is 74–77 min) and repeated doses may be needed, especially when there is any evidence of continuous absorption of the OP compound (Pralidoxime, 2007). The treatment should be continued until no longer needed. This decision can be made on the basis of the clinical status of the patient, relatively high AChE activity in erythrocytes when compared with control values, and the absence of OP compounds and/or OP metabolites in urine (Jokanović, 2009).

In some countries, autoinjectors are available for emergency use containing 2-PAM, either alone or combined with atropine and/or avizafone, a water-soluble prodrug of diazepam (Pralidoxime, 2009). The French army is considering including water-soluble benzodiazepine midazolam in its autoinjector (Masson, 2011). Typical doses are 600 mg of 2-PAM Cl or 500 mg of 2-PAM mesylate given intramuscularly up to three times, depending on symptoms. In severe poisoning, 2-PAM may be given as a continuous infusion of 200–500 mg/h, titrated against response. A maximum dose of 12 g in 24 h has been suggested (Pralidoxime, 2009).

2-PAM has been very well-tolerated in most cases (Pralidoxime, 2007). Adverse effects of 2-PAM in volunteers include dizziness, drowsiness, blurred vision, occasional diplopia, impaired accommodation, nausea, headache, tachycardia, hyperventilation, increased systolic and diastolic blood pressures, and muscular weakness. Tachycardia, laryngospasm, and muscle rigidity have been attributed to giving pralidoxime intravenously too quickly. Large doses of pralidoxime may cause transient neuromuscular blockade (Jager and Stagg, 1958; Sidell and Groff, 1971; Eyer, 2003; Pralidoxime, 2007; Pralidoxime, 2009). Forty to 60 min after intramuscular injection, mild to moderate pain may be experienced at the site of injection. Elevations in AST and/or ALT enzyme levels in blood were observed in one out of six normal volunteers given 1,200 mg of 2-PAM Cl intramuscularly, and in four out of six normal volunteers given 1,800 mg intramuscularly. Levels returned to normal in approximately 2 weeks (Pralidoxime, 2007). 2-PAM should be used cautiously in patients with renal impairment and a reduction in dosage may be necessary. Caution is also required in giving pralidoxime to patients with myasthenia gravis because it may precipitate a myasthenic crisis (Pralidoxime, 2009).

The clinical experience with the use of 2-PAM iodide, given with atropine and diazepam, in the treatment of the victims of Tokyo sarin attack in 1995 was very favorable (Sidell et al., 2009). However, 2-PAM should not be recommended as the drug of choice for the management of poisoning with all OPs due to its lack of efficacy against tabun and soman.

Trimedoxime (TMB-4)

TMB-4 Cl_2 was synthesized in the United States in 1957 (Poziomek et al., 1958). It is the only one of the major bispyridinium oximes with a propylene bridge between the two pyridinium rings. It was shown that TMB-4 is a more potent reactivator of the DFP-inhibited AChE than 2-PAM (Hobbiger and Sadler, 1958) and better reactivator than LüH-6 in the case of the tabun-inhibited enzyme (Hobbiger and Vojvodić, 1966). TMB-4 was the first oxime that was efficient in the treatment of the animals intoxicated with tabun (Schoene and Oldiges, 1973; Maksimović et al., 1980; Bokonjić et al., 1993). It could also protect the animals poisoned with sarin or VX, but not the ones intoxicated with soman (Maksimović et al., 1980; Inns and Leadbeater, 1983; Binenfeld, 1986). Although TMB-4 was the most toxic oxime among the "great four," it was shown in mice that its LD_{50} is three-times, four-times, and eight-times less than that for LüH-6, 2-PAM, and HI-6, respectively (Clement, 1981).

Obidoxime (LüH-6, Toxogonin)

After being introduced into medical practice in 1964, obidoxime showed significant potential as an antidote

in poisonings with OPs (Erdmann and Engelhard, 1964). Given with atropine, obidoxime efficiently protected experimental animals against poisoning with tabun (Inns and Leadbeater, 1983; Maksimović et al., 1989), sarin (Inns and Leadbeater, 1983; Maksimović et al., 1989), and VX (Maksimović et al., 1989). Obidoxime was more effective than TMB-4 as an antidote against intoxication with tabun (Heilbronn and Tolagen, 1965). Similarly to 2-PAM and TMB-4, obidoxime was also inefficient in soman poisoning in mice (Maksimović et al., 1980), guinea pigs (Inns and Leadbeater, 1983), and primates (Hamilton and Lundy, 1989). Obidoxime was more efficient than HI-6 against tabun intoxication. Similarly, obidoxime was more effective as a medical countermeasure after intoxication with most ChE-inhibiting insecticides, whereas HI-6 was considered to be a better drug against soman-inhibited AChE (Aas, 2003). However, LüH6 was not a potent reactivator of tabun-inhibited human AChE (Worek et al., 2004; Lundy et al., 2011). In contrast to TMB-4, obidoxime, when administered with atropine in guinea pigs pretreated with pyridostigmine, could provide some protection against soman as well (Inns and Leadbeater, 1983).

When administered to human volunteers by intramuscular route, 5 mg/kg of LüH-6 produced a plasma concentration more than 4 mg/L, from 5 min after injection to 3 h (Sidell and Groff, 1970). Adverse effects of LüH-6 in male volunteers were described as pallor, nausea, burning sensation, headache, generalized weakness, sore throat, and paresthesia of the face (Simon and Pickering, 1976; Eyer, 2003; Marrs and Vale, 2006). Transient hepatotoxic effects of LüH-6 were reported in approximately 10% of severely poisoned patients (Marrs, 1991; Eyer, 2003).

Obidoxime can be given with atropine in the treatment of OP poisoning in a usual initial dose of 250 mg (4 mg/kg) by slow intravenous injection. This may be followed by intravenous infusion of 750 mg over 24 h, continued until the concentration of OP is below critical levels; alternatively, repeated doses of 4–8 mg/kg may be given at intervals of 2–4 h. It has also been given by intramuscular injection (Obidoxime Chloride, 2009).

Asoxime (HI-6)

The first oxime that could reactivate soman-inhibited AChE and provide at least some protection of the animals experimentally poisoned with this nerve agent was synthesized in 1966. It has been shown that HI-6 is more potent than LüH-6 and HS-6 (1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) in the protection of various rodent species from intoxication with soman (Oldiges and Schoene, 1970; Inns and Leadbeater, 1983; Mesić et al., 1991; Lundy et al., 2011), as well as sarin and particularly VX (Maksimović et al., 1980; Inns and Leadbeater, 1983). HI-6 could not reactivate tabun-inhibited AChE (Clement, 1982; Ćetković et al., 1984; Kassa et al., 2008), and it was inefficient when used as the only oxime against poisoning with tabun (Maksimović et al., 1980; Inns and Leadbeater, 1983; Mesić et al., 1991; Bajgar, 2010). However, other studies found that HI-6 can provide a similar degree of protection against tabun and soman intoxication in primates (Hamilton and Lundy, 1989). It is also suggested that sufficiently high doses of HI-6 could even protect rats from the multiple lethal doses of tabun (Lundy et al., 1989). HI-6 given with atropine protected guinea pigs from poisoning against 5x LD_{50} of soman or cyclosarin (Lundy et al., 2005). It was suggested that HI-6 is superior to the other oximes in treatment of cyclosarin-poisoned animals (Kassa, 2002).

The acute toxicity of HI-6 is the lowest among the six oximes (HI-6, 2-PAM, TMB-4, LüH-6, HLö-7, MMB-4) (Maksimović et al., 1980; Clement, 1981; Rousseaux and Dua, 1989; Bartošova et al., 2006) with LD_{50} value after intramuscular administration of 781 mg/kg in rats.

HI-6 was introduced into clinical practice in Serbia in the early 1980s. It was administered to more than 200 patients poisoned with OP insecticides and to volunteers. A clinical study with HI-6 administered intramuscularly at doses up to 500 mg in 22 healthy volunteers revealed no adverse effects. However, with patients poisoned by several OP insecticides, HI-6 provided rapid reactivation of AChE in almost all cases except in dimethoate and phosphamidon poisoning (Kušić et al., 1985; Jovanović et al., 1990; Kušić et al., 1991; Jokanović and Maksimović, 1995; Jokanović et al., 2010). Similar results regarding HI-6 tolerance were later found in a double-blind, placebo-controlled, single-dose study in 24 volunteers (Clement et al., 1995). Safety of HI-6 administration was in correlation with the recent report of Pohanka et al. (2011), who found that therapeutic doses of HI-6 are safe in dogs but 10-fold therapeutic doses can cause hyperglycemia.

Clinical studies have shown that HI-6 dosed at either 250 or 500 mg by intramuscular route reached plasma concentrations more than 4 mg/L in 4–6 min. This concentration was maintained for 125 min after the lower dose of 250 mg and 200 min after the higher dose of 500 mg (Kušić et al., 1985; Jokanović and Maksimović, 1995).

HI-6 is considered to be a very promising bispyridinium oxime in medical treatment after exposure to most nerve agents. For these reasons, HI-6 is involved in the equipment of the Czech, Slovak, Swedish, and Canadian Armies as an antidote against nerve agent intoxication (Bajgar, 2010; Masson, 2011) and is under development in other countries (Lundy et al., 2011; Masson, 2011; Jokanović, 2012). A disadvantage of HI-6 compared with

other available oximes is the lack of stability in aqueous solutions (Kušić et al., 1985; Aas, 2003).

In addition to the four pyridinium oximes that were introduced to clinical practice, the oximes HLö-7 and MMB-4 require attention after proving to be efficient against warfare nerve agents in animal studies.

HLö-7

Another important "Hagedorn oxime" (after LüH-6, HS-6 and HI-6), HLö-7, was also synthesized in Freiburg, Germany, in 1986 (Löffler, 1986). Only HLö-7 could reactivate AChE inhibited by any of the four major nerve agents *in vitro* and *in vivo* (DeJong et al., 1989; Worek et al., 1994a,b, 1995), as well as AChE inhibited by cyclosarin (Lundy et al., 1992). In addition, HLö-7 more efficiently restored the neuromuscular transmission impaired by *in vitro* superfusion of the neuromuscular preparation with tabun, sarin, soman, or cyclosarin compared with 2-PAM, LüH-6, and HI-6 (Alberts, 1990). It was found that HLö-7 induced a significant reactivation of AChE in mice diaphragms inhibited with tabun, sarin, soman, and cyclosarin (Clement et al., 1992). Both HI-6 and HLö-7 can antagonize sarin-induced hypothermia, proving that, when given with atropine, they can pass the BBB and gain access to the CNS (Clement, 1992; Clement et al., 1992). The toxicity of HLö-7 was 2.5-times higher than that of HI-6 (Clement et al., 1992). The cardiovascular tolerability of HLö-7 was similar but still not as good as HI-6, at least in anesthetized guinea pigs (Worek and Szinicz, 1993). HLö-7 appears to be more effective than HI-6 against tabun and VX poisoning and less effective against sarin, soman, and cyclosarin intoxication (Eyer et al., 1992; Lundy et al., 1992). HLö-7-induced protection against tabun poisoning in guinea pigs was significantly better compared with HI-6, whereas HI-6 was only slightly more efficient than HLö-7 in soman poisoning (Melchers et al., 1994a,b). However, Kuča et al. (2005, 2011) reported that HLö-7 was not a good reactivator of tabun-inhibited AChE *in vitro*, possibly due to the presence of the lone electron pair on the tabun amide group.

The pharmacokinetic profile of HLö-7 was similar to that of HI-6. The mean absorption half-time of HLö-7 was approximately 14 min after intramuscular administration. Maximum HLö-7 concentration in plasma was reached after 30 min, and the half-time of elimination was approximately 45 min (Eyer et al., 1992).

Methoxime (MMB-4)

MMB-4 is a structural analog of toxogonin showing potent antimuscarinic activity (Amitai et al., 1980). It is a bis-pyridinium 4,4′-bis-aldoxime first described by Hobbiger and Sadler (1959). MMB-4 is currently being proposed as a leading candidate for the replacement of 2-PAM as an oxime reactivator of nerve agent–inhibited AChE activity that can prevent lethality after warfare nerve agent exposure (Worek et al., 2010; Lundy et al., 2011; Masson, 2011). So far, the oxime MMB-4 has not undergone any clinical trials (Lundy et al., 2011).

MMB-4 was less toxic in mice (LD_{50} = 441 mg/kg, intramuscular) than obidoxime (188 mg/kg, intramuscular) but more toxic compared with HI-6 (671 mg/kg, intramuscular) (Bartošova et al., 2006). It was shown that MMB-4 was inferior to HI-6 in reactivation of peripheral AChE from soman-poisoned animals, although both oximes were better than 2-PAM. In soman-poisoned rats MMB-4 was not successful in protection or reactivation of soman-inhibited AChE (Shih, 1993). Luo et al. (2008) found that MMB-4 was not a good reactivator of soman-inhibited AChE obtained from human and three monkey species, in which it was found to be less effective compared with HI-6. This is consistent with the findings reported in other studies (Lundy et al., 2011).

MMB-4 was also less effective in reactivation of cyclosarin-inhibited rat brain AChE and sarin-inhibited human or monkey AChE compared with HI-6 (Kuča and Patočka, 2004; Lundy et al., 2011). MMB-4 was found to be a weak reactivator of tabun-inhibited human AChE (Worek et al., 2004). However, experimental data indicate that MMB-4 was superior to PAM-2 in reactivating OP-inhibited AChE and in preventing lethality in OP-poisoned animals (Worek et al., 2010). It was also reported that MMB-4 was the most effective oxime in reactivation of ChE in blood and peripheral tissues in guinea pigs poisoned by sarin, cyclosarin, VX, and VR (Shih et al., 2010). In addition, MMB-4 was reported to be a better AChE reactivator than PAM-2 in paraoxon-poisoned and methylparaoxon-poisoned rats (Petroianu et al., 2006, 2007).

PYRIDINIUM OXIMES IN MANAGEMENT OF POISONING WITH OP PESTICIDES

The efficacy of pyridinium oximes has been demonstrated in experimental conditions and in many cases of patients poisoned with OP insecticides who have been treated in European clinics. In those cases, the recommendations for 2-PAM dosing proposed by the WHO were followed (IPCS, 1998).

Contrary to these findings, reports from Asia indicate that 2-PAM treatment was not sufficiently effective in their patients. However, in their studies 2-PAM was not used as recommended by the WHO (de Silva et al., 1992; Singh et al., 1995; Johnson et al., 1996; Cherian et al., 1997, 2005). In addition, these studies were apparently poorly designed due to suboptimal dose, short duration of treatment, long delay between patient exposure, and

2-PAM administration, and with the chemical structure of OP pesticides not being taken in account (Jokanović, 2009; Jokanović et al., 2010).

A particular problem in interpreting the beneficial role and efficacy of pyridinium oximes in clinical practice is a relative lack of published data, especially those evaluated in controlled clinical trials. Studies related to the efficacy of oximes in a clinical setting showed the heterogeneity of therapeutic approaches (i.e., dose regimen, oxime choice, and final outcome of the treatment) (Jokanović, 2009).

Jokanović et al. (2010) reviewed the experience gained in clinical management of 296 patients poisoned with OP insecticides at Clinic of Toxicology of the National Poison Control Center in Military Medical Academy in Belgrade during the period from 1998 to 2007. Poisoning with dimethyl OP was confirmed in 246 patients (malathion, 153; dimethoate, 69; dichlorvos, 4; fenitrothion, 6; monocrotophos, 8), with diethyl OP in 38 patients (diazinon, 21; parathion, 9; phorate, 1; phoxim, 1; phosalone, 3; chlorpyriphos, 2; quinalphos, 1) and 12 patients in whom OP was not fully identified. The majority of poisonings (92%) was due to deliberate ingestion of OP insecticides. Accidental inhalational poisonings were registered in 3.8% patients, accidental ingestion in 4.1% patients, and there were two cases of deliberate intramuscular and intravenous application of OP. Suicidal poisonings were usually oral, and the severity of poisonings depended on the toxicity, the amount of OP insecticides taken, and the time from exposure to admission to the Clinic of Toxicology and initiation of treatment. In this study, 15.9% of OP-poisoned patients died, which was consistent with the findings of other authors (Eddleston et al., 2009) who reported case fatality generally more than 15%. Approximately 42% of the deceased patients died within 7 days, and lethal outcome occurred within hours in 18% patients due to the severity of poisoning.

In this study, 74 patients were treated with pralidoxime methylsulfate (Contrathion®). The dosage regimen for severely poisoned patients included a loading dose of 400 mg by slow intravenous infusion, subsequently 200 mg/h continuous intravenous infusion administered the first day, and continued as long as clinical signs of poisoning and OP insecticide and/or its metabolite were present in urine and blood. In moderate poisoning, 400 mg of Contrathion® was followed by 400 mg every 3–4 h. However, due to the economic crisis in Serbia, the Clinic of Toxicology did not have oxime available for several years and the patients were treated with atropine and diazepam only. In patients treated with atropine and oxime or atropine only, there were no differences in mortality. Atropine consumption was lower in the group of patients treated with an oxime, but the difference was significant only in the group of severely poisoned patients in whom it was reduced by 30%.

Eddleston et al. (2005) conducted a prospective study on 802 patients self-poisoned with chlorpyrifos, dimethoate, or fenthion. Compared with chlorpyrifos (8.0%), the proportion of fatalities was significantly higher with dimethoate (23.1%) or fenthion (16.2%) than was the proportion requiring endotracheal intubation (chlorpyrifos, 15.0%; dimethoate, 35.2%; fenthion, 31.3%). Patients poisoned by the diethyl OP pesticide (chlorpyrifos) responded well to pralidoxime, whereas those exposed to the two-dimethyl OP pesticides (dimethoate, fenthion) responded poorly. The poor efficacy of 2-PAM in the treatment of human dimethoate and fenthion poisonings was in agreement with experimental studies reported by Jokanović and Maksimović (1995), who found that the antidotal efficacy of obidoxime, trimedoxime, 2-PAM, and HI-6, when given with atropine and diazepam, in rats dosed with 2x LD_{50} of dimethoate was low. However, there was a discrepancy between fenthion-poisoned patients and animals in that 2-PAM was ineffective as an antidote in patients, whereas the four oximes showed considerable efficacy in rats.

In a randomized controlled trial, Pawar et al. (2006) studied the effects of very high doses of 2-PAM iodide (2-g loading dose, then 1 g either every hour or every 4 h for 48 h, then 1 g every 4 h until recovery) in 200 patients with moderate OP poisoning (excluding severely ill patients). The OP pesticides involved were chlorpyrifos (diethyl OP) and dimethoate (dimethyl OP). The dosing regimen was associated with reduced case fatality, fewer cases of pneumonia, and reduced time on mechanical ventilation. This study suggests that large doses of 2-PAM could have a positive effect on patients if they are treated early and have good supportive care.

An interesting clinical trial in which 2-PAM chloride was investigated in 235 patients self-poisoned with OP insecticides was reported by Eddleston et al. (2009). The patients were randomized to receive 2-PAM ($n = 121$) or saline placebo ($n = 114$). 2-PAM produced substantial and moderate erythrocyte AChE reactivation in patients poisoned by diethyl and dimethyl compounds, respectively. Mortality was insignificantly higher in patients receiving 2-PAM: 30 out of 121 (24.8%) receiving 2-PAM died compared with 18 out of 114 (15.8%) receiving placebo. The authors concluded that, despite clear reactivation of erythrocyte AChE in diethyl OP insecticide-poisoned patients, they found no evidence that treatment with 2-PAM improves survival or reduces the need for intubation in patients with OP insecticide poisoning. However, this study has a major methodological problem of randomization; the authors wrote that "more severely poisoned patients were allocated to pralidoxime" than to the placebo treatment. Based on this statement, it is expected that more patients would die in the group treated with 2-PAM. This was supported by another sentence present in this report indicating that "patients died sooner

in the pralidoxime group." In addition, there are other problems with this study, such as the lack of atropine inclusion in both groups. The World Health Organization (1986) recommends treatment of OP-poisoned patients with atropine, 2-PAM, and diazepam, and not just with 2-PAM. There also were a relatively small number of patients included in the study, relatively long and variable periods between self-poisoning and administration of 2-PAM (mean, 4.3 h; intervals, 2.9–7.8 h), which probably had an impact on aging of phosphorylated AChE, and ethical issues such as why the patients in the placebo group were allowed to die without the best possible treatment according to the WHO recommendations. Because of these and other problems, the results of this study shall be considered with caution.

Kušić et al. (1991) tested the oxime HI-6 in OP pesticide poisoning in 60 patients. HI-6 was administered four times per day as a single 500-mg intramuscular injection with atropine and diazepam treatment. Oxime therapy was started on admission and continued for 2–7 days. Most patients were treated with HI-6 and nine severely poisoned patients with quinalphos were treated with 2-PAM chloride (1,000 mg four times per day). HI-6 rapidly reactivated human RBC AChE inhibited by diethoxy OPs (phorate, pyridaphenthion, quinalphos) as well as that inhibited by dichlorvos, a dimethoxy OP. AChE inhibited with other dimethoxy OPs, dimethoate and phosphamidon, was reported to be resistant to HI-6 treatment, whereas reactivation with malathion was slow, with reactivation half-time of 10 h. Both HI-6 and 2-PAM successfully reactivated AChE in quinalphos-poisoned patients, with HI-6 acting as a faster AChE reactivator than 2-PAM.

Willems et al. (1993) reported that ethyl parathion and methyl parathion poisoning could be effectively treated with 2-PAM methylsulfate (plasma concentrations 4 mg/L) and atropine when pesticide concentrations in plasma were relatively low. In severe poisoning with pesticide levels in plasma more than 30 µg/L, high 2-PAM concentrations in plasma (14.6 mg/L) did not provide any improvement. In addition, 2-PAM at concentrations of 6.3 mg/L was not effective in AChE reactivation in dimethoate poisoning when AChE was inhibited with its active metabolite omethoate.

It was reported that in parathion poisoning, an obidoxime dose of 250 mg intravenously administered as a bolus followed by infusion of 750 mg/day was effective, but AChE reactivation after severe poisoning did not occur until the concentration of paraoxon in plasma became low (Thiermann et al., 1997, 1999). Oxydemeton methyl poisoning responded to obidoxime therapy only when the oxime was instituted soon after poisoning. In cases when obidoxime treatment was started too late, there was no reactivation of erythrocyte AChE and one out of six patients died.

Nine patients intoxicated with OP pesticides were treated with 2-PAM methylsulfate using a dose of 4.42 mg/kg as a bolus injection followed by continuous infusion of 1.14 mg/kg/h. In patients poisoned with ethyl parathion or methyl parathion, AChE reactivation could be obtained at low oxime concentrations (2.88 mg/L). In others, however, an oxime concentration as high as 14.6 mg/L was ineffective. The therapeutic effect of the oxime apparently depended on the plasma concentrations of the OP pesticides. Due to AChE reinhibition, reactivation was absent as long as these concentrations remained above 30 µg/L (Aragao et al., 1996).

In a clinical study involving 63 patients poisoned with OP insecticides, patients were divided into three groups: one was treated with atropine only and the other two received atropine and either 2-PAM or obidoxime. Initial and maintenance intravenous doses for 2-PAM were 30 mg/kg and 8 mg/kg/h, respectively; and 8 mg/kg and 2 mg/kg/h, respectively, for obidoxime. The major clinical findings or AChE activities at the time of admission did not show statistically significant differences among the groups. Although the severity of intoxications, based on respiratory complications and duration of hospitalization, was higher in the atropine plus oxime groups, 12% and 50% of patients in the atropine and atropine plus obidoxime groups died, respectively. No mortality was found in the 2-PAM plus atropine group. Incidences of recurrent twitching and convulsions, repeated respiratory arrest, requiring mechanical respiration, requiring intensive care unit therapy, and duration of hospitalization were smaller in the atropine plus obidoxime group than in the atropine plus 2-PAM group. Three of the patients who received the obidoxime combination therapy developed hepatitis and two of them died due to hepatic failure, which may indicate overdosage of obidoxime (Balali-Mood and Shariat, 1998).

In the study reported by Tsai et al. (2007), 2-PAM was administered to 56 patients within 48 h of OP pesticides poisoning. The average duration of 2-PAM treatment was 5.5 ± 4.9 days and the mean total dose was 16.4 ± 12.4 g. The mortality and length of stay did not improve with 2-PAM treatment. Days spent on ventilator support were decreased in patients treated with 2-PAM (6.7 ± 1.9 days) compared with those who were not (23.0 ± 4.8 days). However, there are many limitations in this study. For example, a relatively small number of treated patients, lack of data regarding the OP pesticides involved, dose taken, and AChE inhibition.

AChE inhibited by several OP pesticides, including dimethoate, demethon, triamiphos, ethoprophos, profenofos, fenamiphos, and pyridafenthion, was resistant to any attempt of reactivation with any oxime, probably because of the variations in phosphoryl moiety and distribution of electron charge (Bismuth et al., 1992; Jokanović and Maksimović, 1995; Jokanović, 2009; Jokanović et al., 2010; Jokanović, 2012).

Jokanović and Maksimović (1995) have studied the acute oral toxicity of 25 OP insecticides and one OP fungicide (pyrazophos) in the rat, and the efficacy of antidotal treatment involving TMB-4, LüH-6, 2-PAM, or HI-6 (given with atropine and diazepam 1 min after poisoning) in animals dosed with 2x LD_{50} of the OP insecticides. The success of therapy was dependent on the chemical structure of OPs. The oximes were potent antidotes in poisoning with the insecticides with phosphate structure and provided some extent of antidotal protection in poisoning with phosphonates, phosphorothiolates, phosphorothionates, and phosphorodithioates. However, none of them was an effective antidote against dimethoate and pyridafenthion. The study has shown that TMB-4 was the most effective pyridinium oxime in the treatment of OP insecticide poisoning.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Despite the enormous efforts devoted to synthesis and development of new pyridinium oximes as potential antidotes against poisoning with OPs in the past several decades, there were no major advances in designing compounds that would be effective against the nerve agents and other OPs. Only four compounds so far are applicable in human medicine. However, they differ in their activity in poisoning with warfare nerve agents and OP insecticides. There is still no universal broad-spectrum oxime capable of protecting against all known OP compounds.

HI-6 seems to be a promising choice for nerve agent poisoning because it was able to protect experimental animals from toxic effects and improve survival of animals poisoned with sublethal doses, especially in the cases of soman and cyclosarin. HI-6 was also effective against poisoning with OP insecticides. MMB-4 is currently being proposed as a leading candidate for replacement of 2-PAM as an oxime reactivator of nerve agent-inhibited AChE activity, that can prevent lethality after warfare nerve agent exposure. Available experimental evidence suggests that there are no clinically important differences between pralidoxime, obidoxime, and HI-6 in the treatment of nerve agent poisoning. However, obidoxime appears to be a good choice for the treatment of poisoning with OP insecticides, but it is necessary to rigorously test these statements in properly designed randomized clinical trials.

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CHAPTER

72

Novel Cholinesterase Reactivators

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INTRODUCTION

The continuous threat of organophosphorus (OP) nerve agent misuse supports the development of antidotes with enhanced potency. In this chapter, we are focusing on antidotal therapy with acetylcholinesterase (AChE) reactivators. Although this group of compounds has been known for more than a half-century, there is no single compound that might counteract every nerve agent's lethal effects. This chapter describes in detail the design and synthesis of the AChE reactivators that have been developed within the last two decades. Within the design of novel molecules, special emphasis is placed on the structural requirements (structure–activity relationship) of these compounds. Finally, several new trends in the development of new reactivators are discussed.

OP AChE INHIBITORS

OP compounds are widely used in agriculture as pesticides (e.g., dichlorvos, diazinon, chlorpyrifos, and parathion), industry and technology as softening agents and lubricant additives, and in the medical and veterinary fields as therapeutic agents (Gupta, 2006). Some OPs are declared as chemical warfare agents (CWAs), nerve agents, or both. Sarin, cyclosarin, soman, tabun, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), and Russian VX are well-known members of the OP nerve agent family (Table 72.1; Marrs, 1993; Bajgar, 2004, Gupta, 2006).

The history of nerve agents began prior to World War II in Germany. The first-known nerve agent, tabun (*O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate), was synthesized in the laboratories of IG Farben in Germany by Dr. Gerhard Schrader in 1936. Although the original aim of Schrader's studies was synthesizing pesticides, German authorities identified the deadly potential of OPs, and subsequently, many nerve agents, such as sarin, cyclosarin, and soman, were developed. A few decades later, the nerve agent VX was developed in the United Kingdom. Further-developed nerve agents include intermediate volatile agents, an example of which is Agent GP; and novichok (Figure 72.1; Halamek et al., 2007).

During the Cold War, nerve agents were stored and prepared for potential military use, but were not used in military conflicts. However, they were misused by Saddam Hussein in Iraq in the Kurdish village of Birjinni (1988) and by the Japanese Aum Shinrikyo sect in Matsumoto (1994) and Tokyo (1995; Tu, 2000).

Nerve agents phosphonylate serine Ser203 at the esteratic part of the active site of enzyme AChE (EC 3.1.1.7). AChE plays a key role in the termination of action of a neurotransmitter acetylcholine (ACh) in the peripheral nervous system and central nervous system, and persistent inhibition of AChE can lead to life-threatening consequences (Marrs, 1993).

Depending on the particular nerve agent, AChE is further irreversibly phosphorylated via a time-dependent process called *aging*, defined as dealkylation of the phosphoryl adduct to give a negatively charged adduct that is stabilized by interaction with catalytic His440 (Millard et al., 1999; Carletti et al., 2010). Aged enzymes cannot be restored by any nucleophilic reactivators. Because of the irreversible inhibition of AChE, the enzyme is not able to fulfill its physiological role in the organism—namely, splitting the neuromediator ACh at the synaptic clefts. Subsequently, ACh accumulates at the cholinergic synaptic junctions and persistently stimulates cholinergic receptors (Marrs, 1993).

The acute toxicity of nerve agents is usually attributed to excessive cholinergic stimulation caused by

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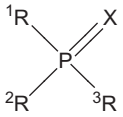
TABLE 72.1 Selected AChE Inhibiting OP Nerve Agents, Pesticides, and Prototype Compounds



Compound	1R	2R	3R	X	Class
Tabun	*O*-Et	N-Me2	CN	O	NA
Sarin	*O*-isopropyl	Me	F	O	NA
Soman	*O*-(3,3-dimethylbut-2-yl)	Me	F	O	NA
Cyclosarin	*O*-cyclohexyl	Me	F	O	NA
VX	*S*-[(2-diisopropyl)aminoethyl]	Me	*O*-Et	O	NA
Russian VX	*S*-[(2-diethyl)aminoethyl]	Me	O-isobutyl		NA
DFP	*O*-isopropyl	*O*-isopropyl	F	O	Pesticide
DDVP	*O*-(2,2-dichloroethenyl)	*O*-Me	*O*-Me	O	Pesticide
Paraoxon	*O*-(4-nitrophenyl)	*O*-Et	*O*-Et	O	Pesticide
Parathion	*O*-(4-nitrophenyl)	*O*-Et	*O*-Et	S	Pesticide

Et, ethyl; Me, methyl.

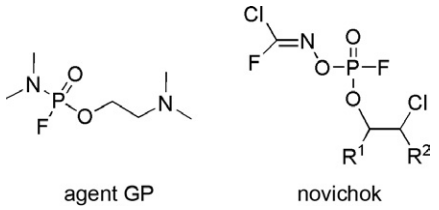


FIGURE 72.1 Proposed structure of new nerve agents (Halamek et al., 2007).

excess accumulation of ACh, followed by subsequent overstimulation of cholinergic pathways and desensitization of cholinergic peripheral and central receptor sites (Bajgar, 2004).

Symptoms of intoxication are as follows: when an individual is exposed to low amounts of a nerve agent, the initial symptoms include runny nose, contraction of the pupils, miosis, deterioration of visual accommodation, headache, slurred speech, nausea, hallucination, pronounced chest pain, and an increase in the production of saliva (muscarinic central and peripheral symptoms). At higher doses, the aforementioned symptoms are more pronounced, and coughing and breathing problems also occur. The individual may then begin to have convulsions (nicotinic symptoms) and may experience impaired ventilation, coma, and death. At even higher doses, an exposed individual would almost immediately go into convulsions and die from respiratory and cardiovascular failure. The initial stages of symptoms of an individual exposed to a nerve agent may vary depending on the particular nerve agent and amount of agent the individual was exposed to (Bajgar, 2004).

ACETYLCHOLINESTERASE (AChE; EC 3.1.1.7)

AChE is a serine hydrolase enzyme that belongs to the esterase family within higher eukaryotes. Enzymes of this family act on different types of carboxylic esters. The biological role of AChE is termination of transmission impulses at cholinergic synapses within the nervous system by rapid hydrolysis of neurotransmitter ACh. The monomer of AChE, with a molecular weight around 60,000, is an ellipsoidal molecule whose size is approximately 45 × 60 × 65 angstrom (Å), consisting of a 12-strand, central, mixed β-sheet surrounded by 14 α-helices (Sussman et al., 1991). Each monomer contains one catalytic center composed of two compartments: the esteratic subsite containing the catalytic triad and the anionic subsite that accommodates the positive quaternary compartment of ACh. In human AChE, the esteratic subsite contains the catalytic machinery of the enzyme: a catalytic triad of Ser203, His447, and Glu334. The anionic subsite is defined by Trp86, Tyr337, and Phe338. Its role is to orient the charged part of ACh that enters the active center. This role is the main function of Trp and Tyr residues (Bourne et al., 2003). The recent rendition of the X-ray structure for AChE places the active catalytic site deep within a gorgelike fold of the protein. The aromatic gorge in the protein is approximately 20 Å deep and penetrates halfway into the enzyme. The active site lies at the base of this gorge only 4 Å above the base, leading some to label this the "active gorge." The aromatic gorge is a more appropriate term because 40% of its content is composed of 14 aromatic residues, which is

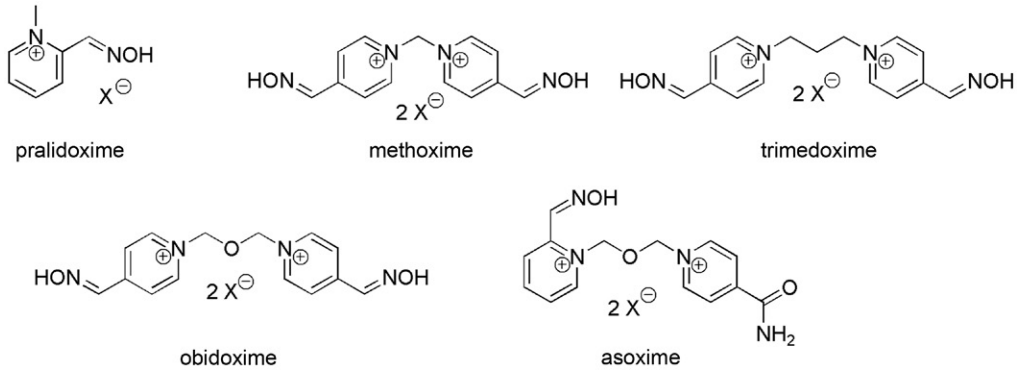


FIGURE 72.2 Standard AChE reactivators (Musilek et al., 2011a).

highly conserved from different species of AChE (Harel et al., 1993). The high aromatic content may explain studies that have proposed hydrophobic and anionic binding sites independently on the active site. Only a few acidic residues are present within the gorge. The aromatic residues play an important role in stabilization of the enzyme–substrate complex. Both electrostatic and hydrophobic effects are important here. The electrostatic potential map of AChE suggests that this enzyme, like the other enzymes with charged substrates, steers its substrate toward its gorge and into the active site.

The second anionic site of AChE, the so-called peripheral anionic site, is located at the active center gorge entry and encompasses overlapping binding sites for different activators and inhibitors. The peripheral anionic site consists of residues Tyr72, Trp286, and Tyr341. Binding of ligands to these residues may be key to the allosteric modulation of AChE catalytic activity (Bourne et al., 2003).

ANTIDOTES FOR AChE INHIBITED BY OP COMPOUNDS

Antidotes developed for treatment of nerve agent intoxication can be divided into two types: prophylaxis, as preexposure administration of antidotes; and post-exposure treatment, consisting of anticholinergic drugs, AChE reactivators, and anticonvulsants. The best-known prophylactic means are carbamates (e.g., pyridostigmine and physostigmine), oximes (e.g., asoxime and the TRANSANT patch), and scavengers (e.g., butyrylcholinesterase, paraoxonase, and phosphotriesterase). The main drugs used for post-exposure treatment are anticholinergic agents (functional antidotes) that antagonize the effects of accumulated ACh at cholinergic synapses, and AChE reactivators (called *oximes* after the functional oxime group) which restore AChE inhibited by the OP inhibitor (causal antidotes). Their effects are synergistic. Centrally active drugs such as benzodiazepines (e.g., diazepam and avizafone) are also used as anticonvulsants (Bajgar, 2004).

Pralidoxime (2-PAM; 2-hydroxyiminomethyl-1-methylpyridinium chloride), obidoxime (1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane chloride), methoxime (MMC-4; MMB-4; 1,1-bis(4-hydroxyiminomethylpyridinium)-methane bromide or chloride), trimedoxime (TMB-4; 1,3-bis(4-hydroxyiminomethylpyridinium)-propane bromide), and asoxime (oxime HI-6; 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane chloride) are considered the most important commercially available AChE reactivators (Figure 72.2). Other AChE reactivators are currently under development in different countries, including HLö-7 (1-[2,4-bis(hydroxyiminomethylpyridinium)]-3-(4-carbamoylpyridinium)–2-oxapropane chloride) and K027 [1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–propane bromide] (Figure 72.3).

From a chemical point of view, standard AChE reactivators are monoquaternary or bisquaternary pyridinium salts bearing a functional oxime group in their molecules. The oxime is able to split the bond between the OP and enzyme. Consequently, free-functioning AChE is released. Unfortunately, none of the abovementioned AChE reactivators is effective against all known nerve agents and OP pesticides (Table 72.2; Kuca et al., 2007a).

DESIGN AND SYNTHESIS OF NEW AChE REACTIVATORS

Nowadays, many scientific institutions around the world are interested in design, synthesis, and improvement of new antidotes against CWAs, especially nerve agents. This is because of the continuous threat of nerve agent misuse by terrorists. Finding a suitable oxime that is sufficiently effective against OP-inhibited AChE (regardless of

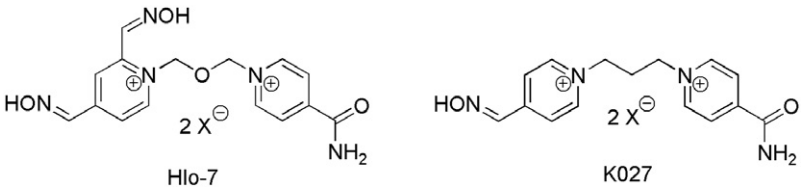


FIGURE 72.3 Selected AChE reactivators that are under development.

TABLE 72.2 Reactivation Potencies of Five Commercially Available Oximes (Kuca et al., 2007a)

	Pralidoxime	Trimedoxime	Obidoxime	Methoxime	Asoxime
VX	34/0	66/10	79/8	59/0	28/13
	++/−	+++/+	+++/+	+++/−	++/+
Russian VX	70/0	30-Apr	66/17	79/36	42/53
	+++/−	++/−	+++/+	+++/++	+++/+++
Sarin	31/0	54/7	26-Mar	43/0	47/50
	++/−	+++/+	++/−	+++/−	+++/+++
Cyclosarin	4/0	0/0	04-Feb	37/90	70/71
	−/−	−/−	−/−	++/++++	+++/+++
Tabun	4/0	28-Oct	37/28	0/0	02-Apr
	−/−	++/+	++/++	−/−	−/−
Soman	0/0	0/0	04-Jan	24-Apr	05-Mar
	−/−	−/−	−/−	++/−	+/−
Chlorpyrifos	38/4	66/38	63/35	45/10	20-Nov
	++/−	+++/++	+++/++	+++/+	++/+

Reactivation (%) at oxime concentration 10 − 3M/10 − 5M.

the type of OP compound) is a very important task. There are several reviews dealing with the synthesis of new cholinesterase reactivators (e.g., Musilek et al., 2007a, 2011a; Mercey et al., 2012a). Several studies related to developments since 2003 in the synthesis of novel reactivators are discussed next.

Pang et al. (2003) prepared bispyridinium dicarbaldoximes (**1**; Figure 72.4) and found a new, efficient method for synthesis of bis-2-hydroxyiminomethylpyridinium salts using bistriflates as alkylating agents. Human erythrocyte AChE inhibited by echothiophate was used for *in vitro* reactivation screening. A compound with three-membered carbon linkers was the most effective from the *p*-series, and a compound with seven carbon connecting chains (called *Ortho-7*) seemed to be the most effective from the *o*-series. These results were demonstrated in the study using 3-D structures.

Kuca et al. (2003a) tried to find a reactivator K048 (**2**; Figure 72.5) with good efficacy against tabun. The *in vitro* test on rat brain homogenate showed that K048 oxime has greater reactivation ability for tabun-inhibited

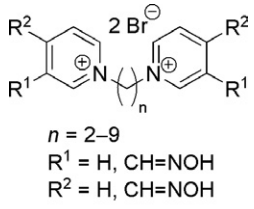


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FIGURE 72.4 Bispyridinium dicarbaldoximes (Pang et al., 2003).

AChE than pralidoxime or asoxime, and is comparable to obidoxime.

Kuca et al. (2003b) prepared a similar potential reactivator of AChE 1-(4-hydroxyiminomethylpyridinium)-3-(carbamoylpyridinium)-propane dibromide (K027; **3**; Figure 72.5) that differs from K048 in length of the connecting chain. Two synthetic pathways were used to reach the proposed structure. The activity of K027 was tested *in vitro* on sarin-inhibited AChE, with promising results.

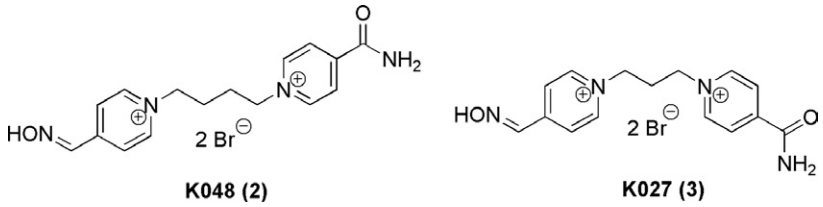


FIGURE 72.5 Reactivator of oximes K048 and K027 for tabun-inhibited AChE (Kuca et al., 2003a,b).

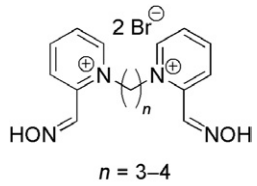


FIGURE 72.6 Synthesis of symmetric bispyridinium dicarbaldoximes (Kuca et al., 2004a).

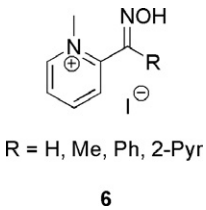


FIGURE 72.7 Monoquaternary compounds prepared by Kuca et al. (2004b).

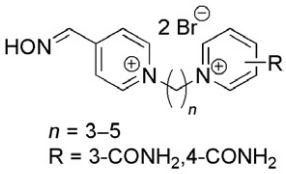


FIGURE 72.8 Reactivators derived from oximes K027 and K048 (Chennamaneni et al., 2005).

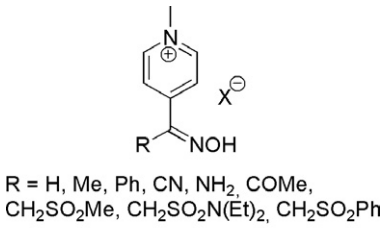


FIGURE 72.9 Structure of compounds prepared by Picha et al. (2005).

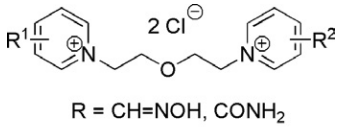


FIGURE 72.10 Structure of reactivators synthesized by Kim et al. (2006).

Kuca et al. (2004a) also synthesized and tested activity of symmetrical bispyridinium dicarbaldoximes (**4–5**; Figure 72.6) *in vitro* against cyclosarin-inhibited AChE. The compound 1,4-bis(2-hydroxyiminomethylpyridinium)butane dibromide (**4**; K033) gave promising results in *in vitro* reactivation of cyclosarin-inhibited AChE compared to commonly used pralidoxime.

In the same laboratory, a new series of four monoquaternary compounds (**6**; Figure 72.7), using the original synthetic strategy was prepared (Kuca et al., 2004b). The reactivation potency was examined *in vitro* on rat brain AChE. All tested compounds were less effective reactivators of sarin, cyclosarin, VX, and tabun-inhibited AChE than pralidoxime.

Chennamaneni et al. (2005) repeated the synthesis of oximes K027 and K048 in experiments conducted in India; moreover, they added four new reactivators derived from these compounds (**7**; Figure 72.8). Consequently, they tested their reactivation activity on tetraethylpyrophosphate-inhibited mouse brain cholinesterases. Their activities were compared to pralidoxime, and all compounds (except the one with a pentylene linker) were found to be more effective. Bispyridinium monooximes with a 3-carbamoyl group were found to be more potent reactivators than corresponding 4-carbamoyl compounds.

Picha et al., (2005) returned to monopyridinium compounds (**8**; Figure 72.9) having a hydroxyiminomethyl group with modified side-chaining, with the aim to improve the nucleophilicity of this group. From the eight oximes prepared and tested, none achieved better reactivation potency compared to pralidoxime, obidoxime, or asoxime (Picha et al., 2005; Jun et al., 2008).

In 2006, a new group of 3-oxapentane linker-containing reactivators (**9**; Figure 72.10) was prepared in South Korea (Kim et al., 2005). Among them, several analogues

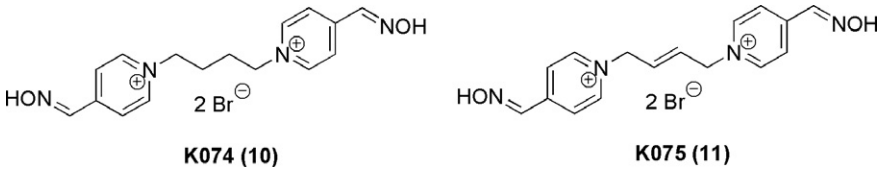


FIGURE 72.11 Structure of oximes K074 and K075 (Kuca et al., 2005a).

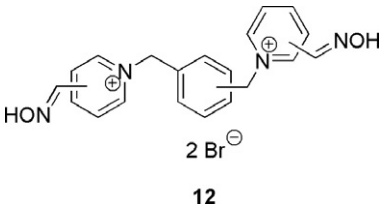


FIGURE 72.12 Structure of xylene containing linker reactivators (Musilek et al., 2005, 2007b).

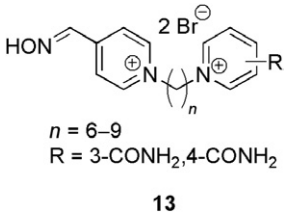


FIGURE 72.13 Structure of monooxime-carbamoyl reactivators (Srinivas Rao et al., 2006).

were found to be promising in cases of paraoxon, diisopropylfluorophosphate (DFP), and tabun-inhibited cholinesterase reactivation (Kuca et al., 2006; Kim et al., 2006).

Very promising compounds (K074-K075; **10-11**; Figure 72.11), which are being tested in many laboratories around the world, were resynthesized in 2005 in the Czech Republic (Kuca et al., 2005a). They showed very promising reactivation activity in the case of tabun and OP pesticide treatment (Musilek et al., 2007c; Lorke et al., 2008a); and this will be discussed more thoroughly later in this chapter.

A new xylene linker connecting two pyridinium rings was studied. In this investigation, 18 new oximes (**12**; Figure 72.12) were prepared and tested for their ability to reactivate tabun and pesticide-inhibited rat brain AChE (Musilek et al., 2005, 2007b; Hrabinova et al., 2006). Several compounds were found to be more promising against tabun, VX, and chlorpyrifos than five commercially available reactivators *in vitro*.

Srinivas Rao et al. (2006) prepared four new series of nonsymmetrical bisquaternary monooximes (**13**; Figure 72.13) with the aldoxime group in position 4 at the pyridinium ring and carbamoyl group in positions 3 and 4 at the second pyridinium ring. This was a follow-up to a similar study done by this group in 2005. The main difference from their previous results was change in the length of the connection chain between two pyridinium rings. Length consisted of six to nine methylene groups. The ability of tested compound to reactivate tetraethylpyrophosphate-inhibited AChE on mouse brain was compared to pralidoxime, and it was found that reactivators with increased linker lengths (from seven to nine methylene groups) potentiated the inhibitory effect of OP.

Musilek and colleagues changed regular connection chains between two pyridinium rings (*n*-methylene or 2-oxapropane) and but-2-ene. When considering steric rearrangement of the but-2-ene linker, there could be two possible geometrical isomers (*E* and *Z*). Both linkers in bisoxime reactivators were used (**14–15**; Figure 72.14). They were tested for reactivation of tabun- and OP pesticide–inhibited AChE. From obtained results, several candidates were considered promising for further evaluation of their reactivation ability (Musilek et al., 2006a–c, 2007e).

Monoquaternary pyridinium compounds with the oxime group in various positions (**16**; Figure 72.15) were prepared with bisquaternary ones (**17**; Figure 72.15) connected via a propane linker. Their reactivation ability was tested on tabun- and OP pesticide–inhibited cholinesterases. As predicted, trimedoxime reached the best activity compared with other oximes synthesized throughout the study (Musilek et al., 2006b,c).

A group of South Korean scientists prepared new oximes with a novel linker having two oxygen atoms in its structure (**18**; Figure 72.16). Their reactivation ability was tested on rat brain homogenate inhibited by agent VX. 1,6-bis(4-hydroxyiminomethylpyridinium)-2,5-dioxahexane dichloride was the most potent and appeared to be the most promising compound as a potential reactivator for AChE inhibited by VX agent. Prepared oximes were tested on housefly AChE and bovine red blood cell AChE inhibited by DFP and paraoxon. In the case of OP pesticide–induced AChE inhibition, the abovementioned oxime achieved promising results (Oh et al., 2006; Yang et al., 2007). Acharya et al. (2008) prepared many other compounds with similar structure. The whole set consisted of 11 compounds that differ in the lengths of their connection chains and positions of oxime groups. They were tested on electric eel AChE inhibited by sarin. Two promising oximes were found that should be considered for further investigation.

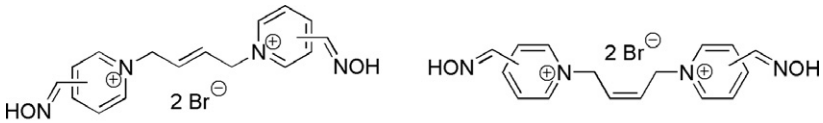


FIGURE 72.14 Oximes with but-2-ene linker in their structure (Musilek et al., 2006a–c, 2007d).

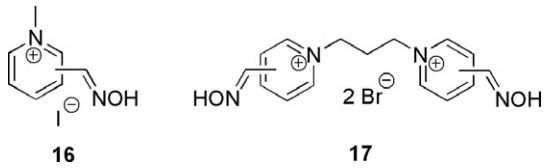


FIGURE 72.15 Monoquaternary and bisquaternary oximes prepared by Musilek et al. (2006b,c).

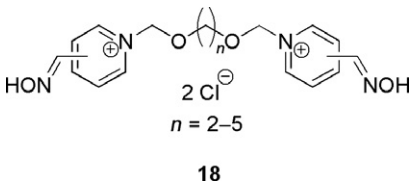


FIGURE 72.16 Structure of oximes prepared in South Korea and India (Oh et al., 2006; Yang et al., 2007; Acharya et al., 2008).

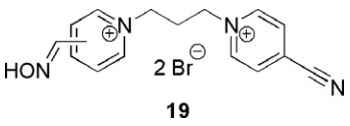


FIGURE 72.17 Structure of oximes with nitril moiety (Musilek et al., 2006d).

Musilek et al. (2006d) prepared bisquaternary reactivators with a carbonitril group instead of the carbamoyl group (**19**; Figure 72.17), which is present in the structure of asoxime, K027, or K048. Only one analogue seemed to be promising against paraoxon-inhibited AChE. None of the tested compounds were able to satisfactorily reactivate tabun-inhibited AChE.

Because asoxime is considered the first choice reactivator, there have been many efforts to improve the way it works. One approach is the choice of the right counteranion in the molecule of reactivator. The anion could influence solubility and stability of the reactivator in solution. In 2007, 12 different salts of asoxime (acetate, bromide, chloride, iodide, maleinate, malonate, mesylate, phosphate, salicylate, sulfate, tartarate, and tosylate) were prepared and tested to evaluate how the anion influences the reactivation process. It was found that there is no difference in reactivation of cyclosarin-inhibited AChE by varying the counteranion (Kuca et al., 2007b).

Because of the presence of promising oximes among those with but-2-ene linker, this pharmacophore was introduced into several other compounds with an oxime group in position 4. Other functional groups were introduced in position 4 at the second pyridinium ring (**20**; Figure 72.18). Thanks to this structural combination, many new promising oximes were found (Musilek et al., 2007f). Among them, oxime K203 (**21**; Figure 72.18) seemed to be the best choice for tabun poisoning. According to the current body of knowledge, it is considered best in terms of reactivating tabun-inhibited AChE (Musilek et al., 2007g).

Because of this promising efficacy, many other compounds with the oxime group on the first pyridinium ring and the carbamoyl group on the other ring were prepared (**22**; Figure 72.19). Among them, no better reactivator of tabun-inhibited AChE was found compared to K203. However, many other promising oximes for treatment of paraoxon-inhibited AChE were investigated (Musilek et al., 2008).

Another five monoquaternary oximes were prepared and tested in Croatia by Odzak et al. (2007) (**23**; Figure 72.20). All oximes reversibly inhibited human AChE. All prepared oximes achieved reactivation of tabun-inhibited AChE within 24 h from 30% to 80% (Odzak et al., 2007).

A South Korean group published a study of synthesis and *in vitro* evaluation of several structurally different cholinesterase reactivators (**24–26**; Figure 72.21). Two from this series (**24**) had already been explored (Pang et al., 2003; Oh et al., 2008). Two other oximes (**25–26**) are very interesting when blood–brain barrier (BBB) penetration is considered. They have either no quaternary nitrogen or only one, where the second ring possesses the tertiary nitrogen without charge. Unfortunately, this structure modification decreased reactivation potency (Oh et al., 2008).

Ohta et al. (2006) published a study of a large group of monoquaternary reactivators (**27**; Figure 72.22) differing in positions of the oxime group and lengths and shapes of their alkylating moiety. Subsequently, Okuno et al. (2008) examined BBB penetration of selected reactivators using *in vivo* rat brain microdialysis with liquid chromatography–tandem mass spectrometry (LC–MS/MS). Increased BBB permeability of modified reactivators was proven, but increased toxicity based on LD_{50} was also found.

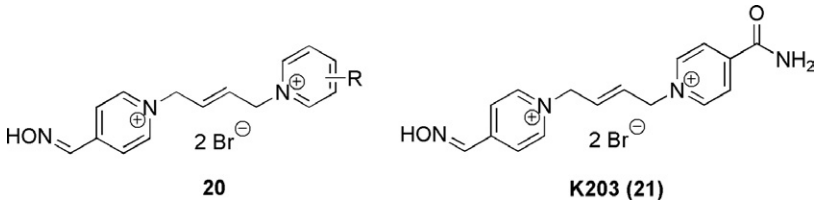


FIGURE 72.18 Structure of promising oximes with but-2-ene linker (Musilek et al., 2007e,f).

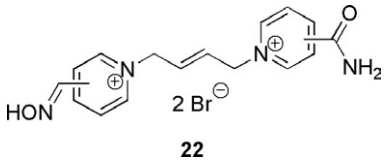


FIGURE 72.19 Analogues of oxime K203 (Musilek et al., 2008).

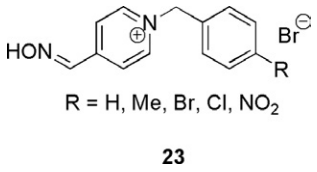


FIGURE 72.20 Monoquaternary reactivators synthesized in Croatia (Odzak et al., 2007).

Other, more recent synthetic results were achieved by Acharya et al. (2008, 2009a,b). The findings of Acharya et al. (2009a) focused on the same topic previously studied by other investigators (Musilek et al., 2005, 2007b; Hrabinova et al., 2006; Acharya, 2008)—namely, a synthesis of series of bispyridinium oximes connected by a xylene linker (**12**; Figure 72.12). The synthesized compounds were tested for their reactivation of sarin-inhibited electric eel AChE. Acharya et al. (2009b) described the synthesis of unique compounds (**28**; Figure 72.23) having *para* and *meta*-xylene, *E*-but-2-ene, and but-2-yne structural motifs in their structure. All these compounds were tested on electric eel AChE inhibited by DFP. Unfortunately, none of the newly prepared compounds could surpass pralidoxime activity.

Jeong et al. (2009) synthesized fluorinated reactivators (**29–30**; Figure 72.24) to increase their BBB penetration. For housefly AChE, some fluorinated compounds turned out to be potent reactivators of paraoxon-inhibited enzyme. BBB penetration was examined only via quantum mechanical calculations that supposed increased lipophilicity of prepared compounds.

Monooxime-monocarbamoyl bispyridinium xylene-linked reactivators (**31**; Figure 72.25) were prepared by Musilek et al. (2010). The compounds were evaluated on the tabun- and paraoxon-inhibited AChE. Compounds did not exceed previously known data for tabun reactivation, but they showed some promising results for paraoxon inhibition. The molecular modeling studies were used to rationalize their binding with active sites of OP-inhibited AChE. Decreased toxicity of prepared compounds (based on LD_{50}) compared to similar bisoximes was proven.

Monooxime bisquaternary reactivators with prop-1,3-diyl linkage and varying moiety on a non-oxime aromatic ring (**32**; Figure 72.26) were prepared by Musilek et al. (2011b). The compounds were examined on tabun- and paraoxon-inhibited AChE. Some of them showed promising reactivation ability for both used OPs. *In vitro* and molecular modeling studies highlighted the previously known oxime K027.

An experiment by de Koning et al. (2011a,b) pioneered a new approach to reactivator development. The chosen peripheral site ligand (PSL) was attached to quaternary pyridinium oxime or non-quaternary keto-aldoxime via an oligo-ethylene glycol linker (**33–34**; Figure 72.27). The prepared compounds did not show promising reactivation ability in case of sarin-, VX-, and tabun-inhibited human AChE compared to commercial standards.

A library of novel AChE reactivators based on imidazole aldoximes and *N*-substituted 2-hydroxyiminoacetamides (**35–36**; Figure 72.28) was screened by Sit et al. (2011). Compounds were screened for reactivation of sarin-, cyclosarin-, VX-, and paraoxon-inhibited human AChE. Reactivators exhibited reactivation activity that was comparable or greater than that of pralidoxime, monoisonitrosoacetone (MINA), and 2,3-butanedione monooxime. Unfortunately, standard substances used in this field (i.e., obidoxime and asoxime) were not compared in this study. More recently, some oximes from this library were tested against tabun-inhibited human AChE (Kovarik et al., 2013). Some selected compounds were able to reactivate tabun inhibition with a performance comparable to pralidoxime, but they were not as effective as the bisquaternary standards trimedoxime and obidoxime. Radic et al. (2012) published presented pyridinium and non-pyridinium oxime reactivators derived from pralidoxime and edrophonium (**37–39**; Figure 72.29). Compounds were tested on cyclosarin-, VX-, and paraoxon-inhibited human AChE and BChE and compared to pralidoxime and asoxime. Some novel compounds were found to be better reactivators than

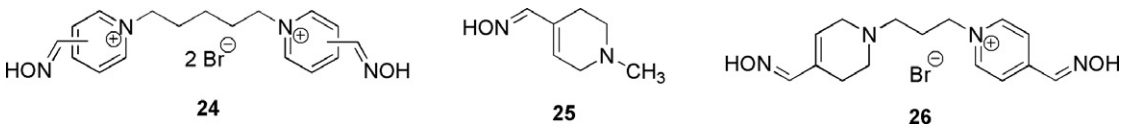


FIGURE 72.21 Structure of reactivators synthesized in South Korea (Oh et al., 2008).

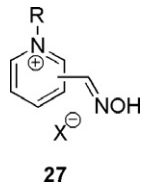


FIGURE 72.22 Structure of monoquaternary oximes from a study in Japan (Ohta et al. 2006).

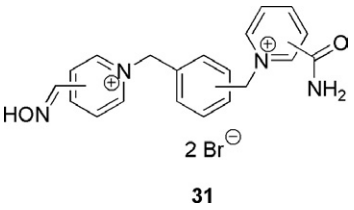


FIGURE 72.25 Monooxime-monocarbamoyl bispyridinium xylene-linked reactivators (Musilek et al., 2010).

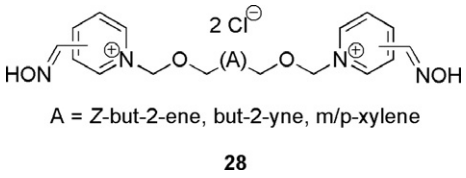


FIGURE 72.23 Structure of reactivators from a study in India (Acharya et al., 2009b).

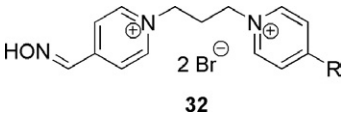


FIGURE 72.26 Monooxime bisquaternary reactivators with prop-1,3-diyl linkage (Musilek et al., 2011b).

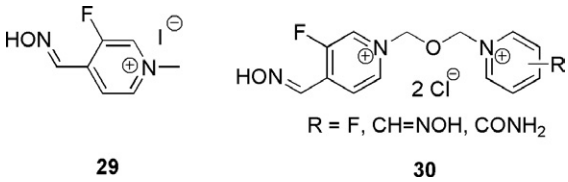


FIGURE 72.24 Fluorinated reactivators from a study in South Korea (Jeong et al., 2009).

pralidoxime, but none of them had better reactivation than asoxime. Some reactivators, together with hBChE injection, improved survival rate of mice intoxicated by sarin and paraoxon.

Kalisiak et al. (2011) introduced amidine oximes (**40**; Figure 72.30) as non-quaternary reactivators. Prepared compounds were tested on human AChE or BChE inhibited by nerve agent– and OP-pesticide model compounds. Novel molecules responded better to non-quaternary oxime MINA, but were worse reactivators than pralidoxime. Kalisiak et al. (2011) prepared cyclic amidine oximes (**41**; Figure 72.30) in a study meant to be an improvement of the previous investigation. The prepared compounds were tested on human AChE- or BChE-inhibited by nerve agent– and OP-pesticide model compounds. Some compounds performed better than MINA or pralidoxime, but bisquaternary standards were not compared in this research. Ongoing research efforts are focused on searching for more convenient PSLs attached to 3-hydroxy-2-pyridinealdoxime moiety that could serve as possible nucleophilic agents and allow preparation of non-quaternary reactivators (Dale and Rebek, 2009; Louise-Leriche et al., 2010; Saint-Andre et al., 2011).

Mercey et al. (2011) prepared two reactivators with 3-hydroxy-2-pyridinealdoxime moiety attached to phenyltetrahydroisoquinoline PSL (**42**; Figure 72.31). Both compounds were tested on human AChE inhibited by VX or tabun, and they were compared to asoxime, obidoxime, and trimedoxime. Reactivation of novel non-quaternary compounds was found to be comparable to existing standards for both nerve agents. Mercey et al. (2012b) broadened the oxime pool with nine phenyltetrahydroisoquinoline conjugates (**43**; Figure 72.32). These novel compounds were tested on tabun-, VX-, and paraoxon-inhibited human AChE. The prepared compound showed increased reactivation potency that was better than or comparable to pralidoxime, asoxime, obidoxime, and trimedoxime.

A series of seven novel, uncharged compounds (Renou et al., 2013) combining 3-hydroxy-2-pyridinealdoxime with different PSLs was prepared and examined for VX- and tabun-inhibited human AChE (**44**; Figure 72.33). Concerning *in vitro* reactivation of VX-hAChE, they were more efficient than pralidoxime, but less efficient than obidoxime and asoxime. Regarding tabun-inhibited AChE, only one compound reached a reactivation potency comparable to pralidoxime.

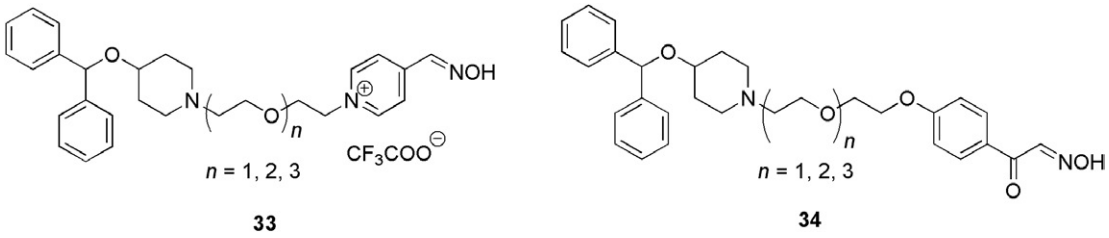


FIGURE 72.27 Monoquaternary and nonquaternary reactivators from the Netherlands (de Koning et al., 2011a,b).

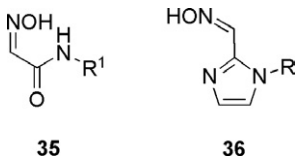


FIGURE 72.28 Imidazole aldoximes from a study in the United States (Sit et al., 2011).

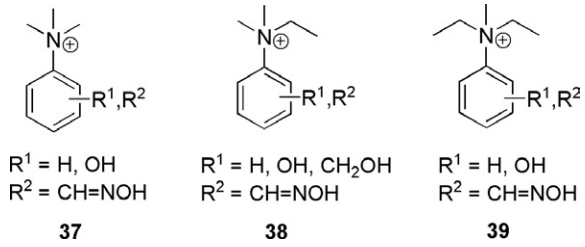


FIGURE 72.29 Reactivators derived from pralidoxime and edrophonium (Radic et al., 2012).

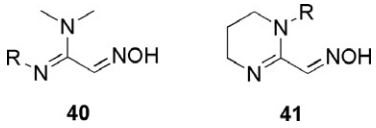


FIGURE 72.30 Amidine oximes from a study in the United States (Kalisiak et al., 2011).

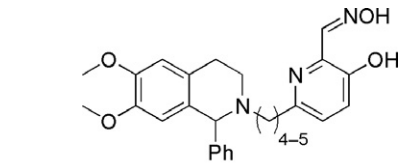


FIGURE 72.31 3-Hydroxy-2-pyridinealdoxime moiety containing reactivators with butane/pentane linker. (Mercey et al., 2011).

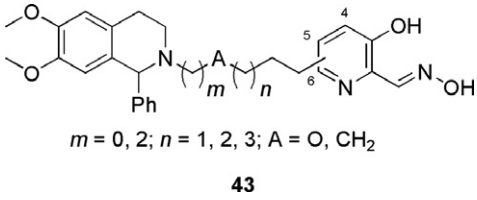


FIGURE 72.32 3-Hydroxy-2-pyridinealdoxime moiety containing reactivators with modified linker. (Mercey et al., 2012a,b).

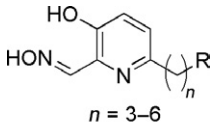


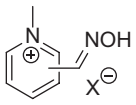
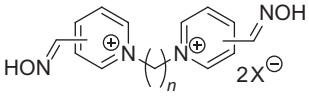
FIGURE 72.33 Reactivators with 3-hydroxy-2-pyridinealdoxime moiety and variable PSL (Renou et al., 2013).

IN VITRO EVALUATION OF SELECTED AChE REACTIVATORS

There are many available techniques to evaluate the reactivation activity of structurally different AChE reactivators (Holas et al., 2012). Probably the most frequent are Ellmann's method (Ellman et al., 1961) and the potentiostatic method (Kuca and Kassa, 2003). Each of these has its advantages and disadvantages. For instance, Ellmann's method uses acetylthiocholine, rather than natural substrate ACh, while the potentiostatic method takes a lot of time and materials.

Although results obtained by both methods are proportionally comparable, due to the influence of structural moieties on final reactivator activity, only one method should be used. Among various scientific groups focused on ChE reactivation, both methods are extensively used. Moreover, species differences in reactivation should be taken into account (Kuca et al., 2005b; Worek et al., 2011). It is difficult or impossible to compare all results among various scientific groups.

TABLE 72.3 Reactivation Ability Influenced by Oxime Group Number and Position

Structure	Reactivation Potency (%)		Structural Factors			
	10^{-5} M	10^{-3} M	No. oxime Groups	Oxime Group Position	Linker Length	No. Quaternary Nitrogen
	9	80	1	2	–	1
	3	0	1	3	–	1
	0	7	1	4	–	1
	6	19	2	2,2′	3	2
	1	16	2	3,3′	3	2
	33	79	2	4,4′	3	2
	10	24	2	2,2′	4	2
	0	6	2	3,3′	4	2
	30	50	2	4,4′	4	2

Source: Source of enzyme—rat brain homogenate; OP inhibitor—chlorpyrifos; time of inhibition—30 min; time of reactivation—10 min; pH 7.6; 25°C; oxime concentrations 10 − 5 M and 10 − 3 M (Racakova et al., 2006).

All results considered in the structure–activity relationship discussed next are trying to compare results obtained in different groups with different methods on different cholinesterase species.

THE STRUCTURE–ACTIVITY RELATIONSHIP OF ACHE REACTIVATORS

Five important structural factors influence the affinity of AChE reactivators toward inhibited AChE: (a) presence of the oxime group; (b) position of the oxime group at the aromatic ring; (c) number of oxime groups in reactivator structure; (d) length and shape of the linker between aromatic rings; and (e) presence of quaternary nitrogen in reactivator molecule.

Presence of the Oxime Group in Reactivator Structure

The presence of the oxime group in the structure of the reactivator is a substantial structural factor. Hydroxyiminoacetones, hydroxamic acids, geminal dioles, and ketoximes used to be prepared as potential nucleophilic agents for breaking the bond between inhibitor and enzyme (Heath, 1961; Picha et al., 2005). The aldoxime group seems to be used predominantly these days, and it is involved in all newly synthesized AChE reactivators (see the section "Design and Synthesis of New AChE Reactivators," earlier in this chapter).

The oxime group in the human body dissociates to oximate anion, which acts as a nucleophilic agent and cleaves the bond between the enzyme and phosphorus atom of the OP inhibitor (Heath, 1961; Bajgar, 2004).

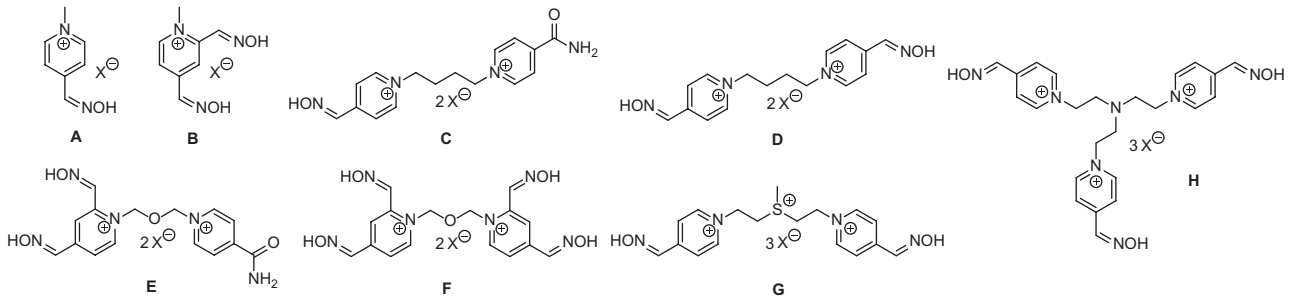
Position of the Oxime Functional Group

The other important structural factor is the position of the oxime group at the aromatic ring. As can be seen in Table 72.3, there are significant differences in reactivation potency of oximes regarding the oxime group position.

There is no applicable rule for oxime position in terms of the ability to reactivate all OP-caused AChE inhibitions. The general finding is that reactivators containing the oxime group in positions 2 and 4 are more effective compared to oximes in position 3, and the reason for that is the difference in pK_a between the number of oximes in positions 2 and 4 (usually between 7 and 9) versus those in position 3 (usually over 9).

Although the rule governing the oxime position for all nerve agents is unrealistic, position of the oxime group and nerve agent being counteracted are related in many ways. It means that the position of the oxime group depends on the nerve agent in question, as well as the length of connecting chain between aromatic rings. For example, cyclosarin-inhibited AChE is properly reactivated by compounds with oxime in position 2 (Kuca and Patocka, 2004). Very good reactivation ability of sarin- and VX-inhibited AChE has been found with oxime groups in positions 2 and 4 (Kuca et al., 2005c,d). On the other hand, reactivators with the oxime group in position 4 are currently considered the most potent for reactivation of tabun-inhibited AChE (Musilek et al., 2007g). Also, OP pesticide–induced poisonings are best treated with AChE reactivators with an oxime at position 4 (Worek et al., 1996; Musilek et al., 2011a). In contrast, uncharged reactivators with oxime in position 2 (Mercey et al., 2011, 2012b) seem to overcome their dependence on tabun- and OP-pesticide reactivation on four-positioned oxime.

TABLE 72.4 Structurally Different Reactivators with Different Amount of Oxime Groups



Compound Type	Reactivation Potency (%)		Structural Factors	
	10^{-5} M	10^{-3} M	No. Oxime Groups	No. Quaternary Nitrogen
A	0	6	1	1
B	0	2	2	1
C	6	25	1	2
D	15	45	2	2
E	2	3	2	2
F	0	1	4	2
G	0	9	2	3
H	0	2	3	3

Source: Source of enzyme—rat brain homogenate; OP inhibitor—tabun; time of inhibition—30 min; time of reactivation—10 min; pH 7.6; 25°C; oxime concentrations 10 − 5 M and 10 − 3 M (Cabal et al., 2004).

Number of Oxime Groups in the Reactivator Molecule

The number of oxime groups in the reactivator molecule is the other structural requirement that needs to be discussed. One molecule of AChE reactivator can contain one, two, three, or four oxime groups with none, one, two, or three quaternary nitrogen in one molecule. Examples of different structures with one, two, three, or four oxime groups are shown in Table 72.4.

As can be seen from Table 72.4, the number of oxime groups does not increase reactivation ability of the potential AChE reactivator. This fact could be connected to increased size of reactivator molecule and limited binding in the AChE active site. Based on this finding, the main role in the reactivation process appears to involve just the first oxime group (with the proper pK_a). Presence of the second or additional oxime group is not necessary for quaternary or uncharged reactivators (Mercey et al., 2011; Musilek et al., 2011a).

Length and Shape of the Linker Between Aromatic Rings

The length of connecting chain between both aromatic rings (for bisquaternary pyridinium as well as uncharged reactivators) plays an important role in potency to reactivate nerve agent–inhibited AChE (Kuca et al., 2003c; Pang et al., 2003; Renou et al., 2013). For *n*-methylene linkage chains, there exists a dependence between length of the connection chain and nerve agent used for inhibition, as shown in Figure 72.34 (Kuca et al., 2003c).

As the figure shows, ideal length of the reactivator linker in bisquaternary oximes for satisfactory activity of tabun-, sarin-, or VX-inhibited AChE is three or four methylene units. On the other hand, one methylene group seems to be the most potent reactivator of cyclosarin-inhibited AChE.

Unfortunately, this rule is satisfied just in the case of *n*-methylene linkage chains and oxime groups in position 4 at the pyridinium ring. Compounds with oxygen, sulfur, and other structural fragments incorporated into the connection chain and with different positions of oxime groups do not fulfill these criteria (Pang et al., 2003; Musilek et al., 2005, 2006a; Oh et al., 2006; Acharya et al., 2008). These variances could be caused by presence of π-electrons or free electron pairs in the connection chain, and subsequent interactions of reactivator molecule with enzyme amino acid residues taking place.

An important structural factor influencing the reactivation process could also be rigidity of the linking chain. Owing to rigidity of the connection chain, the spatial

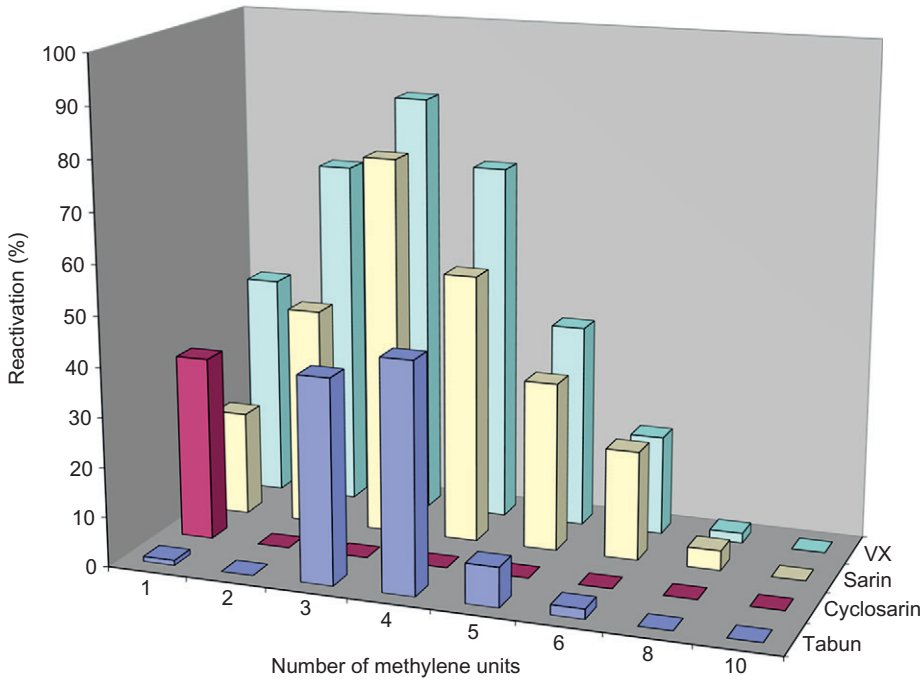


FIGURE 72.34 Dependence of reactivation ability on methylene linker length (Kuca et al., 2003c).

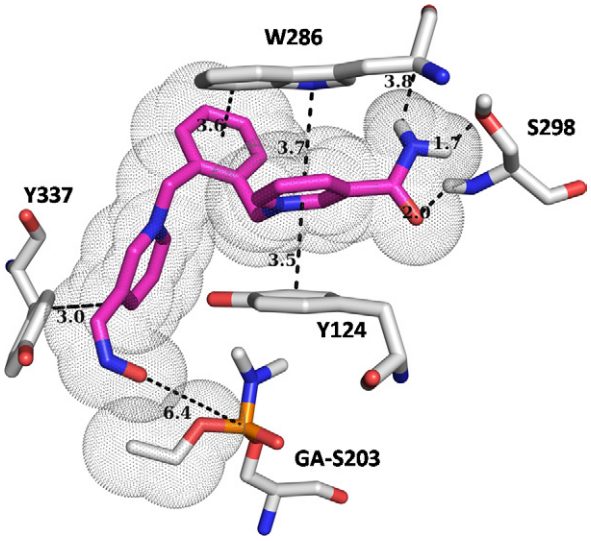


FIGURE 72.35 A spatially rigid linker in the reactivator molecule (Musilek et al., 2010).

orientation of pyridinium rings in the enzyme cavity is limited. Compounds with a certain level of rigidity in the connection chain were synthesized with the aim of elucidating the influence of rigidity on reactivation ability. *Z*- and *E*-but-2-ene and *ortho*-, *meta*-, *para*-xylene moieties were inserted into the linker (Musilek et al., 2005, 2006a, 2007d, 2010). In Figure 72.35, spatial orientation of the reactivator with the rigid linker is shown (Musilek et al., 2010).

Presence of Quaternary Nitrogen in the Reactivator Structure

It is generally known that the anionic center of AChE binds the charged quaternary group of choline moiety of ACh, bisquaternary AChE reactivators (Kuca et al., 2004c), as well as other uncharged ligands, including nonquaternary reactivators (de Koning et al., 2011a,b). Owing to the presence of quaternary nitrogen in the structure of AChE reactivator, quaternary oximes have an increased affinity for both intact and inhibited AChE. In fact, this structural feature allows the molecule of AChE reactivator to enter the enzyme cavity. In contrast, it has been shown that uncharged reactivators with PSL in their molecule may also have an increased affinity for inhibited AChE (Mercey et al., 2011, 2012b).

There has been some interest in the penetration of the quaternary charged compounds (especially AChE reactivators) through the BBB. The partial ability of monoquaternary (Sakurada et al., 2003; Petroianu et al., 2007a) and bisquaternary (Lorke et al., 2007) oximes to penetrate the BBB was published. *In vivo* reactivation of nerve agent–inhibited brain AChE has been determined many times in the past, and this topic is well described in Bajgar et al. (2007) and Lorke et al. (2008b). Commonly used AChE reactivators have one or two quaternary nitrogen in their molecules (e.g., pralidoxime or asoxime). According to our results, affinity of bisquaternary oximes for intact and inhibited AChE is generally higher than monoquaternary ones (Kuca and Kassa, 2004).

Uncharged reactivators were proven to cross BBB more readily than quaternary oximes (Okolotowicz et al., 2014). However, most of them also had poor reactivation ability, increased AChE inhibitory ability, low solubility, and questionable toxicity. The lack of quaternary moiety is responsible for these failings. These findings limit practical use of these compounds and need to be clarified by further development of uncharged oximes. Nevertheless, these uncharged oximes have great potential, so they merit future investigation.

PROMISING OXIMES

Along with those AChE reactivators that are commercially available (namely, pralidoxime, trimedoxime, methoxime, obidoxime, and asoxime), there are several oximes that are seen as promising.

We can also consider development of the new reactivator salt (the known reactivator with a novel counteranion) as the new compound. Asoxime dimethanesulfonate is probably the leading oxime under development in many countries because of its superior stability and solubility compared to asoxime dichloride. It is well known to be a broad-spectrum reactivator for all nerve agents except tabun (Lundy et al., 2006).

In the Czech Republic, great efforts have been focused on the development of new reactivators for use in cases of tabun poisoning. During the last 5 years, three new generations have been prepared. The best reactivators of the first generation (i.e., K027 and K048) are currently being tested in many countries because of their extraordinary potency and low toxicity (Calic et al., 2006; Benko et al., 2007). Subsequently, the second generation of these drugs was developed; among tested compounds, oximes K074 and K075 were recommended as the best. However, their excellent reactivation activity was counteracted by their increased toxicity, so they were excluded from further investigation (Kuca et al., 2007c; Kassa and Humlicek, 2008). Currently, the best third-generation oxime for tabun-inhibited AChE seems to be K203 (Musilek et al., 2007g; Kassa et al., 2008). Except for the promising results of developed oximes obtained with tabun, these oximes seem to be satisfactory in case of OP pesticide poisoning as well (Petroianu et al., 2006a,b). Among them, K027 was tested in the United Arab Emirates as the best oxime (compared with obidoxime, trimedoxime, pralidoxime, and HI-6) against pesticides (paraoxon, methylparaoxon, and DFP) both *in vitro* and *in vivo* (Petroianu et al., 2007b).

Efforts are still being made to compare oxime HLö-7 with asoxime to see if its activity is comparable or better. The structures of both compounds are similar (differing slightly in one added oxime group, at position 4 on the pyridinium ring of asoxime). However, the reactivation potency of HLö-7 is not much better compared to asoxime to perform all steps, which are needed for introduction to the market.

In the United States, methoxime is going to replace pralidoxime, which is now considered an old-fashioned and ineffective antidote. There are many studies focused on this topic (Singh et al., 2007). According to recent research, several laboratories are also focusing on Ortho-7, which has been described by many investigators (e.g., Pang et al., 2003; Ekström et al., 2006; Oh et al., 2008).

The novel classes of uncharged reactivators have been thoroughly investigated in the last decade. Although some potent reactivators were described *in vitro*, there is only limited knowledge on their activity *in vivo*; namely, their increased AChE inhibitory ability, low solubility, and questionable toxicity have to be further improved and clarified.

RECENT TRENDS IN DEVELOPMENT OF NEW ACHE REACTIVATORS

New trends to improve potency of reactivators have various purposes. There are many directions to consider.

The most discussed factor is probably the possibility of a broad-spectrum reactivator. Every new oxime could be tested for its broad-spectrum ability. However, the probability of finding such an oxime is low. Alternatively, a combination of two oximes can be used. Such an approach was originally applied decades ago by Kovacevic et al. (1989). Candidates for this approach include asoxime and various other oximes, which are applicable in case of tabun- or OP-pesticide poisoning.

Another factor that needs to be discussed is BBB penetration, which could be addressed by chemical modification of the reactivator's structure. Examples of this include nonquaternary reactivators and connection of quaternary moiety with lipophilic compounds, which can serve as carriers (e.g., amino acids, steroids, drugs to treat Alzheimer's disease, and saccharides), or the use of special carriers (e.g., nanoparticles and complex systems). However, do we really need to increase oxime levels in the brain? If there are higher concentrations of oxime in the brain, its toxicity could be increased. The uncharged reactivators might have great potential, but these effects should be taken into account and verified. For further details, see Chapter 49.

A new nucleophilic group, instead of the oxime group, could also be considered as a promising approach. However, during the last 50 years, no better nucleophilic group has been found.

New techniques currently used for drug design could be used for this purpose as well. Among them are molecular modeling studies, prediction of new structures using chemometric tools, click chemistry, or random chemistry

approaches, all of which could be applied to achieve the desired goals.

Finally, oximes could be applied for reactivation of AChE as life-endangering enzymes, and their use as butyrylcholinestrase (BChE) reactivators could contribute to finding a pseudocatalytic scavenger. Such a scavenger could neutralize the nerve agent via BChE (as a stoichiometric scavenger) and then inhibited BCHE could be renewed by oxime to be available repeatedly for nerve agent binding.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

As discussed in this chapter, the threat of misuse of OP nerve agents continues. The requirements of agricultural production also demand the use of pesticides, so deaths from these compounds in agricultural workers is increasing. There is no universal oxime that could be applied in every situation of nerve agent or OP pesticide intoxication. The development of new, effective and safe reactivators is necessary.

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CHAPTER

73

Paraoxonase (PON1) and Detoxication of Nerve Agents

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INTRODUCTION

More than 60 years ago, it was discovered that certain organophosphorus (OP) insecticides could be enzymatically hydrolyzed by plasma, and it was determined that this hydrolysis was catalyzed by enzymes that were designated A-esterases (Mazur, 1946; Aldridge, 1953). Aldridge's proposal that an A-esterase hydrolyzed both phenylacetate and paraoxon (the active metabolite of the OP insecticide parathion) was conclusively proven several decades later, when it was shown that recombinant paraoxonase/arylesterase catalyzed both activities (Gan et al., 1991). Studies in the late 1970s and early 1980s indicated that the plasma hydrolytic activity toward paraoxon was polymorphically distributed in human populations (Playfer et al., 1976; Eckerson et al., 1983; Mueller et al., 1983), suggesting a genetically based differential susceptibility to OP toxicity. The molecular basis of the paraoxonase (PON1) activity polymorphisms (Adkins et al., 1993; Humbert et al., 1993) and its role in the toxicity of OP compounds (Davies et al., 1996; Li et al., 2000; Costa et al., 2003a, 2006, 2013) have been elucidated. Furthermore, novel important roles of PON1 in the metabolism of oxidized lipids, in the bioactivation or detoxication of certain drugs and in the hydrolysis of quorum-sensing factor, have also emerged, highlighting the multifaceted roles and importance of this enzyme. These latter aspects of PON1 are not discussed in this chapter, but the reader can refer to other publications (Costa and Furlong, 2002; Mackness et al., 2002; Costa et al., 2003b; Draganov and La Du, 2004; Ozer et al., 2005; Ng et al., 2005; Camps et al., 2009).

PON1 POLYMORPHISMS: DEFINING PON1 STATUS

PON1 is a member of a family of proteins that also includes PON2 and PON3; the genes for these are clustered in tandem on the long arms of human chromosome 7 (q21.22). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoproteins (HDLs) (Sorenson et al., 1999; Deakin et al., 2002). PON1, which received its name from its ability to hydrolyze paraoxon, its first and most studied OP substrate, hydrolyzes the active metabolites of other OP insecticides (e.g., chlorpyrifos oxon, diazoxon) as well as nerve agents such as sarin or soman. However, many other OPs are not hydrolyzed by PON1. Of the three PONs, only PON1 has OP-esterase activity, whereas all three are lactonases, displaying overlapping but distinct substrate specificities for lactone hydrolysis (Draganov et al., 2005). The crystal structure for a recombinant chimeric PON1 indicates that it is a six-bladed β-propeller with two calcium ions in the central tunnel, one of which is essential for enzyme activity and the other is essential for structural stability (Harel et al., 2004). However, recombinant PON1 differs from human PON1 in amino acid sequence and OP-esterase activity (Otto et al., 2009; Trovaslet-Leroy et al., 2011); thus, the tertiary structure of native PON1 remains to be elucidated.

Plasma paraoxonase activity in human populations is polymorphically distributed and individuals with high, intermediate, or low paraoxonase activity can be identified (Eckerson et al., 1983; Mueller et al.,

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1983; Geldmacher-von Mallinckrodt and Diepgen, 1988; Ginsberg et al., 2009), although, as shown later, the resolution of the three phenotypes are not revealed by single substrate assays (Davies et al., 1996; Richter and Furlong, 1999). Studies in the early 1990s led to the purification, cloning, and sequencing of human PON1 (Furlong et al., 1991; Gan et al., 1991; Hassett et al., 1991), and in the molecular characterization of its polymorphisms (Adkins et al., 1993; Humbert et al., 1993). Two common polymorphisms were observed in the PON1 coding sequence: a Gln(Q)/Arg(R) substitution at position 192 and a Leu(L)/Met(M) substitution at position 55 (Adkins et al., 1993; Humbert et al., 1993). The gene frequencies of $PON1_{Q192}$ range from 0.75 for Caucasians of Northern European origin to 0.31 for some Asian populations (Brophy et al., 2002). In addition to these two polymorphisms in the coding region of PON1, several polymorphisms have been found in the noncoding 5 Disease: Basic and Clinical PON1 gene, with the most significant one being the C108T polymorphism at an Sp1 binding site, and with the –108C allele providing levels of PON1 approximately twice as high, on average, as the –108T allele (Leviev and James, 2000; Suehiro et al., 2000; Brophy et al., 2001a,b). Complete resequencing of PON1 from 47 individuals (24 African-Americans and 23 Europeans) has revealed more than 160 single nucleotide polymorphisms (SNPs) (Furlong et al., 2008). Some of the few that have been characterized so far have allowed reconciliation of discrepancies between PCR analysis of codon 192 and determination of PON1 status (Jarvik et al., 2003)

The L/M polymorphism at position 55 does not affect catalytic activity but has been associated with plasma PON1 protein levels, with $PON1_{M55}$ being associated with low-plasma PON1 (Blatter Garin et al., 1997; Mackness et al., 1998); this appears to primarily result from linkage disequilibrium with the low efficiency of the T-108 promoter allele (Brophy et al., 2002), although some data indicate that $PON1_{M55}$ may be somewhat less stable than $PON1_{L55}$ (Leviev et al., 2001; Roest et al., 2007). In contrast, the Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1. This polymorphism is substrate-dependent, because the $PON1_{R192}$ alloform hydrolyzes chlorpyrifos oxon and paraoxon more rapidly than $PON1_{Q192}$ *in vitro* (Li et al., 2000), whereas both PON1 alloforms hydrolyze diazoxon (Li et al., 2000) and phenylacetate (Furlong et al., 2006) with the same efficiency.

Although most studies investigating the association of PON1 polymorphisms with diseases have examined only the nucleotide polymorphisms (Q192R, L55M, C-108T) with PCR-based assays, it has been argued that measurements of PON1 function (plasma activity) are of greater relevance, because they measure the plasma activity of PON1, which is the most important factor that determines rates of detoxication of both endogenous and xenobiotic substrates. This functional genomics analysis is accomplished through the use of a high-throughput enzyme assay involving two PON1 substrates (either diazoxon and paraoxon or a pair of nontoxic substrates) (Richter et al., 2004, 2008, 2009). This approach, which has been referred to as the determination of the "PON1 status" for an individual (Richter and Furlong, 1999), in addition to providing a functional assessment of the plasma PON_{192} alloforms, also provides the plasma level of PON1 for each individual, thus encompassing the two factors that affect rates at which PON1 detoxifies endogenous and exogenous substrates. Rates of detoxication of compounds that are not affected by the Q192R polymorphism are determined by plasma PON1 concentrations. Rates of detoxication of other compounds for which rates are affected by the Q192R polymorphism are determined both by plasma PON1 levels and the-PON1 alloform(s) in plasma. In all cases, it is important to determine PON1 plasma levels and, for some compounds, the Q192R polymorphism as well. For this reason, many epidemiological studies that have analyzed SNPs alone have not provided useful data.

Thus, for adequate risk assessment, it is important to know PON1 levels and activity. In a given population, plasma PON1 activity can vary up to 40-fold (Eckerson et al., 1983; Mueller et al., 1983; Davies et al., 1996; Richter and Furlong, 1999; Jarvik et al., 2000), and differences in PON1 protein levels up to 13-fold are also present within a single $PON1_{192}$ phenotype (QQ, QR, RR) in adults (Davies et al., 1996). Ample evidence is now available from studies investigating the role of PON1 in cardiovascular disease that PON1 status (encompassing genotype and activity levels) is a much better predictor of disease than PON1 genotype alone (Jarvik et al., 2000; Mackness et al., 2001; Bayrak et al., 2012; Zhao et al., 2012).

PON1 AND THE TOXICITY OF OP INSECTICIDES

Evidence that PON1 plays a role in modulating the *in vivo* toxicity of OPs has emerged over the past 20 years, although it had already been noted that species differences in PON1 activity correlated with susceptibility to OP toxicity (Costa et al., 1987; Furlong et al., 2000). A previous study by Main (1956) showed that intravenous administration of partially purified PON1 from rabbit serum would protect rats from the toxicity of paraoxon; therefore, several initial studies followed a similar approach. Administration (by the intravenous route) of purified rabbit PON1 to rats protected the animals from acetylcholinesterase (AChE) inhibition by chlorpyrifos oxon and paraoxon (Costa et al., 1990). Further studies in mice provided evidence that intravenous administration

of pure rabbit PON1 increased serum chlorpyrifos oxonase activity by 30-fold to 40-fold and protected animals against AChE inhibition by dermally applied chlorpyrifos oxon (Li et al., 1993). Because intravenous administration of PON1 increased serum PON1 activity for a short duration ($t_{1/2}$ = ~6 h), additional experiments aimed at investigating other routes of administration for PON1 that would extend its plasma half-life. When PON1 was given by the intravenous and intraperitoneal routes, plasma enzyme activity toward chlorpyrifos oxon increased by 35-fold and the half-life was extended to 30 h. An even longer half-life, albeit with lower peak activity levels, was found when PON1 was administered by the intravenous plus intramuscular route (Li et al., 1993). These studies also showed that rabbit PON1 could confer protection toward the parent compound, chlorpyrifos, and that protection was also present when the enzyme was given 30 min to 3 h after exposure (Li et al., 1995), suggesting a therapeutic potential for exogenous PON1 in cases of OP poisoning, possibly in combination with other conventional treatments.

More recently, *PON1* knockout (*PON1*$^{-/-}$) and *PON1* transgenic mice have provided important new tools to investigate the role of PON1 in modulating OP toxicity. Plasma and liver from *PON1*$^{-/-}$ mice have no detectable hydrolytic activity toward paraoxon and diazoxon, and have very limited chlorpyrifos-oxonase activity (Shih et al., 1998; Li et al., 2000). *PON1*$^{-/-}$ mice do not differ from wild-type animals in their sensitivity to demeton-*S*-methyl, an OP insecticide with a structure similar to malathion and that is not a substrate for PON1 (Li et al., 2000). In contrast, *PON*$^{-/-}$ mice have a dramatically increased sensitivity to chlorpyrifos oxon and diazoxon, and a slightly increased sensitivity to the toxicity of the parent compounds chlorpyrifos and diazinon (Shih et al., 1998; Li et al., 2000). A surprising finding is that the lack of PON1 did not affect the sensitivity of mice to paraoxon, the substrate for which the enzyme was named, despite the absence of any paraoxonase activity in plasma and liver (Li et al., 2000).

When *PON1*$^{-/-}$ mice were given exogenous PON1, resistance to OP toxicity was also restored (Li et al., 2000). In these studies, purified human $PON1_{Q192}$ or $PON1_{R192}$ was injected, by the intravenous route, into *PON1*$^{-/-}$ mice, and the effects of various OPs on brain and diaphragm AChE activity were determined (Li et al., 2000). $PON1_{R192}$ provided significantly better protection than $PON1_{Q192}$ toward chlorpyrifos oxon, a finding also confirmed by a study by Cowan et al. (2001), who administered recombinant adenoviruses containing *PON1*-LQ or *PON1*-LR genes to BALB/c mice before challenge with chlorpyrifos oxon. Li et al. (2000) also showed that $PON1_{R192}$ and $PON1_{Q192}$ were equally effective in protecting against the toxicity of diazoxon, but neither alloform afforded protection against paraoxon toxicity.

Kinetic analyses of substrate hydrolysis by purified human $PON1_{R192}$ or $PON1_{Q192}$ provided an explanation for the *in vivo* finding. The catalytic efficiency of both PON1 alloforms toward chlorpyrifos oxon was very high, with the $PON1_{R192}$ alloform having the highest catalytic efficiency. In the case of diazoxon, catalytic efficiency was still high, although lower; however, there was little difference between the two $PON1_{192}$ alloforms. The $PON1_{R192}$ alloform was also much more efficient than the $PON1_{Q192}$ alloform in hydrolyzing paraoxon; however, its overall catalytic efficiency was too low to protect against exposure (Li et al., 2000). This latter finding confirms the hypothesis that PON1 may not degrade paraoxon efficiently at low concentrations *in vivo* (Chambers et al., 1994; Pond et al., 1995).

Further experiments were performed in *PON1* transgenic mice (i.e., mice expressing either human PON_{Q192} or human $PON1_{R192}$) on the knockout background (*Tg-hPON1*$_{R192}$ and *Tg-hPON1*$_{Q192}$) or mice expressing the human $PON1_{R192}$ on top of murine PON1. The latter mice, whose serum paraoxonase activity was 3.5-fold higher than that of wild-type mice, showed similar sensitivity to paraoxon as wild-type mice (Li et al., 2000), further confirming the inefficacy of PON1 for detoxifying paraoxon *in vivo*. *Tg-hPON1*$_{R192}$ mice were significantly less sensitive to the toxicity of chlorpyrifos oxon and of chlorpyrifos than *Tg-hPON1*$_{Q192}$ mice, despite having the same level of PON1 protein in liver and plasma (Cole et al., 2005). Thus, these further experiments in transgenic mice confirmed all previous observations. In addition, administration of a recombinant human PON1 to *PON1*$^{-/-}$ mice protected them against the toxicity of diazoxon when given either after or before the enzyme (Stevens et al., 2008).

Altogether, these animal experiments indicate that PON1 can modulate the acute toxicity of OPs in a compound-specific manner. Thus, in the case of chlorpyrifos oxon, both the level of expression and the Q192R genotype are important determinants of susceptibility, highlighting the importance of assessing PON1 status in potentially exposed individuals. With regard to diazoxon, protection or susceptibility is dictated primarily by the level of expression of PON1, independently of the Q192R genotype, stressing the importance of knowing PON1 levels. Perhaps ironically, PON1 status does not appear to play a role in modulating sensitivity to paraoxon toxicity due to the low catalytic efficiency of paraoxon hydrolysis.

There is limited evidence that PON1 status may affect susceptibility to OP insecticides in humans. A series of studies has investigated the role of PON1 in modulating chronic central and/or peripheral nervous system abnormalities, at times referred to as "dipper's flu," associated with exposure of sheep dippers to diazinon. Diazoxonase activity was initially found to be lower

in cases than referents (Cherry et al., 2002); however, in a follow-up study in the same populations, serum PON1 activity toward diazoxon did not differ between cases and controls. Although when the two groups were divided into quintiles according to serum diazoxonase activity, sheep dippers in the lowest quintile had a greater risk of reporting ill health than those in the other quintiles (Mackness et al., 2003). Additional investigations suggested that the risk associated with PON1 polymorphisms may vary with genotypes of CYP2D6 (which bio-activates diazinon) (Povey et al., 2007). On the basis of these and of similar findings (O'Leary et al., 2005; Mackenzie Ross et al., 2010; Cherry et al., 2011), it would appear that ill health in sheep dippers was more pronounced in individuals who had a lower ability to detoxify diazoxon.

A few other studies are suggestive of a role of PON1 status in modulating susceptibility to various effects of OPs (Lee et al., 2003; da Silva et al., 2008; Lacasana et al., 2010; Singh et al., 2011). However, these studies lack detailed information on exposures, which hampers interpretation of the findings. Exposure to diazinon and to chlorpyrifos, but not to parathion, was found to be associated with an increased risk of Parkinson disease in carriers of the $PON1_{M55}$ variant (low PON1 activity) (Manthripragada et al., 2010). Exposure to diazinon and chlorpyrifos was also associated with an increased risk of brain tumor in children with low PON1 activity (Searles Nielsen et al., 2005). Finally, a study of pesticide handlers in Washington State, exposed to various OPs (most notably chlorpyrifos), low PON1 catalytic efficiency (Q192), and low plasma activity were associated with increased degrees of plasma butyrylcholinesterase (BChE) inhibition from baseline levels (Hoffman et al., 2009).

Overall, human studies available to date provide limited initial evidence that low PON1 status may increase susceptibility to adverse effects of certain OP insecticides. However, further studies are certainly warranted.

PON1 AND THE TOXICITY OF NERVE AGENTS

Several OP nerve agents, such as soman, sarin, tabun, and VX, have been shown to be metabolized *in vitro* by PON1 (Davies et al., 1996; Rochu et al., 2007; Valiyaveettil et al., 2010). Information is scarce regarding VX (Peterson et al., 2011), but more information is available regarding the other compounds, particularly soman and sarin. Both sarin and soman are hydrolyzed to a higher degree by $PON1_{Q192}$ than by $PON1_{R192}$ (Davies et al., 1996), and plasma somanase activity and sarinase activity (both in U/L) were found to be 2,143 and 335 for QQ homozygotes and 992 and 38 for RR homozygotes, respectively (Davies et al., 1996). Thus, homozygotes for the $PON1_{Q192}$ allele hydrolyze sarin approximately 10-times better than individuals homozygous for the $PON1_{R192}$ allele, whereas the ratio is approximately two for soman (Davies et al., 1996). Results of a kinetic study confirmed these findings; catalytic efficiency for sarin was determined as 0.91 and 0.07 (ratio = 13) for $PON1_{Q192}$ and $PON1_{R192}$ homozygotes, respectively, whereas values for soman were 2.8 and 2.1 (ratio = 1.3) (Rochu et al., 2007). Soman exists as four stereoisomers (C+P+, C+P−, C−P+, C−P−) (Beschop and deJong, 1988), with both P− isomers displaying the highest *in vivo* toxicity; wild-type recombinant human PON1 was shown to stereo-selectively hydrolyze soman, with a sixfold overall difference in catalytic efficiency (P+ > P−) (Yeung et al., 2007). Further evidence that purified human PON1 and recombinant PON1 can hydrolyze soman and sarin has been provided by Valiyaveettil et al. (2010), who also reported hydrolysis of tabun.

In vivo studies in animals have shown that intravenous administration of naked DNA bearing the human $PON1_{Q192}$ cDNA to mice could elevate plasma somanase activity by approximately twofold (Fu et al., 2005). The pcDNA/PON1-treated mice survived in greater number with a pcDNA/PON1 dose-dependence and for a longer period after an acute dose of soman (0.2 mg/kg, subcutaneous) (Fu et al., 2005). More recently, Valiyaveettil et al. (2011a,b) have reported that PON1 could protect guinea pigs from the acute toxicity of sarin and soman. In one study, small quantities of purified human or rabbit PON1 were administered intravenously to guinea pigs 30 min before exposure to sarin or soman by microinstillation inhalation (846 and 841 mg/m^3, respectively, equivalent to 1.2 × LC_{t50}) (Valiyaveettil et al., 2011a). Although there was only a modest increase in plasma PON1 activity, PON1 from both species significantly increased survival time and antagonized several effects of the nerve agents, including AChE inhibition in brain. In a companion study, human recombinant PON1 expressed in *Trichoplusia ni* larvae was given intravenously to guinea pigs 30 min before exposure to sarin or soman (Valiyaveettil et al., 2011b). Protective effects were essentially identical to those of the other study. In apparent contrast to these findings, a more detailed study by Hodgins et al. (2013) reported that administration of wild-type human PON1 purified from *T. ni* larvae (Otto et al., 2010) or induced via adenoviral infection in mice protected against the toxicity of the OP insecticides diazoxon and chlorpyrifos oxon, but not of several nerve agents (tabun, soman, sarin, cyclosarin). These findings re-emphasize the fact that human PON1 has a low catalytic activity toward nerve agents and suggest that there is a need for developing novel human PON1 mutants with enhanced nerve agent–hydrolyzing activity. These studies also suggest that the $PON1^{-/-}$ mouse may be a much better model than the guinea pig for two

reasons. First, is no background OP hydrolase activity in the plasma of *PON1*$^{-/-}$ mice, allowing for the pharmacokinetics to be clearly delineated. Second, much less PON1 (purified or recombinant) is required to increase the plasma level of mice to quite high levels of activity (Li et al., 1993, 1995, 2000; Stevens et al., 2008). Once the efficacy of a given PON1 variant has been demonstrated in the mouse model and protocols have been developed to produce large quantities of rPON1, it will be feasible to study the efficacy of the variants in other animal models. The toxicity of exogenous PON1 described by some workers is most likely due to residual detergent or other toxins in the PON1 preparations. Removal of such contamination should allow for the injection of much higher quantities of rPON1.

There are currently two quite different approaches underway for engineering recombinant PON1 variants with higher catalytic efficiency for treating OP exposures. One initiated by Tawfik et al. begins with the chimeric PON1 that they engineered for solubility and the determination of the crystal structure (Harel et al., 2004). The goal of this approach is to make the sequence of variants with high catalytic efficiency of agent hydrolysis (Aharoni et al., 2004) more "human-like" to minimize immunogenicity (Sarkar et al., 2012). A second approach is to begin with the native human rPON1 and introduce the minimal number of mutations required for the desired catalytic efficiency (Stevens et al., 2008). These two approaches are complementary and inform each other.

The 1995 terrorist attack in the Tokyo subway system that left 12 people dead and more than 5,000 injured (Suzuki et al., 1995; Nagao et al., 1997) provided the opportunity to investigate the role of PON1 in modulating the toxicity of sarin in humans. The prevalence of the $PON1_{R192}$ genotype in the Japanese population is 0.66 compared with 0.25–0.30 in the Caucasian population (Yamasaki et al., 1997; Brophy et al., 2002). Thus, Japanese individuals may have been more prone to sarin toxicity because of the low sarin-hydrolyzing ability of the $PON1_{R192}$ allozyme. However, among 10 of the victims of the Tokyo attack, seven expressed the $PON1_{192Q}$ genotype, with six Q/R heterozygotes and one Q/Q homozygote (Yamada et al., 2001). Thus, the genotype that confers high hydrolyzing activity toward sarin did not appear to provide protection from acute sarin poisoning. However, several issues need to be considered. First, only the Q192R genotype of those 10 individuals was analyzed, with no information regarding their PON1 status. In a Caucasian population, the range of sarinase activity among individuals with the QQ or QR genotype ranged from approximately 0–758 U/L (Davies et al., 1996). Second, exposure to sarin in these seven QQ or QR individuals was indeed massive, because it caused death instantly or, with one exception, in less than 48 h (Yamada et al., 2001). Such high-dose exposure would be expected to overcome any potential protection afforded by the $PON1_{Q192}$ genotype. Third, and most importantly, the catalytic efficiency of sarin hydrolysis by PON1, even in QQ homozygotes, is low; the situation is thus similar (albeit reversed) to that of paraoxon, with one $PON1_{192}$ alloform hydrolyzing sarin with better efficiency, but still not sufficiently high enough catalytic efficiency to provide protection against nerve agent exposure.

A few studies have also investigated PON1 polymorphisms in US and UK troops that were deployed in the Persian Gulf area during 1990 and 1991. Individuals who served in the Gulf War were potentially exposed to a wide range of biological and chemical agents, including sand, smoke from oil well fires, solvents, petroleum fuels, depleted uranium, anthrax and botulinum toxoid vaccinations, insecticides, pyridostigimine bromide, and nerve agents (IOM, 2000, 2003). A large number of these veterans have reported a range of unexplained illnesses, including chronic fatigue, muscle and joint pain, loss of concentration, forgetfulness, and headache, symptoms that are often referred to as Gulf War syndrome (IOM, 2000, 2003). PON1 genotypes and plasma enzyme activity were investigated in a group of 25 ill US Gulf War veterans and 20 controls (Haley et al., 2000). $PON1_{R192}$ homozygotes or $PON1_{Q/R192}$ heterozygotes were more likely to have neurologic symptoms than individuals homozygous for PON_{Q192}. Furthermore, low activity of the plasma $PON1_{Q192}$ isoform appeared to correlate better with illness than the PON1 genotype or the activity levels of the $PON1_{R192}$ genotype (Haley et al., 2000). This study would suggest that low PON1 status may represent a risk factor for illness in Gulf War veterans, although such findings necessitate further confirmation in a larger population (Furlong, 2000).

A similar study of a group of 152 UK Gulf War veterans, who self-reported the presence of symptoms associated with the Gulf War syndrome, yielded somewhat different results (Mackness et al., 2000). Plasma paraoxonase activity and levels of PON1 protein were lower in veterans than in a control group, and these decreases were independent of the *PON1* genotype (Mackness et al., 2000). Thus, although in both studies a reduced plasma paraoxonase activity was found, in one case it was attributed to an over-representation of the low-activity PON1 isozyme (Haley et al., 2000), and in the other it was common to all *PON1* genotypes (Mackness et al., 2000). Although the latter study suggests that this group of veterans may have a decreased capacity to hydrolyze some OP insecticides, such as chlorpyrifos oxon, its significance is hampered by the lack of information regarding the extent of exposure to such compounds among veterans (Costa et al., 2003a). An additional study compared *PON1* genotypes and plasma paraoxonase activity in groups of UK veterans

from the Persian Gulf War who were symptomatic by self-reporting ($n = 115$), healthy Persian Gulf War veterans ($n = 95$), symptomatic Bosnia peacekeeping veterans ($n = 52$), and symptomatic nondeployed military controls ($n = 85$) (Hotopf et al., 2003). No differences in genotype distribution or PON1 activity were found between healthy and ill Gulf War veterans. However, individuals who were deployed to the Gulf had 25–35% lower median PON1 values than the other two groups, and these differences were not explained by differences in $PON1_{192}$ genotypes between groups. Thus, $PON1_{192}$ genotype and activity were not associated with Gulf War syndrome but appeared to be the results of deployment in the Persian Gulf. Possible explanations suggested for such findings were the potential exposure of those who served in the Gulf to yet unknown agents that led to a long-term decrease in PON1 activity, and/or an overrepresentation in those two groups of individuals with the –108T allele, which is associated with lower PON1 levels (Hotopf et al., 2003).

PON1 AS A THERAPEUTIC AGENT

In recent years there has been much interest in developing new means of antagonizing the toxicity of OPs, particularly of nerve agents (Masson and Rochu, 2009; Nachon et al., 2013). Current therapy for OP poisoning relies on the use of atropine, a cholinergic muscarinic antagonist, and on oximes, such as pralidoxime (2-PAM), to reactivate phosphorylated AChE before "aging occurs" (Lotti, 2000; Eyer et al., 2007). Anticonvulsant drugs such as diazepam may also be utilized to control OP-induced convulsions (Lotti, 2000). More efficacious pyridinium 4-aldoximes, such as obidoxime and trimedoxime, have also been developed; however, their ability to reactivate phosphorylated AChE is not fully exploited because of the formation of phosphoryloximes, which have very high anticholinesterase activity. Interestingly, phosphoryloximes are rapidly hydrolyzed by PON1, with the $PON1_{Q192}$ allozyme being approximately 50-fold more active than $PON1_{R192}$ (Stenzel et al., 2007), suggesting that PON1 status would also influence the effectiveness and safety of these oximes (Eyer et al., 2007).

Although current therapies are effective in preventing lethality, it has been suggested that they may not prevent behavioral deficits, incapacitation, loss of consciousness, or the potential for permanent brain damage caused in particular by OP nerve agents (Lenz et al., 2007). Complementary approaches have thus focused on the use of human proteins that would act as biological scavengers for OP compounds. Such biological scavengers should have no effects on their own, should not present an antigenic challenge to the immune system, should act rapidly and specifically, and should remain in circulation for a prolonged period of time (Lenz et al., 2007; Suzuki et al., 2010). Out of this general concept two parallel approaches have emerged, one relying on stoichiometric bioscavengers and the other relying on catalytic bioscavengers. Among stochiometric bioscavengers, B-esterases, such as AChE and BChE, which react with OPs but do not catalyze their hydrolysis, have been utilized in several studies. Administration of these two proteins to rodents has been shown to afford protection toward twofold to fivefold LD_{50} doses of sarin, soman, or VX (Wolfe et al., 1987; Doctor and Saxena, 2005; Lenz et al., 2007; Saxena et al., 2011). Of particular interest in this regard is human BChE, either isolated from human plasma or recombinant (Cerasoli et al., 2005).

Although stoichiometric scavengers may offer some protection toward OP toxicity, high doses are needed to neutralize an equimolar amount of nerve agent (Ashani and Pistinner, 2004; Valiyaveettil et al., 2012). In contrast, a catalytic scavenger would afford similar or even higher protection at relatively low doses, because one molecule of a catalytic scavenger can hydrolyze a very large number of molecules of an OP (Sweeney and Maxwell, 2003; Stevens et al., 2008; Valiyaveettil et al., 2012). For example, it has been estimated that 3 μM sarin would be neutralized by 765 mg of human BChE, but only 120–550 mg of $huPON1_{Q192}$ (depending on time) (Rochu et al., 2007), or a much lower dose of engineered recombinant human PON1 with significantly increased catalytic efficiency of agent hydrolysis. A number of arguments would support the use of PON1 as a catalytic bioscavenger (Nachon et al., 2013). PON1 is a human protein, suggesting the possible absence of immunological response. However, reports of PON1-induced cytotoxicity in macrophages needs to be further investigated (Mackness and Mackness, 2010). Experiments using mice and guinea pigs have clearly shown that administration of purified human PON1 or of recombinant PON1 protects animals against the toxicity of specific OP compounds, provided that the catalytic efficiency is sufficient (Li et al., 2000; Cowan et al., 2001; Stevens et al., 2008; Duysen et al., 2011; Valiyaveettil et al., 2011a,b; Hodgins et al., 2013). In this regard, novel ways to deliver PON1 are being investigated in addition to administration of purified enzyme (Li et al., 2000) or of an adenoviral vector (Cowan et al., 2001; Duysen et al., 2011; Hodgins et al., 2013). For example, Gaidukov et al. (2009) showed that administration of BL-3050, a highly stable engineered human PON1-HDL complex, protected mice against the toxicity of chlorpyrifos oxon and did not cause any apparent adverse effect on its own. In another study, human PON1 was re-engineered as an IgG-PON1 fusion protein in which the 355-amino-acid human PON1 was fused to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody against the human insulin receptor (Boado et al., 2011).

This fusion protein, designated as HIR-Mab-PON1, was able to enter the brain when injected intravenously into nonhuman primates. This would be of much interest because PON1 is basically absent from brain cells (Giordano et al., 2011), whereas OPs easily penetrate the blood–brain barrier.

PON1 has been shown to hydrolyze soman, sarin, and tabun, although its catalytic efficiency toward OP nerve agents, in particular sarin, is low. It has been calculated that, to provide a valuable medical countermeasure against intoxication by nerve agents, the catalytic efficiency of PON1 will need to be enhanced by one or two orders of magnitude (Rochu et al., 2007). Some direct evolution variants of a recombinant PON1 were found to exhibit a 10-fold faster detoxication of cyclosarin and soman (Amitai et al., 2006). Other huPON1 mutants with enhanced catalytic activity have also been described, although not in relationship to OP nerve agents (Yeung et al., 2004). Directed evolution of a chimeric PON1 made via mammalian gene shuffling, combined with high-throughput screening, led to an evolved variant of chimeric PON1 with a 100,000-fold higher catalytic efficiency toward the most toxic enantiomer of a coumarin analog of cyclosarin (Gupta et al., 2011). However, chimeric PON1 is expected to be immunogenic; hence, its practical application is doubtful. Nevertheless, further efforts to develop human PON1 variants with increased catalytic efficiency toward nerve agents are warranted.

An additional approach would be that of stimulating the biosynthesis of natural PON1. Several compounds have been shown to stimulate the expression of PON1 (Costa et al., 2005, 2011), among them being low doses of ethanol, some fibrates, statins, or dietary polyphenols such as quercetin (Gouedard et al., 2004a). The red wine ingredient resveratrol has been shown to increase PON1 activity nearly threefold *in vivo* (Gouedard et al., 2004b) and less than twofold in liver cells *in vitro* (Curtin et al., 2008). Interestingly, in the latter study, the increased PON1 afforded protection of cells against the toxicity of soman and sarin (Curtin et al., 2008). Modulation of PON1 activity by drugs or dietary agents is of interest; however, more information of the regulation of the PON1 gene is needed to devise approaches that would offer substantially larger increases in PON1. However, it also has been argued (Valiyaveettil et al., 2012) that a moderate increase of PON1 (less than twofold) may perhaps be sufficient to provide some protection against some of the symptoms of OP exposures, as shown by the protection seen against sarin and soman in guinea pigs (Valiyaveettil et al., 2011a,b). Nevertheless, the low catalytic efficiency of nerve agent hydrolysis by native human PON1 may not be adequate to provide efficient protection against nerve agent exposure. The recent report by Hodgins et al. (2013) supports the point that the catalytic efficiency for hydrolysis of a specific OP must be sufficiently high to protect against exposure to that specific OP.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Evidence from *in vitro* studies shows that PON1 can hydrolyze a number of OP compounds, including nerve agents. *In vivo* studies using rodents have also indicated that PON1 status can influence the acute toxicity of certain OPs, and that administration of exogenous PON1 can protect animals from OP toxicity. In case of nerve agents, the catalytic efficiency of PON1 is relatively low, and PON1 status does not greatly influence an individual's susceptibility to toxicity. However, recombinant variants can be engineered with enhanced catalytic activity toward nerve agents. The nonglycosylated, engineered recombinant PON1s with high catalytic efficiency for OP hydrolysis should be excellent candidates for use as catalytic biological scavengers, with both prophylactic and therapeutic applications. As previously suggested (Suzuki et al., 2010; Costa et al., 2011; Hodgins et al., 2013) efforts in this direction and in attempting to increase endogenous PON1 should proceed in parallel, because a combined strategy may prove successful. The increase in endogenous PON1 levels should be efficacious for exposures to the OPs known to be hydrolyzed at rates sufficient to provide protection (Li et al., 2000; Hodgins et al., 2013).

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CHAPTER

74

The Role of Carboxylesterases in Therapeutic Intervention of Nerve Gases Poisoning

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INTRODUCTION

Carboxylesterases (CarbEs or CEs) constitute an important group of enzymes that plays a major role in the hydrolytic biotransformation of a large number of drugs. In the present context, we discuss their ability to act as scavengers toward the highly toxic organophosphorus (OP) nerve agents. The scavenger function of CarbE in blood plasma is the main reason for the variation in nerve gas toxicity among the species, both in the presence and absence of carbamate prophylaxis and oxime treatment. The various effects of CarbE proteins in plasmas of different species are due to their content, not necessarily their apparent enzymatic activity. The fact that plasma CarbE in rodents is a natural scavenger toward nerve gases allows for explaining the species differences between the toxicities of nerve agents. This is of vital importance when comparing and analyzing OP toxicity data for experimental animals, such as rodents, and species that do not have this specific blood protein, such as rhesus monkeys and humans. The plasma CarbE scavenger function in rodents allows for estimating the protection factor of therapeutic intervention of nerve gas poisoning in humans. Although experimental protection factors with such interventions in rodents may be relatively low due to their natural scavenger content in plasma, it is worth noting that the estimated factors appear to be high for species (including humans) that do not have endogenous plasma CarbE. Treatment of nerve gas poisoning, therefore, might strongly benefit from further exploration of the use of CarbE and other scavengers.

ENZYMOLOGY

Classification

Aldridge (1953) separated esterases into two groups. The first was A-esterases, which are capable of hydrolyzing OP compounds, and the second was B-esterases, which could bind the OP compounds. This group includes other esterases as well, such as CarbE, acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and chymotrypsin. They all have an active group with serine that binds to the OP compounds. CarbEs are also connected to the alpha/beta hydrolase fold, which is common to several hydrolytic enzymes of widely differing phylogenetic origins and catalytic functions. The core of each enzyme is similar: an alpha/beta-sheet, not barrel, of eight beta-sheets connected by alpha-helices. These enzymes have diverged from a common ancestor and comprise a large family of proteins. They are important in hydrolyzing substrates containing ester or amide groups to free acids and alcohols. They are found in several tissues, including the liver, small intestine, kidney, lung, and plasma. At present, the database for this family contains over 450 orthologous or homologous members with 44 distinctive functions. Mammalian liver CarbEs belong to a family of proteins encoded by multiple genes. The isoenzymes were initially classified by their substrate specificity and isoelectric point (pI; Mentlein et al., 1987). However, this classification is now ambiguous because of overlapping substrate specificities, and it is imprecise because CarbEs as glycoproteins exhibit multiple bands with different pI values upon

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electrophoresis. They are very difficult to classify due to their wide substrate specificity. Satoh and Hosokawa (1998) separated all mammalian CarbEs into five main groups based on their molecular properties. The two most important groups are CES1 and CES2. CES1 is the largest group, comprising members that have more than 60% homology to human liver CarbE. It contains the liver enzymes of humans, monkeys, rats, and mice. CES1 enzymes preferentially hydrolyze substrates with lower alcohol groups esterified to a large acyl group. Examples of substrates are cocaine, meperidine, methylphenidate, and temocapril (Zhang et al., 1999), which contain methanol or ethanol linked to a large acyl group. CES2 includes human, monkey, mouse, rat, and hamster intestinal CarbE. In contrast to CES1, CES2 enzymes hydrolyze esters with a higher alcohol group and a relatively small acyl group. Examples of substrates are heroin, 6-acetylmorphine, and methylprednisolone, which contains acetate or succinate as acyl groups. Other categories are CES3, which includes ES-male and human hCE3 and is expressed at low levels in the liver and the gastrointestinal tract; CES4, which includes a protein excreted in cat urine with Mol.wt. 70 kD (Miyazaki et al., 2003); and CES5, which contains protein with a much lower Mol.wt., (namely, 46.5 kD) and a different structure.

Active Site

Cohen et al. (1955) demonstrated that all the esterases in Aldrige group B contain an active serine that binds to the OP group or participates in ester hydrolysis. Further, comparison of the structure of a large number of esterases and lipases confirmed that they all contained a catalytic triad consisting of Ser, His, and Glu (and sometimes Asp instead of Glu). This catalytic triad were preserved in AChE, BuChE, a series of lipases, and CarbE (Cygler et al., 1993). Two adjacent Gly molecules formed an oxyanion hole. Frey et al. (1994) showed for chymotrypsin that the formation of a low-barrier hydrogen bonds facilitates a nucleophilic attack of the serine-OH group on the acyl carbonyl group of peptide. In the same way, these substances allow reactions between substrate esters or OP esters and the serine-OH of ChEs or CarbEs. When the catalytic triad consisting of Ser203, Glu336, and His450 in CarbE was transferred to The 203, Als 336, and Ala 450, respectively, the mutant reduced CarbE activity (Satoh and Hosokawa, 1998). The specificity of CarbE differs from AChE and BuChE in active site volume, electrostatic character, and cation-pI binding. The active site gorge of the enzymes has different sizes. For torpedo AChE, it is 296 D3; for BuChE, it is 496 D3; and for CarbE, it is 3014 D3. This explains why diisopropylfluorophosphate (DFP) cannot inhibit AChE, but it can inhibit BuChE and CarbE. In cholinesterase (ChE), there are 7 anionic groups to establish catioinic substrates, whereas CarbE has only 2. Further, AChE has 14 aromatic residues lining the active site gorge, BuChE has 7, and CarbE has only 2 (Ripoll et al., 1993; Shafferman et al., 1994). This explains the attraction of AChE and VX for ChE and not for CarbE.

The steps for CarbE interacting with substrate or soman (as an example of an OP inhibitor) are the following:

1. The ester bond or the P—F bond is attacked by the serine-OH.
2. The hydrogen bond between the negatively charged oxygen of a tetrahedral complex and the NH groups of glycine stabilize the charged carbonyl oxygen or phosphate oxygen of the substrate or inhibitor.
3. The ester bond or P—F bond breaks and the leaving group (alcohol or F) diffuses away.
4. A water molecule attacks the serine-O-acyl or O-phosphoryl group on the enzyme. Then, histidine delivers a hydrogen atom to the serine-O-acyl, which releases the acyl or phosphoryl group.

This reaction is fast for esters, but slow for soman. In human CarbE1, replacement of histidine for V1466 and glutamate for L363 leads to an enzyme with the same binding to OPs, but with an increased spontaneous reactivation of the enzyme (Hemmert et al., 2011). The two residues are positioned on different sides of the active site and are in an ideal position to direct a water molecule to the serine-OH. This results in an enzyme that is a significantly better scavenger for sarin, cyclosarin, and soman.

In the case of AChE, there is an intermediate step where serine-O-phosphoryl is dealkylated or aged. This is a process where the alkoxy group of the soman residue on the enzyme is hydrolyzed and the soman residue is resistant to the last hydrolysis step. The orientation of the sequence in CarbE allows the alkoxy group in soman residue and other nerve agents to be farther from the His group (7 Å instead of 3.5 in AChE), and therefore, it is not dealkylated (Maxwell and Brecht, 2001).

Substrate Specificity

Several different carboxylic esters may be hydrolyzed by CarbEs; among them, several may also be hydrolyzed by BuChE and A-esterases. For CarbEs, the aromatic butyryl esters are better substrates than the acetyl esters, whereas the opposite holds for A-esterase (Augustinsson, 1959; Aldridge 1953; Ecobichon, 1970). The hydrolysis of two simple aliphatic and aromatic butyryl esters (namely, methyl butyrate and 4-nitrophenyl butyrate) is catalyzed by CarbE, with only minimal contributions by ChE and A-esterase in a series of rat tissues, except

for duodenum, based on the use of specific inhibitors of the three enzymes (Sterri et al., 1985b). The two substrates, therefore, are appropriate to use for comparative evaluation of rat tissue CarbEs with respect to both inter- and intra-tissue diversity. Thus, the ratio between activities toward the two substrates in different tissues correlated to the plasma ratio seem to separate CarbEs of rat tissues into two main groups. The CarbEs of cerebrum, kidney, and duodenum display similar relative activity as plasma, with a preference for 4-nitrophenyl butyrate. In contrast, liver, lung, heart, and diaphragm constitute the other group with a far higher (13- to 17-fold) relative preference for methyl butyrate (Sterri et al., 1985b). This may indicate that the two groups are predominantly constituted by CES1 and CES2 CarbEs (as described in the section "Classification," earlier in this chapter), with preference for a substrate with lower (methanol) or higher (4-nitrophenol) alcohol esterified to the butyric acid. Also, the two CarbE isoenzymes of rat plasma and the three isoenzymes of guinea-pig plasma seem to differ with respect to their relative specificity for methyl butyrate and 4-nitrophenyl butyrate. For each species, the ratio between substrate activities of the plasma isoenzymes differs fivefold to sevenfold in favor of 4-nitrophenyl butyrate, as measured in the top fraction of activity peaks separated by chromatofocusing (Sterri and Fonnum, 1987, 1989).

Inhibition of CarbE with OP Compounds

Paraoxon, sarin, and soman inhibit AChE, BuChE, and CarbE almost to the same extent. DFP inhibits BuChE and CarbE to the same extent and 1,000-fold more than AChE. Diphenyl p-nitrophenyl phosphinate inhibit CarbEs over 1,000-fold more than the ChEs. This explains why bis-p-nitrophenylphosphate is a specific inhibitor of CarbEs.

A problem for CarbE as a scavenger for nerve gases is that VX and echothiophate are both 10,000-fold better inhibitors of AChE than CarbE. This is due to the anion attraction of ChE as described in the section "Active site," earlier in this chapter. OP-inhibited CarbE differ from OP-AChE in that they do not age. Instead, they spontaneously reactivate similar to a slow substrate. The half-life for spontaneous reactivation of sarin-inhibited CarbE is 2 h, whereas for soman or paraoxon, it is 20 h and for DFP, it is 40 h (Maxwell and Brecht, 2001). This agrees with results from a series of experiments with repetitive injection of 0.5x LD_{50} of soman in guinea pigs at different intervals, which demonstrated that soman was completely removed within 24 h and plasma CarbE (tributyrinase) activity was fully recovered (Sterri et al., 1981). The slow reactivation of DFP from CarbE explains its low tolerance to repetitive administration.

Reactivation by Oxime of Nerve Gas-Inhibited CarbE

The original finding by Askew (1956) that diacetylmonoxime (DAM) has an antidotal effect for sarin poisoning in several species, including guinea pigs, was due to the reactivation by DAM of sarin-inhibited CarbE in plasma (Myers, 1959; Polak and Cohen, 1970a; Cohen et al., 1971). In addition, they found that DAM reactivation had less of an effect on sarin-inhibited erythrocyte, brain, and lung CarbEs than on plasma CarbE of both rat and mouse (Myers, 1959).

Similar different sensitivities to DAM reactivation are reported for various soman-inhibited CarbEs. Following *in vitro* inhibition by soman, the CarbE isoenzymes with low pI in plasma of both rats and guinea pigs are partially reactivated (50–60%) by DAM within 5 min. and are not further reactivated during the next 30 min. (Sterri and Fonnum, 1987; Sterri and Fonnum 1989). The two isoenzymes in rat plasma cannot be discriminated based on DAM reactivatability (Sterri, 1989), whereas the one with high pI (6.1) in guinea-pig plasma is about half as sensitive to DAM reactivation than the other two (Sterri and Fonnum, 1987). Also, two out of three CarbE isoenzymes in rat small intestine display similar reactivatability as the plasma CarbEs (Sterri, 1989), whereas none of the three CarbE isoenzymes in guinea-pig liver can be reactivated by this oxime after soman inhibition (Sterri and Fonnum, 1987). The latter results correspond well with the poor effect of DAM on soman-inhibited commercial CarbEs from porcine liver (Fonnum et al., 1985).

ORIGIN OF PLASMA CARBE

It follows from the role of CarbE in nerve gas poisoning that the regulation of plasma CarbE is of great interest. Possible tissues of origin may include the liver (Yan et al., 1995) or the intestine (Sterri, 1989).

Interestingly, based on the results from immunoblots with hydrolase S antibody and messenger RNA (mRNA) levels detected by Northern blot, the 67- and 71-kDa proteins in the liver and serum, respectively, seem to be coregulated by the treatment of rats with various xenobiotics, including phenobarbital (approximately 1.7-fold induction; Yan et al., 1995). Such coregulation are not supported by the results obtained on plasma and liver CarbE activities with methyl butyrate or 4-nitrophenyl butyrate following phenobarbital treatment of rats by Sterri et al. (1985b). In this study, the induction was 1.5-fold to 1.8-fold in liver and maximally 1.2-fold in plasma. However, phenobarbital induction of CarbE in mice led to similar enhanced activities in both liver and plasma (Clement, 1983).

Some interesting results on soman toxicity, thyroid deficiency, and plasma CarbE were reported by Swisher

et al. (1986), which observed reduced effects of soman intoxication in hypothyroid rats with respect to both seizure activity and lethality. The resistance to soman was ascribed to increased binding of soman to plasma proteins since both ChE and CarbE (tributyrinase) of plasma displayed nearly twofold higher activity in hypothyroid than euthyroid rats. We therefore investigated hypothyroid rats with respect to the possible parallel coregulation of CarbE and ChE in plasma and liver. Plasma ChE is undoubtedly synthesized in liver (Augustinsson, 1948; Koelle, 1963), and the ChE activity in rat plasma and liver is known to differ between males and females (Leeuwin, 1966; Andersen et al., 1983; Edwards and Brimijoin, 1983; Sterri et al., 1985a) and to respond upon thyroidectomy (Leeuwin, 1966). When male and female rats received rat chow supplemented with 0.05% thiouracil for 22, 30, or 38 days, they became hypothyroid within 3 weeks (Stakkestad and Bremer, 1983). The results of CarbE activities in rat plasma and liver are presented in Figure 74.1. The activities with methyl butyrate increased significantly in liver but did not change in plasma of both males and females after 22 and 38 days of thiouracil feeding, whereas the hydrolysis of 4-nitrophenyl butyrate varied only slightly and inconsistently. At the same time, the ChE activities of plasma and liver decreased in females and increased in males.

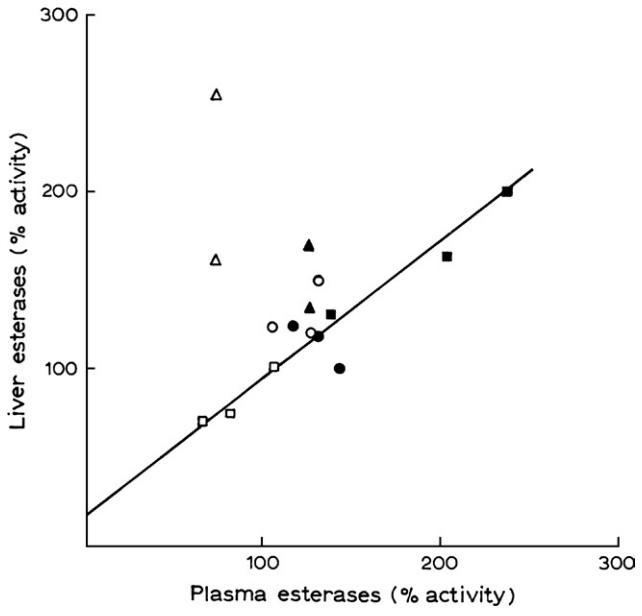

FIGURE 74.1 CarbE and ChE activities in liver versus blood plasma of thiouracil-fed rats. The animals received rat chow supplemented with 0.05% thiouracil for 22, 30, or 38 days. Methyl butyrate hydrolysis (▲△) and 4-nitrophenyl butyrate hydrolysis (●○) were measured in liver homogenate and plasma in accordance with Sterri et al. (1985b), and AChE hydrolysis (■□) by the method of Sterri and Fonnum (1978). The activities are percent of corresponding control (= 100%) activities in male (closed symbols) and female (open symbols) animals fed standard rat chow.

The results show that the ChE activities in plasma and liver of both male and female rats are strongly coregulated, whereas the CarbE activities of the two tissues are definitely not coregulated (methyl butyrate hydrolysis) or are only marginally influenced by thyroid deficiency (4-nitrophenyl butyrat hydrolysis). This investigation, therefore, does not indicate that rat plasma CarbE may be synthesized in the liver.

Results concerning the various CarbE isoenzymes with respect to reactivatability by DAM after soman inhibition (see the section "Reactivation by oxime of nerve gas-inhibited CarbE," earlier in this chapter) might suggest that plasma CarbE has an intestinal origin. Two isoenzymes in plasma of both rat and guinea pig are reactivated to a similar degree as two isoenzymes in the rat small intestine, and quite different from the three liver isoenzymes of guinea pig, which are not sensitive to DAM (Sterri and Fonnum, 1987; Sterri, 1989). Since the results from rat plasma and the small intestine are obtained with the same nerve gas and the same oxime under the same conditions, they may reflect similarities or differences, respectively, in enzymatic properties. It is interesting in this context that the DAM-sensitive isoenzymes of rat plasma and small intestine display correspondingly low isoelectric points (4.0–4.8; Sterri, 1989), whereas higher pI values (5.0–6.4) are reported for 6 CarbE isoenzymes of rat liver (Mentlein et al., 1987). The conclusion, therefore, may be that depending on the species, the plasma CarbE may come from the intestine or the liver.

ROLE OF PLASMA CARBE

Scavenger Function

The early results by Myers (1959) concerning prophylactic action of DAM against sarin poisoning in rats suggested that most of the sarin required to kill a rat actually may be used up by the inhibition of plasma CarbE, and the sarin may be destroyed in the bloodstream before it reaches target AChE levels in the vital organs of the rat. The importance of plasma CarbE for detoxification of sarin in the blood was also suggested by results from 32p-sarin injection and its distribution in the rat (Polak and Cohen, 1969, 1970a,b). Similar conclusions with respect to the function of plasma CarbE were based on results from a series of experiments with acute or repetitive injection of soman in rodents (Sterri et al., 1980, 1983, 1985a; Fonnum and Sterri, 1981; Sterri, 1981; Sterri and Fonnum, 1984; Fonnum et al., 1985). The results showed that the reaction rate between plasma CarbE and soman or sarin was rapid enough to inactivate a large proportion of the OP compound before it leaves the blood. Also, the plasma CarbE seemed to

be spontaneously and sufficiently recovered within 24 h and is then ready to detoxify another dose of soman or sarin—a point that is especially relevant in cases of repetitive exposure.

A scavenger function of plasma CarbE that is able to prevent highly toxic OP compounds from reaching AChE in the brain and diaphragm requires that an adequate amount of CarbE is present in the plasma. This is fulfilled for rodents since CarbEs have been found by several studies to be especially abundant in rodent plasma (Myers, 1952; Aldridge, 1953; Goutier, 1956; Augustinsson, 1959; Christen and Cohen, 1969; Polak and Cohen, 1970a). In fact, rat and guinea-pig plasma may contain as much as 2.8–3.0 μM or 0.5–0.6 μM of CarbE catalytic centers, respectively, as found with 32p-sarin binding to plasma proteins and subsequent polyacrylamide gel electrophoresis or reactivation of CarbE by lowering the pH (Christen and Cohen, 1969; Christen et al., 1969; Cohen et al., 1971). CarbE, therefore, is able to bind and detoxify the highly toxic OP compounds at the active site in equimolar proportions.

The various isoenzymes of plasma CarbE might have different importance with respect to their scavenger function, and it is not possible to predict the scavenger effect from assaying of substrate activities. But results from comparing male and female rat isoenzyme activity and developing of isoenzyme activities with age in rats showed a better correlation to soman toxicity for 4-nitrophenyl butyrate than for methyl butyrate as substrate (Sterri and Fonnum, 1989)

Toxicity of Nerve Gases

Several investigations have demonstrated that the concentration of CarbE protein in rodent plasma is critical for tolerance to acute exposure to highly toxic ChE inhibitors such as soman. For example, a strong correlation exists between the LD_{50} of soman and the plasma CarbE content in developing young rats (Fonnum et al., 1985; Sterri et al., 1985a). In these rats, the plasma CarbE activities with 4-nitrophenyl butyrate increased from negligible (5 days old) to adult (31 days old); and in the same period, the acute LD_{50} of soman increased eightfold to tenfold (while toxicity decreased) and reached adult value in the 31-day-old rats. In addition, young rats (14 days old) with low content of their own plasma CarbE, displayed strongly reduced mortality to soman poisoning when a partly purified CarbE protein from rat liver was prophylactically supplied by intravenous (IV) injection into the rats' blood before the exposure to soman (Fonnum et al., 1985). Also, a linear correlation between soman toxicity and changes in plasma content of CarbE in aging rats is reported by Maxwell et al. (1988).

The specific CarbE inhibitors tri-O-cresyl-phosphate (TOCP) (Mendel and Myers, 1953) and 2-[O-cresyl]-4H-1,2,3-benzodioxa-phosphorin-2-oxide (CBDP; Casida et al., 1961) have been used by several investigators to evaluate the effect on LD_{50} of highly toxic OP compounds. In mice, the LD_{50} of soman was reduced to about 1/18 by pretreatment with 35–50 mg/kg CBDP (McKay et al., 1971; Boskovic, 1979), whereas pretreatment with 100 mg/kg TOCP reduced the LD50 of soman to one-third in guinea pigs and rats (Fonnum and Sterri, 1981; Sterri, 1981; Sterri et al., 1981). In rats, the LD_{50} of sarin was reduced to about 1/5 by pretreatment with 40–50 mg/kg TOCP (Myers, 1959; Polak and Cohen, 1969). Inhibition of CarbE with CBDP showed that soman displayed the same toxicity with different species (Maxwell et al., 1988). Interestingly, for malathion, a more than 100-fold increase in acute toxicity by pretreatment with CarbE inhibitor tri-o-tolyl-phosphate was reported by Murphy et al. (1976). However, malathion is a less toxic OP compound that will be hydrolytically detoxified by the enzymatic activity of CarbE due to its content of carboxylic ester group.

The scavenger function of plasma CarbE may explain various aspects of soman toxicity and soman inactivation in rodents, but it also may be of value for an adequate understanding of soman toxicity in other species. Rats, guinea pigs, and primates (including humans) are species with high, medium, and minor (marmosets), or zero (rhesus monkeys and humans) concentrations of plasma CarbE, respectively (Myers, 1952; Aldridge, 1953; Christen et al., 1969; Christen and Cohen, 1969; Cohen et al., 1971). Sterri and Fonnum (1989) presented a concept based on the presence of CarbE, which in principle could explain the differences in LD_{50} of soman among the species. The predicted LD_{50} values of soman for rats, guinea pigs, marmosets, and rhesus monkeys (or humans) agreed well with the experimental values for subcutaneous LD_{50} of soman in rats (Sterri et al., 1980, 1985a), guinea pigs (Sterri et al., 1981), marmosets, and rhesus monkeys (Dirnhuber et al., 1979). General knowledge of the experimental LD_{50}s of soman suggests that rodent plasma CarbE may be a main scavenger for soman during inhalation, IV, and subccutaneous administration, whereas additional dermal or hepatic factors may influence the LD_{50} of soman by percutane or intraperitoneal administration (Sterri, 1989).

The CarbE concept described by Sterri and Fonnum (1989) assumes that the concentration of target AChE in the nervous tissue is small, so it can be regarded as constant in the different species, whereas the concentration of CarbE in plasma differs. A strongly simplified visual representation of their proportional contribition to LD_{50} of soman in rats is shown in Figure 74.2. The concentrations of target AChE and plasma CarbE in rats is represented in the figure by the quadrangles 1 and 2, respectively, and their proportions are similar to those previously presented in the CarbE concept (Sterri and

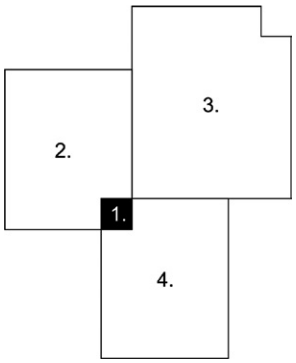

FIGURE 74.2 Visual representation of soman LD_{50} in rats as proportionally influenced by (1) target AChE, (2) plasma CarbE, (3) carbamate prophylaxis, and (4) other therapeutic regimen (at rat plasma CarbE conc.). The concentration of target AChE (1) or its enhancement by carbamate prophylaxis (3) is constant among the species, whereas the concentration of plasma CarbE (2) varies. The representation is based on the CarbE concept outlined by Sterri and Fonnum (1989) and Sterri (1989). LD_{50} decreases (and toxicity increases) when plasma CarbE (2) is decreased or absent.

Fonnum, 1989). At a glance, one can see from Figure 74.2 that without therapeutic intervention, the LD_{50} of soman as represented by (1 + 2) is strongly influenced by the concentration of plasma CarbE (2). Also, it is reduced in species with lower CarbE concentrations than rats, until it is exclusively represented by the target AChE concentration (1) in species such as rhesus monkeys and humans, which do not have CarbE in their plasma.

This concept works well with OPs that are very toxic, such as sarin, soman, and paraoxon. It does not work for less toxic OPs such as DFP because their toxicity is so low that the level of OP in plasma overwhelms the level of CarbE (Dettbarn et al., 1999). It does not work for VX and echothiophate because they are very good inhibitors of AChE and poor inhibitors of CarbE

Therapeutic Intervention

The effect on soman toxicity by pyridostigmine prophylaxis in different species could in principle also be explained by the CarbE concept of Sterri and Fonnum (1989). This means that the protection factors of carbamate prophylaxis, as calculated for the different species, were similar to the experimental protection factors of pyridostigmine prophylaxis against soman as observed with rats and guinea pigs (Gordon et al., 1978), as well as marmosets and rhesus monkeys (Dirnhuber et al., 1979). Thus, due to the CarbE concept, we were fairly convinced that the human species would achieve similar protection by pyridostigmine prophylaxis as rhesus monkeys, since both species lack the plasma CarbE. The proportional contribution by pyridostigmine prophylaxis to the LD_{50} of soman in rats is displayed in Figure 74.2, as represented by quadrangle 3. Since this prophylaxis leads to protection of a constant factor (namely, target AChE), the contribution to LD_{50} by pyridostigmine is regarded to be constant in the species (30× target AChE concentration) in accordance with the CarbE concept (Sterri and Fonnum, 1989). At a glance, one can see from Figure 74.2 that the protection factor of carbamate prophylaxis, which is the LD_{50} with prophylaxis (1 + 2 + 3) divided by that without prophylaxis (1 + 2), is relatively low in rats due to the large concentration of plasma CarbE (2), whereas in species such as rhesus monkeys and humans, the factor would be equal to 30 (see Figure 74.2, 1 + 3) divided by (1) since plasma CarbE is not present. Any other form of therapeutic regimen can be treated in a similar way to obtain the protection factor in humans.

This concept was later confirmed by studies by Maxwell and Brecht (1991) and Maxwell et al. (1993). When animals such as rats, guinea pigs, and rabbits with widely different levels of CarbE were poisoned with soman, therapeutic regimens gave the same level of protection in all the animals pretreated with CBDP.

Since plasma CarbE is established as a functional scavenger for highly toxic OP compounds, this has inspired other investigators to use artificially supplied scavengers to improve human protection against nerve gases. Efficient protection has been observed against OP poisoning by prophylactic injection of CarbE, AChE, or BuChE into the bloodstream of rodents and primates (Fonnum et al., 1985; Wolfe et al., 1987; Raveh et al., 1989, 1997; Broomfield et al., 1991; Maxwell et al., 1991, 1992; Doctor et al., 1993). In the future, new scavengers that could mimic the function of plasma CarbE might hopefully be developed. The consequence of this concept is that for evaluating toxic doses in humans, one should use animals with no plasma CarbE. This can be achieved by using plasma CarbE knockout mice, as described by Duysen et al. (2012), or rodents whose plasma CarbE is inhibited by TOCP or CBDP (Sterri et al., 1981; Fonnum and Sterri, 1981; Maxwell and Brecht, 1991; Maxwell et al., 1993; Boskovic, 1979).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Plasma CarbE has proved to play an important role in nerve gas poisoning, both with and without therapeutic intervention, and it has been shown in nature that an effective scavenger of nerve gases can be present in the blood. In accordance, both CarbE and other B-esterases have proved to be effective as artificially supplied scavengers, but since they are highly molecular proteins, some problems may be difficult to overcome for practical use. It is tempting to speculate, as has been

done previously (Sterri 1989), that a low-molecular scavenger associated with the large amount of albumin in human plasma would be a better alternative. Perhaps resources should be directed toward investigating these possibilities.

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CHAPTER

75

Catalytic Bioscavengers: The New Generation of Bioscavenger-Based Medical Countermeasures

Patrick Masson

INTRODUCTION

Organophosphate thioesters/oxoesters (OPs) were discovered in the middle of the nineteenth century. They are mostly pesticides (parathion, malathion, chlorpyriphos, dichlorvos), and some of them have been used as drugs or pro-drugs (echothiophate, métrifonate, cyclophosphamide), flame retardants, and antiwear agents (tricresyl phosphate [TCP]). Other OPs are potent chemical warfare agents (G agents: tabun, sarin, soman, cylclohexyl-sarin; and V agents: VX, VR, CVX). The mysterious nerve agents (NAs) called "novichoks" (A-230, A-232, A-234) are also OPs (Tucker, 2006). Highly toxic OPs may be formed by cytochrome P450-mediated metabolic oxidation of thionophosphoesters, such as paraoxon from parent compound parathion (Foxenberg et al., 2007), or 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP or cresyl-saligenin phosphate [CSP]) by cyclization of tri-*ortho*-cresyl phosphate (TOCP), a TCP isomer (Eto et al., 1962). High-temperature pyrolysis of synthetic oils may lead to toxic OP such as trimethylolpropane phosphate (TMPP) (Masson et al., 2012). Most of these compounds are potent irreversible inhibitors of cholinesterases (ChEs) (Figure 75.1) (Costa, 2006) and of many other serine hydrolases. However, TMPP is not an inhibitor of ChEs; it is a gamma aminobutyric acid (GABA) antagonist so toxic that it has been called "the poor man's nerve agent."

There are two types of ChEs, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC.3.1.1.8). AChE plays a major role in the cholinergic system terminating the action of the neurotransmitter acetylcholine (ACh). Inhibition of AChE leads to accumulation of ACh in synapses and blockade of cholinergic transmissions in peripheral and central nervous systems. Inhibition of peripheral and central AChEs is the main cause of acute toxicity of OPs (Maxwell et al., 2006). Irreversible inhibition of other enzymes, including various serine hydrolases, plays a role in subacute toxicity and noncholinergic toxicity of OPs (Casida and Quistad, 2004, 2005).

Significant progress has been made in the past 25 years in emergency treatments of acute poisoning and management of poisoned casualties (Eyer et al., 2007; Thiermann et al., 2007; Wetherell et al., 2007; Eddelston et al., 2008; Masson, 2011; Moshiri et al., 2012). However, classical pharmacological approaches are reaching their optimum limit. Recent attacks in Syria, where nerve agents (NAs) were used, revealed the limitations of current medical treatments (Dolgin, 2013). In addition, due to accumulation of OP in depot sites and subsequent slow release from these sites, blood and tissue ChEs may remain inhibited for long periods of time. Therefore, persistence of certain OPs in the body after initial exposure complicates treatments. This is particularly well-documented for severe intoxications by parathion (Willems and de Bisschop, 1993).

Endogenous enzymes are involved in natural defenses against OP toxicity. The presence of detoxifying enzymes in skin contributes to reduce the OP dose that penetrates into the body (Schallreuter et al., 2007). Numerous secondary targets of OPs found in various tissues are detoxifying enzymes and they certainly play a role in the natural defenses against OPs (Wang et al., 1998; Nomura et al., 2005, 2008). Liver enzymes play an essential role in detoxification. In particular, oxidases, such as glutathione *S*-transferases (GSTs), play a role in degradation of

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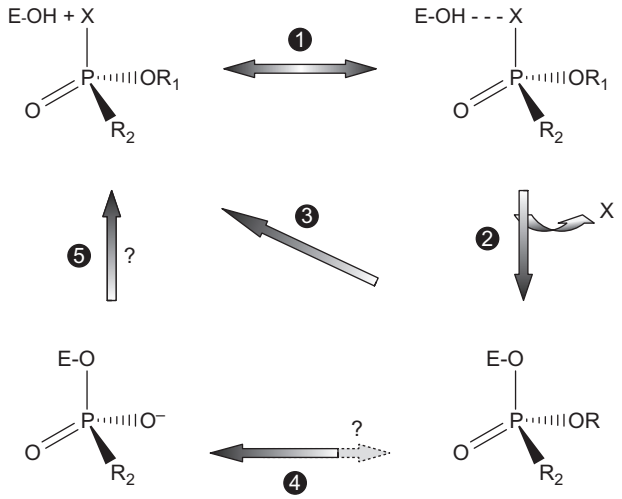

FIGURE 75.1 Mechanism of inhibition of cholinesterases by OPs. After formation of reversible complex between ChE and OP (step 1), the active serine (esteratic site, E-OH) is phosphylated; the reaction leads to release of leaving group X (step 2). The phosphylated enzyme can be reactivated by nucleophilic agents, such as quaternary oximes (2-PAM [Contrathion or pralidoxime], TMB-4, MMB-4 [methoxime], Obidoxime [toxogonin], HI-6, Karboxim), used as antidotes in emergency treatment of OP poisoning (Lundy et al., 2006; Worek et al., 2007; Worek and Thiermann, 2013) (reaction 3). Water is a nucleophile that is too weak for fast spontaneous reactivation of phosphylated ChE. The phosphyl–ChE conjugate may undergo spontaneous dealkylation through alkyl-oxygen bond scission ("aging") (Kovach 2004; Masson et al., 2010), resulting in irreversibly inactivated ("aged") enzymes (step 4). The dealkylation reaction can be very fast ($t_{1/2}$ = 3 min at 37°C for human AChE phosphonylated by soman). At the moment, drug-mediated reactivation to E-OH through realkylation (R_1) of aged ChE (reverse reaction 4 and subsequent reaction 3) or direct displacement of the aged adduct (reaction 5) are not possible.

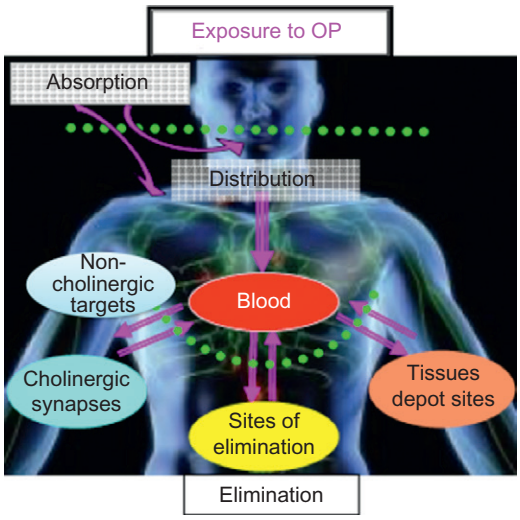

FIGURE 75.2 Biological fate of organophosphorus compounds in humans. Routes of penetration of OPs are absorption through the skin, eyes, and/or respiratory tract (nerve agents, pesticides), or ingestion (self-poisoning). OP molecules distribute from the blood compartment into tissues, including depot sites, biophases (cholinergic synapses and secondary targets), and sites of elimination (liver and kidneys). Cholinesterases are the main biological targets (acute toxicity); reaction with secondary targets (carboxylesterases, serine-amidases, peptidases, and other proteins) may be responsible for non-cholinergic sublethal effects of OPs and chronic toxicity at low-dose exposure (Casida and Quistad, 2004; Costa, 2006).

alkyl/aryl chains (Casida and Durkin, 2013). However, as mentioned, liver cytochrome P450 enzymes activate phosphorothioates and lead to oxon forms much more toxic than parent compounds, but cytochrome P450 also dearylate aryl-containing OPs and participate in detoxification of OPs (Furlong, 2007; Ellison et al., 2012). Natural blood bioscavengers significantly contribute to reduce the amount of OP molecules reaching physiological targets. It has been shown that animal species in which the concentrations in paraoxonase-1 (PON-1; EC 3.1.8.1) or in carboxylesterase (CarbE; EC 3.1.1.1) are high are relatively resistant to OPs (Kaliste-Korhonen et al., 1996). Conversely, *knockout* mice for PON1 are very sensitive to OPs (Shih et al., 1998). Albumin displays low esterase activity and slowly reacts with carbamyl-esters and phosphoryl-esters. However, its concentration in blood and lymph is so high (≈0.6 mM) that it likely plays a role in detoxification of carbaryl at toxicologically relevant concentrations (Sogorb et al., 2007). Thus, plasma albumin could also play a role in detoxification of certain OPs (Li et al., 2008; Tahroni et al., 2007).

Toxicity of OPs can be countered by reducing skin absorption and lowering OP concentrations in the blood compartment, thus preventing the transfer of OP molecules toward cholinergic synapses and other biological targets (Figure 75.2). Neutralization of toxicant molecules can be achieved by using stoichiometric traps or catalysts acting on exposed surfaces (e.g., active TSPs) or in the bloodstream (bioscavengers). The concepts of bioscavenger and development of this approach in prophylaxis and post-exposure treatment of OP poisoning have been exposed in several recent reviews (Masson and Rochu, 2009; Masson and Lockridge, 2010; Nachon et al., 2013).

STOICHIOMETRIC SCAVENGERS

The first molecules that have been studied with the purpose of making stoichiometric scavengers were cyclodextrins (Désiré and Saint-André, 1986), neutralizing antibodies (Glikson et al., 1992), and activated charcoal. Although the interest of charcoal is much debated, hemodialysis on a charcoal cartridge was successfully used in a Tokyo casualty who was resistant to the classical treatment for sarin poisoning (Yokoyama et al., 1995). Transfusion of human plasma has been used for treating OP poisoning. The effects of fresh-frozen plasma (plasmapheresis) on ChE levels and outcomes in OP-poisoned

patients have been evaluated (Güven et al., 2004; Qiu et al., 2011). An *in vitro* study also suggests that plasma (freshly prepared and fresh-frozen plasma) therapy may be an effective adjunctive treatment method against G agents (Wille et al., 2013). Plasma BChE and possibly other abundant OP scavenging proteins in plasma (e.g., albumin, PON-1) may have contributed to these results. At this point, it must be noted that unlike plasma of most model animals, human plasma does not contain CarbE (Li et al., 2005).

At the end of the 1980s, research of scavengers mostly focused on enzymes that specifically react with OPs. ChEs (Wolfe et al., 1987) and CarbE (Redinbo and Potter, 2005; Fleming et al., 2007; Hemmert et al., 2010) have been proposed as stoichiometric scavengers. Human BChE has been proven to be an effective stoichiometric bioscavenger for pretreatment and post-exposure treatment of NA and OP pesticide poisoning (Allon et al., 1998; Doctor and Saxena, 2005; Lenz et al., 2007; Saxena et al., 2008a,b; Mumford et al., 2013; Rosenberg et al., 2013, 2014). Human plasma-derived BChE was granted Investigational New Drug (IND) status by the FDA in 2006 for protection against NAs (Lenz et al., 2007). Clinical trials using volunteers (phase I) started a few years ago. However, enzymatic stoichiometric neutralization of several OP LD_{50} needs administration of huge amounts of enzymes, approximately 3 mg/kg of highly purified plasma BChE (i.e., 200–300 mg for humans against 2× LD_{50} of soman) (Ashani and Pistinner, 2004). Large-scale production of enzymes under good manufacturing practices (GMP) conditions at a reasonable cost was the subject of intense research during the past 10 years. However, although several thousand tons of outdated human plasma are available in the United States for preparing the enzyme, 1 L of human plasma provides less than 1 mg of GMP BChE.

Several industrial GMP processes have been proposed for mass production of human BChE. The first one is purification of the natural enzyme from human plasma (Cohn Fraction IV). This process has been developed by Saxena et al. (2008a,b), Lockridge et al. (unpublished), and Baxter Healthcare Corporation (www.baxter.com). The second method has been developed by Nexia (www.nexiabiotech.com); it uses the recombinant human enzyme produced in milk of transgenic goats. Several grams of enzyme can be secreted in 1 L of milk. This enzyme has been named Protexia™. Since 2005, Pharmatheme (www.pharmathene.com) developed Protexia™ PEGylated derivatives of this enzyme (Huang et al., 2007) and fusion proteins (Huang et al., 2008). However, production of recombinant human BChE in milk of transgenic mammals has been discontinued due to high coast and pharmacokinetics problems. Therefore, Pharmatheme and other companies are working on large-scale production of recombinant human BChE expressed in human cells or in Chinese hamster ovary (CHO) cells (Rosenberg et al., 2010). Production of human BChE in transgenic tobacco has also been made possible (Geyer et al., 2010; Larrimore et al., 2013). PlantForm in Canada (www.plantformcorp.com) has been engaged in development of recombinant human BChE in tobacco that mimics stability and pharmacokinetics of plasma-derived enzyme.

Certain secondary targets of OPs are potential bioscavengers. In particular, because of the high number of amino acid residues in human serum albumin that covalently bind OP molecules (five tyrosines and two serines) (Ding et al., 2008), it may be hypothesized that reactivity of these residues could be enhanced by genetic engineering and/or on specific chemical modification. However, direct tyrosine nitration (to lower pK_a of tyrosine) did not lead to expected improvement in reactivity, possibly because of steric hindrance (Masson et al., unpublished). Engineered albumins could lead to a new generation of stoichiometric bioscavengers. However, conversion of albumin into a catalytic bioscavenger would need to increase its catalytic efficiency by several orders of magnitude, a challenge that seems to be unrealistic (Li et al., 2008).

Low molecular stoichiometric scavengers could be an economic alternative to enzyme-based stoichiometric scavengers. Several serine-containing and tyrosine-containing hexapeptides from a random library of peptides have been selected because they form phosphoester bond with a fluorescent analog of sarin (Landry and Deng, 2008; Zhu et al., 2008). Finally, a way to increase the endogenous stoichiometric bioscavenger capacity could be to enhance the endogenous expression of BChE and AChE. This could be achieved by administration of proline-rich polypeptides derived from ChE collagenic tail peptide (ColQ PRAD) or peptides derived from transmembrane anchor (PRIMA) that promote folding, tetrameric assembly, and exportation of the enzymes from cells (Rotundo, 2011).

PSEUDOCATALYTIC BIOSCAVENGERS

Because the main limitation of the stoichiometric bioscavenger approach is the cost of the huge dose of enzyme to be administered for challenging the OP molecules, a way to circumvent this problem is to *in vivo* reactivate the administered enzyme, turning the stoichiometric bioscavenger into a pseudocatalytic bioscavenger.

Certain ChE mutants sensitive to OPs do not "age" after phosphylation; they are fully reactivatable (Figure 75.1, reaction 3). Such ChE mutants, such as the human AChE mutant Y337A/F338A (Cochran et al., 2011) when associated with oximes (e.g., 2-PAM, HI-6), act as pseudocatalysts in displacing the OP moiety bound to the enzyme. These enzyme reactivator-coupled systems could lead to a new family of pseudocatalytic bioscavengers (Taylor et al., 2007; Kovarik et al., 2007, 2013;

Mazor et al., 2008). Success of this approach requires implementation of new oximes displaying higher affinity for phosphylated ChEs (lower K_D), higher reactivation constant (k_r), and a long circulation time in the bloodstream. Study with wild-type human BChE showed that the geometry of oxime function access to the phosphorus atom of conjugate is an important criterion for a fast reactivation. Several libraries of positively charged and uncharged molecules have already been made by *in silico* design or click chemistry and tested with success *in vitro* against tabun and soman (Kovarik et al., 2013; Radić et al., 2013; Renou et al., 2013).

CATALYTIC SCAVENGERS

Catalytic scavengers are enzymes or artificial catalysts capable of degrading OPs. These catalysts detoxify OPs by hydrolyzing the phosphoester (organophosphorus acid anhydride hydrolase, OPAA) activity, also called "phosphotriesterase" activity, OP hydrolase (OPH, OPase) activity, or lead to less toxic compounds by degrading their alkyl/aryl chains through oxidation.

The catalytic bioscavenger concept, second-generation bioscavengers, is based on the idea of continuously trapping and degrading OPs with a turnover in the bloodstream before OP molecules reach their central and peripheral neuronal and neuromuscular targets. Then, prophylactic injection of enzymes capable of hydrolyzing OP quickly (alone or in association with current prophylactic countermeasures, i.e., pyridostigmine bromide, PANPAL, and other reversible ChE inhibitors and anticholinergic drugs) (Masson, 2011) would allow first responders, firemen, explosive ordnance disposal technicians, and medical personnel to operate safely in contaminated environments on contaminated casualties. Intravenous or intramuscular administration of bioscavengers to chemical casualties is expected to greatly improve the efficacy of implemented pharmacological countermeasures (Ashani et al., 1998; Saxena et al., 2006).

These enzymes could be used also for protection of skin (Fisher et al., 2005), and for decontamination of skin, mucosa, and open wounds (Lejeune and Russell, 1999; Gill and Ballesteros, 2000). Genetically engineered bacteria producing OPHs could be introduced in water effluents of decontamination units and could purify contaminated water before recycling or washing up in the environment (Chen and Mulchandani, 1998).

Research of catalytic antibodies has made some progress (Jovic et al., 2005; Smirnov et al., 2012). In particular, resolution of the three-dimensional structure of a "reactibody" fragment with an OP (Smirnov et al., 2011) has opened the possibility of rational design of more active catalytic antibodies (Kurkova et al., 2012). However, the turnover of catalytic antibodies remains extremely slow, and their specificity is too narrow for practical interest as medical countermeasures.

Research of artificial enzymes acting as catalytic scavengers (e.g., functionalized β-cyclodextrins) is promising. Cyclodrextrin derivatives bearing a nucleophilic group such as isodozobenzoic acid or an oxime were made (Ramaseshan et al., 2006; Estour et al., 2013; Kalakuntla et al., 2013; Bierwisch et al., 2014). These compounds display very interesting catalytic properties against certain NAs, particularly cyclosarin (Müller et al., 2013). However, engineering (computer design and/or directed evolution) of enzymes capable of degrading OPs remains the most promising short-term research field.

REQUIREMENTS

The general requirements for the use of enzyme-degrading OPs as medical countermeasures against OP poisoning are as follows. These enzymes must have a large activity spectra and, ideally, enantioselectivity for toxic stereoisomers. Their mass production under GMP conditions must be realizable at a reasonable cost. Long-term storage without activity loss (in solution, lyophilized, or adsorbed/bound on a matrix) must be possible under field conditions. Conformational stability can be optimized by chemical modification or addition of stabilizers likes polyols. Otherwise, thermostable enzymes from hyperthermophilic bacteria (Merone et al., 2005; Elias et al., 2008) and expressed in *E. coli* or mutated/evolved highly stable enzymes from mesophilic bacteria are promising alternatives.

Other conditions depend on the type of administration, delivery system, or galenic formulation of these enzymes. Enzymes can be injected intravenously or intramuscularly. Other routes have been considered, such as the intranasal route against aerosolized or gaseous NAs. For instance, pretreatment with aerosolized forms of BChE have been shown to protect against aerosolized paraoxon (Rosenberg et al., 2013). For parenteral administration, the toxicant concentration in blood has to be considered. Even in the most severe case of poisoning, this [OP] concentration is always very low. For example, the sarin concentration in serum of casualties after Matsumoto and Tokyo chemical attacks have been estimated between 1.5 and 30 nM at 14 h after exposure (Polhuijs et al., 1997). Therefore, the [OP] concentration in plasma is always well below the K_m of the enzyme for OP substrates. This determines first-order kinetics for hydrolysis of OP in blood (Masson et al., 1998, 2008) as described by Eq. (75.1):

$$v = k_{cat}/K_m \cdot [E] \cdot [OP] \tag{75.1}$$

In Eq. (75.1), the product of the bimolecular rate constant (k_{cat}/K_m) and the enzyme active site concentration ([E]) is the first-order rate constant. The amount of enzyme to be injected for degradation of toxic molecules in a very short time depends on the enzyme efficiency (i.e., k_{cat}/K_m). The higher the catalytic efficiency, the lower the enzyme dose to be administered. The enzyme concentration that reduces the OP concentration to a nontoxic concentration in time t is:

$$[E] = \frac{X}{k_{cat}/K_m \cdot t} \tag{75.2}$$

X is the factor by which [OP] is reduced ($X = \mathrm{Ln}[OP]_0/[OP]_t$) (Masson et al., 2008). In this equation, it is assumed that stability and pharmacokinetics of the administered enzyme have been optimized, and that the enzyme concentration [E] does not decrease during the time course of the reaction with OP. The efficiency and stereo-specificity of a given enzyme can be increased by several orders of magnitude by mutagenesis or chemical engineering (Griffiths and Tawfik, 2003; Hill et al., 2003; Gupta et al., 2011; Tsai et al., 2012).

The second constraint is to maintain the bioscavenger concentration [E] in the bloodstream as high as possible for a long time. [E] is controlled by the enzyme pharmacokinetics/pharmacodynamics and/or the frequency of repeated administrations. Increasing the size of the enzyme by polymerization, decreasing glycosylation microheterogeneity, and chemical modifications of solvent-exposed surface ("capping") improve the biological life of injected enzymes (Cohen et al., 2006). Fast clearance of glycoproteins is often due to glycosylation defects; fast clearance of nonglycosylated enzyme may result from their too small size (Jackson et al., 2010). Glycosylation defects can be corrected by chemical modifications such as PEGylation (Chilukuri et al., 2005), polysialylation of purified expressed enzyme (Ilyushin et al., 2013), or by selection of appropriate expression system (Chitlaru et al., 1998), including overexpression of an additional glycosylation enzyme that increases sialylation content of the expressed glycoprotein (Schneider et al., 2013). Size defects can be corrected by conjugation to polyethylene glycol, dextran, other macromolecules, or fusion to albumin (Huang et al., 2008). All these modifications reduce renal clearance and increase plasma retention.

Administration of homologous enzymes does not induce immunologic response after a second injection (Sun et al., 2009). However, immunotolerance of injected heterologous enzymes is a major issue. Bacterial enzymes and heterologous mammalian enzymes are not suitable for use in humans, but conjugation to dextran or polyethylene glycol (PEGylation) is often sufficient to reduce antigenicity and to slow clearance after multiple injections (Novikov et al., 2010; Trovaslet-Leroy et al., 2011; Sun et al., 2013).

Extracorporeal dialysis has been successfully implemented in a patient for blood decontamination after the Tokyo subway attack (Yokoyama et al., 1995). Incorporation of OP-degrading enzymes in the medical dialysis system could greatly improve the efficiency of dialysis. Enzymes can be immobilized on dialysis cartridges (Klein and Langer, 1986). Accessibility of OP molecules to an enzyme active center must not be altered by the immobilization method or by matrix effects. The enzyme concentration per surface unit has to be maximized to reduce diffusion constraints. In that case, both the reactive surface of the matrix and k_{cat}/K_m would have to be as high as possible and the flow rate would have to be reduced to increase the efficiency of the reactor. First-order degradation kinetics takes place under the particular conditions of immobilized enzymes in the continuous-flow system. Immunocompatibility problems are theoretically suppressed, thus permitting the use of unmodified nonhuman enzymes.

Finally, *in situ* transient production of enzymes, if the need arises, will be possible by gene therapy in the future. Promising results have been published using short-induction gene vectors (adenoviral systems) for human PON-1 (Cowan et al., 2001; Bradshaw et al., 2005; Fu et al., 2005; Miyoshi et al., 2007; Guns et al., 2008; Duysen et al., 2011), human AChE (Li et al., 2006), human BChE (Chilukuri et al., 2009; Parikh et al., 2011), mutated human BChE that displays high cocaine esterase (Gao et al., 2013), and human prolidase (Aleti et al., 2013).

Enzymes in skin and eye lotions, immobilized in foams and on tissues for skin and eye decontamination (Gordon et al., 2003) or in TSPs (Braue et al., 2002) act under conditions where local OP concentrations can be very high. In these cases, enzyme reaction order in OPs tends to zero, so that reaction rate is close to maximum velocity:

$$v \rightarrow V_{max} = k_{cat} \cdot [E] \tag{75.3}$$

The enzyme efficiency depends on its concentration and its catalytic constant k_{cat}. Thus, for external use, enzyme preparations have to be highly concentrated with high molecular catalytic activity. Co-immobilization of different enzymes could be an easy way to extend the spectra of agents to be degraded. This should allow simultaneous detoxification of G and V agents, as well as other potential chemical warfare agents, pesticides, and so-called "nontraditional nerve agents." Exposure to multiple agents has to be considered. In this view, it should be remembered that during the war between Iran and Iraq, tabun and other OPs have been combined with mustard gas in some attacks (UN Reports, 1984, 1987). In asymmetric conflicts and eschatological and mafia-like terrorism, the most extreme scenarios have to be taken into account.

POTENTIAL ENZYMES

Phosphotriesterases

Bacterial PTEs

Bacterial PTEs (EC 3.1.8.1) detoxify OPs (Ghanem and Raushel, 2005; Theriot and Grunden, 2011). These enzymes have been isolated from numerous sources. Four enzyme families showing different folds or topologies can be described: TIM-barrel fold, β-lactamase fold, pita bread fold, and β-propeller fold (Bigley and Raushel, 2013). They are encoded by the OP degradation (*opd*) gene found in species of *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*), *Flavobacterium Sp., Agrobacterium radiobacter* (Horne et al., 2002), *Pseudomonas pseudoalcaligenes* (Gotthard et al., 2013), and genes similar to *opd* were also located in archaea (Merone et al., 2005). PTEs belong to a superfamily of amidohydrolases (Holm and Sander, 1997; Bigley and Raushel, 2013).

Brevundimonas diminuta PTE is a 72-kDa dimeric bimetallic enzyme with Zn^{2+} involved in the catalytic process (Carletti et al., 2009). Substitution of the native Zn^{2+} ions in the active site with Mn, Co, Ni, or Cd ions results in the almost full retention of catalytic activity. After the first determination of the three-dimensional structure of *P. diminuta* PTE (Benning et al., 1994), a series of crystal structures and kinetic and spectroscopic experiments were described. Nevertheless, the enzyme mechanism of bacterial PTEs is still debated and the functional roles of divalent metal cations and amino acids in the active center are not yet fully understood (Aubert et al., 2004; Jackson et al., 2006, 2008; Chen et al., 2007; Samples et al., 2007; Wong and Gao, 2007; Bigley and Raushel, 2013).

These enzymes that are primarily lactonases are now called PTE-like lactonases (PLLs). The lactonase activity plays a role in bacterial communication (*quorum* sensing) (Dickschat, 2009). Virulence and formation of biofilms are regulated by concentration of lactones (*N*-acetyl-homoserine lactones) in the medium. Thus, the lactonase activity by hydrolyzing lactones acts as a *quorum* quencher, which in turn inhibits bacterial communication (Amara et al., 2011). The PTE activity is an undiscriminating activity believed to have evolved from lactonases (Afriat et al., 2006; Elias et al., 2008; Afriat-Jurnou et al., 2012; Hiblot et al., 2012).

Whereas the catalytic efficiency of *Brevundimonas diminuta* PTE for hydrolysis of paraoxon, the best substrate identified so far, is approaching the diffusion-controlled limit, it is slow against OP NAs (Table 75.1). Meanwhile, directed evolution of *Brevundimonas diminuta* PTE showed that only three amino acid changes dramatically enhanced the catalytic efficiency for an analogue of soman by approximately three orders of magnitude (Hill et al., 2003). Further studies combining rational design and directed evolution led to randomized libraries of mutants and selection of variants with greatly improved catalytic activity against S_p enantiomers of NA chromogenic analogues, including VX and VR analogues, and racemic real NAs (Table 75.1) (Tsai et al., 2010, 2012). There have been numerous studies highlighting the potential of this enzyme for decontamination or skin protection in addition to OP detection (Lejeune and Russell, 1999; Gill and Ballesteros, 2000; Ghanem and Raushel, 2005; Létant et al., 2005; Karnati et al., 2007). Administration of PTE before or after OP exposure was shown to improve pretreatment and current treatment of OP intoxication (Doctor and Saxena, 2005). However, to prevent abnormally fast pharmacokinetics and/or immunological response due to injection of a bacterial enzyme, PTE could be PEGylated (Jun et al., 2007) or encapsulated. First attempts at using PTE encapsulated within sterically stabilized liposomes were promising, providing protection of rats from multiple LD_{50}s of OP pesticides (Petrikovics et al., 2004). An alternative route could be the blood detoxification by extracorporeal circulation through a cartridge containing PTE immobilized in hollow fibers (unpublished results). PTEs possibly could also be used for skin protection as active components of TSPs or covalently coupled to the cornified layer of epidermis (Parsa and Green, 2001).

Brevundimonas diminuta PTE was also entrapped in additives for latex coating of biodefensive surfaces. Such PTE-based additives for paints and coatings were shown to retain catalytic parameters and stability of the enzyme (McDaniel et al., 2006). For decontamination of OPs in the environment and remediation, an alternative approach, phytodegradation by transgenic plants (e.g., tobacco) expressing a bacterial PTE, has been considered as a potentially low-cost, effective, and environmentally friendly method (Wang et al., 2008). It should be mentioned that His-tagged PTE (Efremenko et al., 2007) was reported to degrade NAs, including VX, at a high rate. Because the enzyme was not genetically modified, it is suggested that the presence of His tag plays a role in this amazing activity. Although neither three-dimensional structure nor molecular dynamics studies are available for this enzyme, it can be hypothesized that the His tag may increase the enzyme flexibility, which in turn should increase the enzyme capability to accommodate numerous OP molecules and improve its catalytic activity.

Highly stable promiscuous lactonases/PTEs from hyperthermophilic archae have been isolated in hot springs and volcano *solfatare*. Their three-dimensional structures, evolution, stability, and catalytic properties of several have been determined (Elias et al., 2008; Zhang et al., 2012; Hiblot et al., 2012; 2013a,b; Gotthard et al., 2013; Porzio et al., 2013). These enzymes have

TABLE 75.1 Catalytic Efficiency (k_{cat}/K_m, $M^{-1}\,min^{-1}$) of Different Natural and Engineered OPHs Toward Different OPs

Source of Enzyme	Paraoxon	DFP	Tabun (GA)	Sarin (GD)	Soman (GD)	Cyclosarin (GF)	Echothiophate	VX
Human PON1 Q192	6.8×10^{5}[a]	4×10^{4}[b]		9.1×10^{5} [c]	2.8×10^{6}[c]			+[d]
Human PON1 R192	2.4×10^{6}[a]			7×10^{4}[c]	2.1×10^{6}[c]			+[d]
Human rPON1 in 293T					6.2×10^{5}–4.1×10^{6}[e]			
Mammalian rPON1 G3C9	7.2×10^{5}[f]							
Mammalian rPON1 V346A					8.7×10^{4}[g]	3.6×10^{5}[g]		
Chimeric rPON1 IIG1			2.6×10^{6}[h]	9.5×10^{6}[h]	6.4×10^{7}[h]	8.4×10^{7}[h]		1.43×10^{2}(*)[h]
Human BChE G117H	5.7×10^{3} [i]	5.2×10^{3} [i]		1.6×10^{2} [j]	–		1×10^{4}[i]	1.5×10^{3} [j]
Blowfly CaE G117D	2×10^{5}[k]							
Human CaE1 (V146H/L363E) *B. fasciatus* AChE HQT	64 [i]	7.6×10^{2} [i]				5.3×10^{3}[l]	24 [h]	
*Loligo vulg*aris DFPase		7.8×10^{7} [m]		2.4×10^{6}[m]	2.4×10^{6}[m]			0 [m]
P. diminuta PTE	2×10^{9} [n]	5.8×10^{8} [o]		4.8×10^{6}[p]	6×10^{5} [p]	5×10^{3}		4×10^{4} [q]
P. diminuta PTE (H257Y/L303T) *Alteromonas* sp. JD6.5 OPAA		44.6×10^{7}[s]		12×10^{7}[r]	3×10^{6}[r] 14.6 [q]	4.8×10^{6}[r]		
Alteromonas sp. JD6.5 cloned				5.8×10^{6} [s]	1×10^{7} [s]	6.2×10^{7} [s]		
Alteromonas undina			21.8	30.4	1.6×10^{2}	1.3×10^{2}		
Sulfolobus solfataricus (W263F) PTE NG108-15 hybrid cells	7.2×10^{5}[t]				2.5×10^{3}			

[a] *Smolen et al., 1991.*
[b] *Masson et al., 1998.*
[c] *Davis et al., 1996.*
[d] *C.A. Broomfield, unpublished result.*
[e] *Yeung et al., 2008, with the four soman stereoisomers.*
[f] *Harel et al., 2004.*
[g] *Amitai et al., 2006.*
[h] *Worek et al., 2014 (*, under second-order conditions).*
[i] *Poyot et al., 2006.*
[j] *Lockridge et al., 1997.*
[k] *Newcomb et al., 1997.*
[l] *Hemmert et al., 2011.*
[m] *Hartlieb and Rüterjans, 2001.*
[n] *Kuo et al., 1997.*
[o] *Lai et al., 1995.*
[p] *Dumas et al., 1990.*
[q] *Rastogi et al., 1997.*
[r] *Tsai et al., 2012.*
[s] *Cheng et al., 1999.*
[t] *Hiblot et al., 2013a,b*

been conveniently expressed in *E. coli* and mutated (site-directed mutagenesis and directed evolution) to improve their catalytic properties against OPs (Merone et al., 2010; Hiblot et al., 2012; 2013a,b) (Table 75.1). Because of their high stability that allows long-term storage at room temperature, fieldable uses for decontamination are possible. Other extremophile PTEs have been isolated from radio-resistant bacteria, *Deinococcus radiodurans*, and *Agrobacterium radiobacter.* Three-dimensional structure and catalytic mechanism of these enzymes have been determined and used for structure-based random mutagenesis rational design to improve their catalytic

efficiency against OPs (Hawwa et al., 2009; Jackson et al., 2009; Ely et al., 2012).

Human Paraoxonase (PON-1)

The human paraoxonase-1 (PON-1) is a 45-kDa calcium-dependent enzyme bound to high-density lipoprotein (HDL) particles in association with other apolipoproteins. PON-1 shows a genetic polymorphism; the most prominent allele determines the Q192R allozyme, which can have a substantial impact on PON-1 activity against OPs and arylesters (Smolen et al., 1991) (Table 75.1). The enzyme was shown to be involved in the protection against atherosclerosis (Watson et al., 1995; Shih et al., 1998), and thus became a player in vascular physiology. Albeit its primary function is a lipophilic lactonase (Khersonsky and Tawfik, 2005; Ben-David et al., 2013), PON-1 displays two random activities, PTE and arylesterase.

Abundant biochemical, biological, and toxicological information have been collected in the past two decades, leading to partial characterization of the enzyme function (Costa and Furlong, 2002; Mackness et al., 2008), but recurrent attempts at solving the structure of human PON-1 failed. Through chemical modification and site-directed mutagenesis studies, some amino acid residues have been identified as essential for activity (Josse et al., 1999). Finally, molecular modeling (Fokine et al., 2003; Yeung et al., 2004) and crystal structure of a hybrid rPON-1 (a synthetic construct issued from shuffling of rabbit, mouse, rat, and human PON-1 genes expressed in *E. coli*) (Harel et al., 2004), showed that human PON-1 is a six-bladed β-propeller protein with a structure very similar to that of *Loligo vulgaris* DFPase (Katsemi et al., 2005). A catalytic mechanism for this eukaryotic calcium-dependent PTE was described (Blum et al., 2006). This mechanism involves a calcium-coordinated aspartate residue as the nucleophile that attacks the OP phosphorus atom. Alternatively, another more realistic mechanism was recently proposed; it involves the activation of a water molecule into a hydroxide ion that attacks the phosphorus center (Elias et al., 2013). This mechanism is consistent with the common mechanism that has been proposed for PON-1 (Ben-David et al., 2013), PTEs (Aubert et al., 2004), and PLLs (Elias et al., 2008).

As a naturally occurring enzyme present in plasma, human PON-1 is the most promising catalytic scavenger candidate for pretreatment and therapy of poisoning by OP (La Du, 1996; Rochu et al., 2007a; Worek et al., 2014). Thus, the enzyme has been the focus of intensive research to improve its efficacy and functionalization. To provide a valuable medical countermeasure against intoxication by NAs, the catalytic efficiency of PON-1 has to be enhanced by only one or two orders of magnitude. Chimeric PON-1 mutants obtained by directed evolution and exhibiting enhanced OPH activity (Amitai et al., 2006; Ashani et al., 2011; Worek et al., 2014) show that this goal could be reasonably achieved soon. However, instability of PON-1 mutants could impinge on their biotechnological development. As an HDL-bound protein, PON-1 requires association with apolipoprotein partners to retain its stable active conformation (James and Deakin, 2004; Gaidukov and Tawfik, 2005; Rochu et al., 2010) and long residence time in the bloodstream (Valiyaveettil et al., 2012). Highly purified human plasma PON-1 was found to be associated to human phosphate binding protein (HPBP) (Rochu et al., 2007b). HPBP is an apolipoprotein that binds inorganic phosphate in blood. HPBP was serendipitously discovered (Morales et al., 2006). This protein belongs to the family of DING proteins: named after their DINGGG N termini (Berna et al., 2009). Its three-dimensional structure and complete amino acid sequence were solved (Morales et al., 2006; Diemer et al., 2008). The conditions found to separate HPBP and PON1 *in vitro* indicated that HPBP is strongly associated with PON-1 (Renault et al., 2006). Moreover, the stabilization of the active form(s) of human PON-1 by HPBP suggests that HPBP could be a functional chaperone for PON1 (Rochu et al., 2007b,c; Cléry-Barraud et al., 2009).

A gene issued from HPBP amino acid sequence was synthesized (Diemer et al., 2008), and the protein was expressed in *E. coli*. However, attempts at co-crystallization of the PON-1-HPBP complex have failed so far. Yet this crucial phase is the first step of the staircase leading to the design and development of stable human PON-1 mutants with enhanced catalytic efficiency against toxic OP stereoisomers (S_p) of G NAs.

Site-directed mutagenesis of human PON-1 based on molecular modeling has led to double mutants capable of hydrolyzing G and V NAs (Kirby et al., 2013). However, the catalytic efficiency of these mutants is modest and enantioselectivity is not changed compared with the wild-type enzyme. Directed evolution strategy has been more successful in producing chimeric PON-1 capable of degrading toxic isomers of coumarinyl NA analogs as well as real G NAs. In particular, enantioselectivity of evolved PON-1 was completely reversed. For instance, the activity against S_p-cyclosarin was enhanced by 10^5-fold (Ashani et al., 2011; Gupta et al., 2011; Goldsmith et al., 2012). The most active evolved PON-1 mutant, IIG1 (Table 75.1), administered at a dose of 1 mg/kg was shown to prevent 2× LD_{50} cyclosarin systemic toxicity in guinea pigs (Worek et al., 2014). This mutant was also shown to detoxify most G NAs at high rates under first-order conditions but was ineffective against VX under second-order conditions.

Gene therapy could also be considered to challenge OPs by using a mutated PON-1 gene coding for an enzyme with high OPH activity against NAs. Several approaches with different gene delivery vectors in mice showed increased PON-1 level serum that reduced or

even prevented the entry of OP into the brain and reduced atherosclerosis signs (Cowan et al., 2001; Bradshaw et al., 2005; Fu et al., 2005; Guns et al., 2008; Duysen et al., 2011; Hodgins et al., 2013). Local delivery of PON-1 gene using Sendai virus vector inhibited neonatal hyperplasia after arterial balloon injury in rabbits fed a high-fat diet (Miyoshi et al., 2007). However, wild-type human PON-1 does not have sufficient catalytic efficiency against NAs to provide *in vivo* protection against 2× LD_{50} of G agents. Thus, enhanced expression of mutated PON-1 by gene therapy could be beneficial for the different functions of the enzyme. Meanwhile, the complex and defectively identified PON-1 activity makes it apparent that strategy for repetitive administration of high concentrations in humans must be undertaken cautiously. For further details on paraoxonase, see chapter 73.

Other Enzymes

Other enzymes are involved in biodegradation of OPs; some are hydrolases such as prolidases, senescence marker protein (SMP), and platelet-activating factor (PAF-AH); others are oxidases, such as cytochromes P450, GSTs, laccases, and peroxidases.

Other Mammalian PTEs

Prolidases (PROL; EC 3.4.13.9) were first isolated from halophilic bacteria (*Alteromonas haloplanktis* and *A. sp.* JD6.5). This bacterial metallo-enzyme (binuclear Mn^{++} center) has a "pita bread" structure (Vyas et al., 2010). Prolidase from *A. sp.* JD6.5 is an OPAA that displays high activity against soman ($k_{cat} = 3{,}100\,s^{-1}$), but it is inactive against VX (Cheng et al., 1999). Thermostable prolidases from hyperthermophilic archaes *Pyrococcus furiosus* (Theriot et al., 2010) and *Pyrococcus horikoshii* (Theriot et al., 2011) hydrolyze P-F and P-O bond in NAs. Evolved mutants of these enzymes capable of degrading OPs over wide temperature ranges, as engineered hyperthermophilic PLLs do, should have future use for biodecontamination under mild conditions. PROL was also isolated from human liver, kidney, erythrocytes, and skin, and was expressed in *E. coli* (diTargiani et al., 2010; Costance et al., 2012; Chandrasekaran et al., 2013). This enzyme displays a catalytic activity against sarin and soman (Wang et al., 1998; diTargiani et al., 2010) and exhibits sequence homology with the *Aleromonas haloplanktis* prolidase (Wang et al., 2006). Gene-delivered human liver PROL using adenovirus produced high level of active enzyme but protected mice only against 1× LD_{50} of DFP (Aleti et al., 2013).

SMP-30 was isolated first from rat liver (Kondo et al., 2004). It is a six-bladed-β-propeller metallo-lactonase structurally close to PON-1 and DFPase with random PTE activity (Chakraborty and Bahnson, 2010). The human and mouse enzymes display modest PTE against G agents (diTargiani et al., 2010; Belinskaya et al., 2012). Engineering of this enzyme is still in its beginnings.

PAF-AH is a group of 45-kDa lipoprotein (HDL and LDL)-associated phospholipase A2. In humans, they are present in plasma and in the brain. These enzymes that react with numerous OPs are structurally related to neuropathy target esterase (NTE) (Karasawa et al., 2003; Quistad et al., 2004, 2005; Epstein et al., 2009). Site-directed mutagenesis of human plasma PAF-AH for making an OPH for the purpose of NA detoxification has been undertaken (Kirby et al., 2012). However, research of these enzymes as possible catalytic bioscavengers is still in its infancy.

Oxidases

GSTs (EC 2.5.1.18) are 20- to 30-kDa enzymes that catalyze glutathione conjugation (nucleophilic attack of the thiol group) to electrophilic substrates. They are involved in cellular detoxification processes of endogenous compounds and of numerous xenobiotics, and their role in resistance to insecticides in insects is well established. OP detoxification by GSTs results from a regioselective dealkylation of alkyl or aryl side chain (Maturano et al., 1997). These enzymes exhibit wide genetic polymorphisms. Some GST allelozymes from *Drosophila melanogaste*r and *Musca domestica* flies are highly active against OP insecticides and have been cloned and expressed in *E. coli* (Fournier et al., 1992). There is evidence that GSTs contribute to OP detoxification in humans (Fujioka and Casida, 2007).

Laccases (EC 1.10.3.2) are fungal phenol oxidoreductases that have been used for detoxification of numerous xenobiotics, including dyes and pesticides (Richardt and Blum, 2008). The laccases from *Pleurotus ostreatus* and *Chaetomium thermophilium* were found to rapidly degrade VX and VR in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a mediator (Amitai et al., 1998). Laccases from *Trametes versicolor* and *Coriolopsis polyzona* with ABTS display similar properties against V agents (Trovaslet et al., 2010). The heme-containing chloroperoxidase (EC 1.11.1.X) from *Caldaromyces fumago*, with peroxide as co-substrate, is another efficient VX-degrading enzyme (Amitai et al., 2003).

These enzymes are promising for destruction of chemical weapons stockpiles, soil remediation, decontamination of materials, protective equipments, and water polluted by pesticides and NAs (Russel et al., 2003). In particular, phosphorothiolates such as VX are almost resistant to PTEs. Thus, oxidative cleavage of the P-S bond could be achieved by oxidases like laccases. These enzymes could be used in association with other OP-degrading enzymes for skin decontamination or in topical skin protection formulations. Although no work has been performed on combined action of oxidases and hydrolases, oxidation of P-bonded alkyl/aryl chains by oxidases is expected to alter enantioselectivity of PTE

for parent OPs. Therefore, biopharmaceutical formulations of combined oxidases and PTEs may improve the efficiency of PTE-based catalytic bioscavengers.

Engineered ChEs and CarbEs

As seen in Figure 75.1, OPs may be regarded as pseudo-substrates of ChEs and CarbEs. When ChEs and CarbEs react with carboxyl-ester substrates, the acyl-enzyme intermediate is a transient, with the acyl group being rapidly displaced by a water molecule. On the contrary, in the case of phosphyl-esters, the stereochemistry of the phosphyl-enzyme intermediate restricts the accessibility of water to the phosphorus atom. Thus, hydrolysis of phosphylated intermediate is very slow, and the enzyme remains inhibited (Järv, 1989). It was postulated that introduction of a second nucleophile pole in the active center could activate a water molecule. This water molecule could subsequently attack the phosphorus atom on the back face, leading to breakage of the P-serine bond.

The determination of the three-dimensional structure of AChE from *Torpedo californica* (Sussman et al., 1991) opened the way to rational redesign of CarbEs. Then, the possibility to convert a ChE into an OPH was demonstrated a few years later by Millard et al. (1995). Human BChE was chosen as the model enzyme because its active center is larger (500 $Å^3$) and less stereo-specific than that of AChE (300 $Å^3$). Molecular modeling based on the structure of the *Torpedo* AChE model was used for making the first mutants of human BChE. The second nucleophile pole was created in the oxyanion hole of the active center; a glycine residue was replaced by a histidine. The first mutant, G117H, was capable of hydrolyzing paraoxon, sarin, echothiophate, and VX (Millard et al., 1995; Lockridge et al., 1997) (Figure 75.3). However, this mutant was irreversibly inhibited by soman because the "aging" process of the conjugate was faster than the dephosphonylation reaction (Figure 75.1, reaction 4). The mechanism of aging (i.e., dealkylation of an akyl chain on the phosphylated serine adduct) is almost completely elucidated. The dealkylation reaction involves a carbocationic transient that is stabilized by active site residues E197 and W82 and water molecules (Kovach, 2004; Masson et al., 2010). Mutation of E197 into D, Q, or G considerably reduced the rate of aging. As expected, the double mutant G117H/E197Q was capable of hydrolyzing soman (Millard et al., 1999). However, the catalytic activity of this mutant was too slow to be of pharmacological interest.

Construction of transgenic mice *knockout* for AChE and carrying the G117H mutant of human BChE were found to be less sensitive to OP than in wild-type animals (Wang et al., 2004). Although transgenic mice expressed G117H mutant in all organs, unlike resistance of the blow fly, their resistance to OP cannot be explained by OP hydrolysis that was too slow, but rather by hydrolysis of excess ACh in cholinergic synapses.

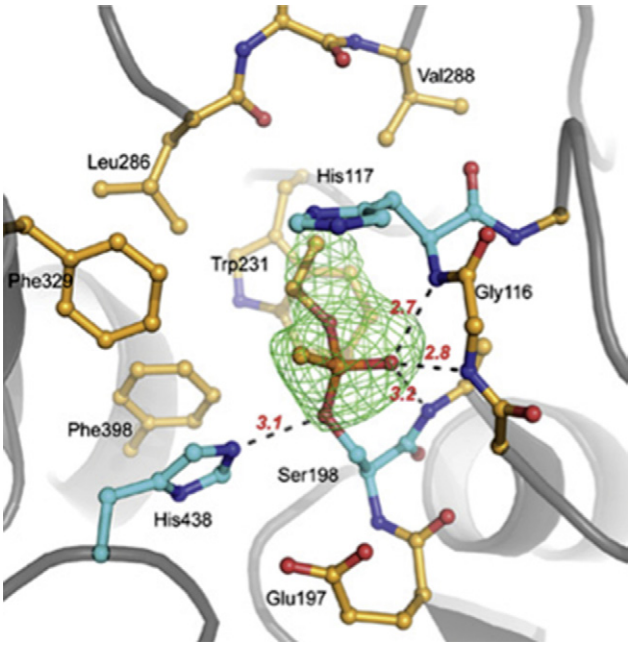

FIGURE 75.3 X-ray structure of the active center of G117H mutant of human butyrylcholinesterase conjugated with VX at 2.1 Å resolution (Nachon et al., 2011). The enzyme was phosphonylated by soaking the crystal in 1 mM VX for 2 min. X-ray data were collected at European Synchrotron Research Facility (ESRF; Grenoble, France). Two catalytic triad residues, active serine (Ser198) and histidine (His438), and the mutated residue (His117) in the oxyanion hole are shown as sticks as well as other important active center residues. His117 is mobile enough to activate a vicinal water molecule and stabilize the trigonal bipyramidal dephosphonylation transition state.

More than 60 double or triple mutants based on G117H were made (Schopfer et al., 2004), and certain mutants were eventually designed using the X-ray structure of human BChE (Nicolet et al., 2003). At the same time, mutants of human AChE and *Bungarus fasciatus* AChE were made using the same strategy (Poyot et al., 2006). Unfortunately, none of these muteins was more active than the G117H mutants. Actually, there is evidence that mutations at position G117 cause dislocation and loss of functionality of the oxyanion hole (Masson et al., 2007). For a historical review of this quest, see Masson et al. (2008).

The crystal structure of the G117H mutant conjugated to echothiophate and VX was solved (Nachon et al., 2011) (Figure 75.3). Works are in progress to understand the mechanism of dephosphorylation of this mutant (Amitay and Shurki, 2011; Lushchekina et al., 2011; Nachon et al., 2011; Yao et al., 2012). Thus, computer-assisted design of new OPH mutants of BChE is conceivable (Lushchekina et al., 2011). This new approach called "intelligent" directed mutagenesis design is based on simulation of

transition states. Simulation of activation transition state approach was already successfully applied to the design of other BChE mutants. Using the three-dimensional structure of human BChE, molecular dynamic simulations of deacylation transition state has allowed highly active mutants against (-)-cocaine to be made (Zheng and Zhang, 2008; Liu and Zhan, 2012; Zhan et al., 2014). This strategy applied to G117H-based mutant of human BChE (i.e., simulation of dephosphylation transition states) is expected to indicate how to optimize interactions favoring productive crossing of the energetic barrier for dephosphylation. Then, enzyme mutagenesis could lead to new generations of BChE mutants capable of hydrolyzing OPs at a high rate. Directed evolution of ChEs could be an alternative to computer-based methods. However, functional expression of ChEs is difficult in yeast and has failed in bacteria so far.

Bioavailability and biological stability of mutated ChEs for injection are important issues. First, pharmacokinetic studies of highly purified human BChE injected in the rat showed that the half-time ($t_{1/2}$) of enzyme in the bloodstream depends on sialylation of enzyme carbohydrate chains (Douchet et al., 1982). It is well-known that rapid elimination of asialoglycoproteins from the circulation is due to their capture by specific receptors located on the surface of hepatocytes. These receptors recognize galactosyl residue, the carbohydrate that precedes sialic acid at the terminus of complex glycans. Studies with other natural and recombinant ChEs confirmed the importance of sialic acid (*N*-acetylneuraminic acid) residues ending glycans (Kronman et al., 1995; Saxena et al., 1998; Cohen et al., 2007; Kronman et al., 2007). It was found that $t_{1/2}$ is inversely proportional to the number of unoccupied attachment sites of sialic acid (Kronman et al., 2000). To increase $t_{1/2}$ of administered recombinant ChEs, all galactosyl residues have to be sialylated. Full sialylation of recombinant enzymes can be achieved using an expression system capable of synthesizing glycans similar to natural human glycoprotein glycans and adding inhibitors of sialidase in the cell culture medium. Co-expression of the enzyme of interest and sialyltransferase in HEK 293 cells was found to lead to fully sialylated recombinant human AChE (Kronman et al., 2000). Alternatively, *in vitro* sialylation of purified enzymes is possible with a sialyltransferase or using a chemical method (Gregoriadis et al., 1999) and even by cross-linking polysialic acid chains (Ilyushin et al., 2013). PEGylation has also been proven to be an effective chemical modification for increasing circulatory half-life of administered recombinant ChE (Cohen et al., 2007; Huang et al., 2007; Kronman et al., 2007). Recently, a 150-kDa recombinant fusion protein human albumin–human BChE showed a substantially improved pharmacokinetics when administered to juvenile pigs: $t_{1/2}$ ≈32 h against ≈3 h for recombinant 70% tetrameric BChE (Huang et al., 2008).

Engineering of CarbEs to make enzymes capable of hydrolyzing NAs has made recent progress. In fact, the discovery of a blowfly (*Lucilia cuprina*) resistant to OPs because it carries a mutated CarbE (CarbE: LcαE7), G137D, at a position homologous to G117, stimulated research of G117H-based mutants of BChE. Although the OPH activity of the G137D is low, it is balanced by the abundance of the enzyme in the insect organs (Newcomb et al., 1997). The three-dimensional structure of this enzyme was recently solved (Jackson et al., 2013). Knowledge of this structure is a good starting point for engineering of mutants of LcαE7 with improved catalytic activity against OPs. Another interesting CarbE is the human CarbE1. The three-dimensional structure of human CarbE1-NA conjugates (with soman, tabun, sarin, cyclosarin) was solved (Fleming et al., 2007; Hemmert et al., 2010). The enzyme was shown to reactivate spontaneously after phosphonylation by the most toxic P_s stereoisomer of sarin (Hemmert et al., 2010). These results guided the computer-modeling design of the first CarbE1 mutants displaying OPH activity against soman and cyclosarin (Table 75.1) and enhanced the rate of spontaneous dephosphonylation after sarin inhibition (Hemmert et al., 2011). Thus, CarbEs can be reasonably considered as novel catalytic bioscavenger candidates.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Enzymes that neutralize or degrade OPs can be purified from natural sources, such as human plasma. Recombinant enzymes can be produced using procaryotic expression systems (*E. coli*), eucaryotic expression systems (yeast, insect, mammalian cell cultures), transgenic animals (worm, rabbit, goat), or transgenic plants (tomato, potato, tobacco), and also acellular biosynthetic systems. The goal of current research regarding protein engineering is to improve mass production of stable muteins at low cost. OPH improvement of *in vitro* and *in vivo* catalytic properties toward NAs and pesticides is still the main issue. In addition, improvement of thermodynamic stability (storage stability in solution or in dry forms) and *in vivo* operational stability, improvement of immunotolerance, and bioavailability are other goals. For this purpose, the different strategies of enzyme engineering have been implemented. They consist of research of new natural enzymes, particularly in collections of bacterial strains (Otto et al., 2013) and in extreme environments (Feerer et al., 2007), or identification of such enzymes from genomic sequences of extremophiles, followed by expression of the synthetic gene in a mesophilic bacterial host, characterization of catalytic properties, and X-ray structure determination (Hiblot et al., 2013a,b). Potential extremozymes, PLL and

PROL, have been discovered in halophilic, hyperthermophilic, piezophilic, and radio-resistant bacteria, and archaes. Other enzymes of interest are in insects resistant to OP pesticides and among secondary targets of OPs in human. Site-directed mutagenesis and directed evolution approaches in combination with chemical modifications and medium manipulations have been used with success to improve the desired properties, particularly stereo-selectivity, high k_{cat}/K_m, and broad-spectrum activity of selected enzymes (Bershtein and Tawfik, 2008; Goldsmith et al., 2012; Bigley et al., 2013). Computational redesign (molecular modeling and transition state simulations) of known enzymes is another promising strategy. It has already been successfully implemented: the active site of mouse adenosine deaminase, a zinc enzyme, was redesigned for hydrolysis of OPs. After maturation using directed evolution, the novel enzyme displayed a k_{cat}/K_m activity more than 10^7-fold the activity of the wild-type enzyme against R_p model OP (Khare et al., 2012). Finally, pharmacokinetic, toxicokinetic, and immunological studies of animal models allow validation of enzymes of interest.

Catalytic bioscavengers will be part of the arsenal of medical countermeasures for prophylaxis and post-exposure treatments of OP poisoning in the near future. Multiple enzyme associations (enzyme cocktails) will extend the activity spectrum of injected catalytic bioscavengers as well as the efficacy of active components in TSPs, chemical-protective clothing suits, and decontamination tools.

Gene therapy will offer the possibility of transitory production of human or humanized OP-degrading enzymes in the body. However, the road to gene therapy is still long. Regardless of ethical issues, further works are needed to engineer safe vectors that do not produce toxic viral proteins and/or induce immune response.

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SECTION IX

DECONTAMINATION AND DETOXIFICATION

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CHAPTER

76

Rapid Decontamination of Chemical Warfare Agents from the Skin

Edward D. Clarkson and Richard K. Gordon

BACKGROUND: THE NATURE OF HUMAN SKIN

Human skin, the largest organ in humans, developed as a physical barrier to the environment (keeping things out); however, it also maintains the aqueous nature of the human body (keeping things in). Mammalian skin consists of three major layers: the stratum corneum, epidermis, and dermis. The stratum corneum, the thin outer layer of keratin-filled dead cells (corneocytes) bounded by densely cross-linked protein and embedded in crystalline lamellar lipids, represents the major barrier protecting the body from loss of internal components and entry of undesirable external materials. The epidermis, the layer underneath the stratum corneum, contains cells that change from viable keratinocytes to corneocytes (which are anucleated cells without cytoplasmic organelles) as they migrate from the dermis to the stratum corneum. It also contains a large number of specialized dendritic cells. Smaller amounts of specialized cells are integral to the epidermis, including the pigmentation melanocytes, the immunological Langerhans cells, and the sensory Merkel cells. Throughout the epidermis, sebaceous glands, sweat glands, and hair can be found. The next inward layer, the dermis, contains hair follicles with associated sebaceous glands, ecrine sweat glands and ducts, dendritic cells, and a vascular network, including subepidermal capillaries, vascular plexi associated with the sweat glands, and dermal papillae associated with the hair follicles. Capillaries are responsible for transporting any chemicals that enter the skin systemically. Recent reviews of the skin structure and permeation are available (Menon, 2002; Hadgraft and Lane, 2005; Godin and Touitou, 2007; Wester and Maibach, 2000).

The stratum corneum, composed of keratinized dead cells that are continually being replaced, is the first major barrier to chemical agents. The barrier qualities of the stratum corneum depend on a number of factors, including its location on the body, its thickness, and how much hair is present. Thus, hair follicles and sweat glands can either provide channels through the stratum corneum, and thereby bypass its barrier attributes, or at least provide increased surface area for compounds to penetrate, since a number of compounds were shown to penetrate faster in hair follicle–rich areas (Illel et al., 1991). Maibach et al. (1971) studied three radiolabeled pesticides, parathion, malathion, and carbaryl, for their permeability at 13 different anatomical sites in humans. Variations in percutaneous penetration were observed; greater penetration of pesticides occurred at the abdomen and the dorsum of the hand.

Because it is often difficult to determine which specific nerve agent was used, most poisoning from pesticides or chemical warfare agents (CWAs) will initially be treated in a generally supportive manner, not by administering a specific antidote. Thus, decontamination is the most important early intervention (Simpson and Schuman, 2002). Although Maibach et al. (1971) present relatively recent human *in vivo* studies, most testing of decontamination products is performed either *in vivo* with animal models (Braue et al., 2011a,b; Clarkson et al., 2012) or *in vitro* using human skin samples (Mircioiu et al., 2013).

The lipid matrix is another important feature for barrier function in the epidermis. The arrangement of lamellarlike sheets yields a barrier to hydrophilic compounds and transcutaneous water transport. Extraction of those lipids from skin with organic solvents reduces barrier function (Hadgraft, 2001). The lamellae, which

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have little phospholipids as they are catabolized, ultimately contain mainly ceramides, cholesterol, and fatty acids (Wertz and Downing, 1989; Bouwstra and Ponec, 2006). The resulting matrix is composed of nonpolar compounds enriched in cholesterol, which are adapted to protect the dermis from water loss. While extracting these lipids may increase the penetration of aqueous moieties, in the case of organophosphates (OPs—note the organic nature of the CWAs, as described later in this chapter), the hydrophobic nature of skin likely facilitates the partition of these chemical agents through the lipid matrix, which then enter the subepidermal capillaries for dissemination throughout the body. Further insight into the barrier properties of skin can be observed in some disease states, including psoriasis, where an increase in epidermal cell replication yields an irregularly stacked stratum corneum and abnormal capillaries in the dermis. This leads to an increase in drug penetration such as hydrocortisone (Kranz et al., 1977). No studies have evaluated pesticide or CWA penetration in people with psoriasis.

Aging contributes to decreased lipid barrier protection, decreased intercellular cohesion, and increased absorption of toxic material. This barrier is also complicated by environmental effects such as exposure to sun, disease, and other aging processes that include many changes to the structure of the skin. Examples of such changes are decreased amounts of collagen, loss of melanocytes, decreased number of glands and hair follicles, reduced blood flow (Yates and Hiley, 1979), and the loss of lipid content in the stratum corneum (Elias and Ghadially, 2002). Another study found that 11 of 14 pesticides showed different rates of skin penetration in aged rats compared to young rats (Shah et al., 1987). Generally, decreased absorption occurred in studies of aged skin (Fisher et al., 1992; Farage et al., 2007).

Percutaneous absorption *in vivo* leads to the delivery of the chemical or drug to the microcirculation in the dermis. The period of time necessary for entrance to the blood supply and circulation throughout the body depends on the diffusion parameters and the interaction with the lipid matrix (Roberts, 1997). Thus, chemicals exhibiting a longer lag time to pass through the skin should be less toxic if quickly removed, as opposed to rapidly penetrating compounds. Another aspect of percutaneous absorption is the number of exposures to the chemical. Some chemicals, such as azone (1-dodecylazacylohepan-2-one), alter the organization of the skin so that an increase in absorption or a synergistic effect is observed with each exposure (Ademola et al., 1993). Chemicals that don't alter the skin's structure would not be likely to increase their bioavailability and absorption; rather, they provide an additive response (Bucks et al., 1985).

BACKGROUND: NERVE AGENTS

Nerve agents are among the most toxic of the known chemical agents. Nerve agents are OPs that bind irreversibly to acetylcholinesterase (AChE; Taylor et al., 1999) and to the bioscavenger butyrylcholinesterase (BChE; Wolfe et al., 1992) in both the peripheral nervous system (PNS) and central nervous system (CNS). AChE is responsible for terminating the action of the neurotransmitter acetylcholine (ACh) by hydrolysis. OP-inhibited AChE results in an excess of ACh and overstimulation of muscarinic and nicotinic receptors. Characteristic signs of nerve agent poisoning and cholinergic overload include hypersecretion and respiratory distress. When the nerve agent is transported past the blood–brain barrier (BBB), convulsions can lead to coma and death. OPs pose a hazard in both their vapor and liquid states. Notably, AChE inhibitors are used not only as a therapy for treating glaucoma, myasthenia gravis, Alzheimer's disease, and atropine poisoning, but also in potentially hazardous ways, as pesticides to kill insects and as CWAs by terrorists and in warfare to kill humans (Sidell, 1997; Leikin et al., 2002; Martin and Lobert, 2003).

The nerve agents include the G-type agents tabun (GA; ethyl *N,N*-dimethyl-phosphoramidocyanidate), sarin (GB; isopropyl-methylphosphonofluoridate), and soman (GD; 1,2,2-trimethylpropyl methylphosphonofluoridate), and V-type agents such as *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothiolate (VX). The V-type nerve agents are several orders of magnitude less volatile than the G-type agents and act primarily as a liquid via the percutaneous route; for example, VX is several orders of magnitude more lethal percutaneously than GB (Reutter, 1999; Braue et al., 2011a) or GD (Braue et al., 2011b; Clarkson et al., 2012).

Log *P* data (octanol:water partition coefficients and a reflection of lipid solubility) of nerve agents were used to both predict absorption through the skin and determine the distribution of OP compounds in tissues. Then, the data were correlated with toxicity as measured by the onset of fasciculation in guinea pigs. An excellent correlation (r=0.95) was established between the measured log *P* value and the rate of onset of local fasciculations, reflecting absorption in the skin, penetration to blood, and dissemination to muscle tissue throughout the animal (Czerwinski et al., 2006).

Maxwell and Lenz (1992) reported that, in general, AChE and BChE are more reactive with cationic nerve agents such as VX, while neutral agents that contain less than two bulky groups (e.g., GA, GB, and GD) were equally reactive with the cholinesterase (ChE) enzymes (Maxwell and Lenz, 1992). In general, since AChE has a smaller active site than BChE, the size and ionic character of the active sites determine the specificity of these

esterases for the agents. The estimated ranked percutaneous nerve agent LD_{50}, listed in descending order, are VX, GD, GA, and GB, and this reflects their volatility. The ranked volatility for these agents, in ascending order, is VX, GA, GD, and GB.

Using parathion as a model simulant for the nerve agent VX, the *in vitro* percutaneous absorption through unprotected human skin and clothed and uniformed skin was determined. The percent of a parathion dose that is absorbed through the unprotected skin was significantly greater than that observed through dry, uniformed skin, while absorption was higher through the wet (sweaty) uniform. These results suggested that military uniforms and other clothing worn in public places provide protection to this simulant (and by analogy to VX); but absorption through cloth and skin quantitatively occurred more readily with wet clothing than dry. Thus, even with clothing, an immediate response and decontamination of skin and clothing are required (Wester et al., 2000).

In conclusion, because of the extreme toxicity of nerve agents, the search for medical decontamination countermeasures to OPs is of paramount importance. Rapid removal from the skin would prevent penetration to the general circulation and the resulting decrements of cholinergic toxicity, which ultimately leads to seizure, death, or both in untreated individuals. In the development of medical decontamination countermeasures to nerve agent poisoning, different nerve agent administration routes likely will have different requirements for effective treatment. The window of opportunity for decontamination treatment following agent exposure is limited. The signs of poisoning develop within minutes, and if decontamination is delayed, toxic levels of the nerve agents are likely to be disseminated via the bloodstream after the agent has been absorbed. Decontamination will prevent continued absorption of the agent, reducing the need for further medical management (Table 76.1; Hamilton et al., 2004; Clarkson et al., 2004, 2012).

TABLE 76.1 VX Applied to Pig Skin (Ear)

Decontamination	
Delay[a] (min)	**Signs**
0	–
15 (No decon)	+++
15 (decon)	+

[a]*Delay in decontamination (Hamilton et al., 2004).*

BACKGROUND: VESICATING AGENTS (DISTILLED SULFUR MUSTARD, HD; IMPURE SULFUR MUSTARD, H; LEWISITE, L)

Sulfur mustard (HD), a synthetic vesicating agent, was a major CWA during World War I and continues to be a threat today (Reutter, 1999; Bismuth et al., 2004; Ghanei and Harandi, 2007). Its simple and cheap chemical synthesis makes it readily accessible to terrorists, as well as for use by the military. Sulfur mustard is an alkylating agent that causes damage by disrupting nucleic acids and proteins, impairing cell homeostasis and eventually causing cell death, although the significance of the multiple pathways is unclear (Smith et al., 1995). Whole-body exposure results in cutaneous (liquefaction necrosis of the epidermis), respiratory (injury to the laryngeal and tracheobronchial mucosa), and ocular effects (severe conjunctivitis). In contrast to HD agents, there is no delay with lewisite, which produces immediate burning of the skin and eyes. Compared with the G-nerve agents, sulfur mustard has a relatively low acute lethal toxicity; that is, its toxicity as an incapacitating agent is of much greater concern than its capacity to kill. Furthermore, HD persists in the soil and other materials for hours to weeks (Devereaux et al., 2002).

The skin is an important port of entry for vesicating agents. The agent's lipophilic nature and the propensity of skin to exclude aqueous compounds, but not lipophilic substances, make the skin an unwitting transport system. An increase in ambient temperature causes increased penetration (which was used effectively in World War I, where mustard was mostly disseminated at night and warmed by the early-morning sun). It has been estimated that 80% of liquid mustard evaporates and 20% penetrates the skin. Of that 20%, 12% is retained in the skin matrix, while 8% is absorbed systemically, so only large dosages of mustard will produce significant systemic toxicities (Cullumbine, 1947; Dacre and Goldman, 1996).

Mustard skin lesions first present as erythema followed by blisters (Somani and Babu, 1989). Erythema usually begins 2–24 h after contact, followed by acute itching, which diminishes as the characteristic blisters appear. These blisters initially appear as small vesicles within the area of erythema 18 h after contamination; these vesicles then coalesce to form the characteristic pendulous blisters containing large volumes of clear yellow fluid. Blisters are not painful *per se*, but they may be uncomfortable and may feel tense. Warm, moist areas, such as genitalia and axilla, are more likely to exhibit bullous lesions. By 48 h post-exposure, blistering is clearly evident, and a new round of blisters appears.

As the skin layer is disrupted, the large blisters break, leading to erosion, full-thickness skin loss (in which subcutaneous fat may be visible, but bone, tendon, and muscle are not exposed), ulceration, necrosis, and (72 h post-exposure) formation of an eschar. The eschar sloughs in a 4- to 6-day time period, finally leaving a pigmented scar (Reid et al., 2000, 2007). A burn caused by a blister agent is much slower to heal than a thermal burn, likely because of the multiple mechanisms by which the agent affects biological tissue, as discovered during World War I and reestablished in Iranian casualties from the Iran–Iraq war (1980–1988). The site of healed mustard burns is hypersensitive to mechanical trauma. In a comparison of cutaneous lesions in 500 mustard-exposed Iranian veterans and 500 unexposed veterans, a correlation was observed between exposure and severe dry skin, hyperpigmentation and hypopigmentation, local hair loss, eczema, chronic urticaria, and other skin lesions. Histopathological examination of skin biopsies has revealed nonspecific findings, including epidermal atrophy, keratosis, and basal membrane hyperpigmentation (Balali-Mood and Hefazi, 2006).

MODEL SYSTEMS TO MEASURE ABSORPTION, REMOVAL, AND DECONTAMINATION

Rats

Many different animal models have been used to assess the percutaneous absorption of toxic chemicals. There is little question that while *in vivo* human studies are best for predicting the absorption of percutaneously applied CWAs, ethics preclude conducting such studies. Rats have been widely used in the study of skin contamination, wounds, and healing and the efficacy of different decontamination modalities (Shah et al., 1987; Baynes et al., 1997; Wester and Maibach, 2000).

Guinea Pigs

While rats are often selected for their availability, low cost, small size, and thorough biological characterization, rats are not the ideal CWA model because they contain a high amount of carboxylesterase (CarbE), a potential hydrolytic enzyme for OPs (Sweeney and Maxwell, 2003). Unlike rats, humans have small amounts of this enzyme relative to AChE and BChE. To overcome this limitation, the guinea pig, which exhibits low CarbE, has been developed as a model for CWA exposure (Fonnum et al., 1985). Guinea pigs have been evaluated for skin damage from burns and are often used as a wound-healing model for sulfur mustard (Ramos et al., 2008), as well as for skin irritation to toxic industrial chemicals (TIC) (Weaver et al., 2003; Kennedy, 2007). Guinea pigs have also been used to study absorption of CWAs through the skin (Wormser et al., 2002; Dalton et al., 2006) and uptake of radioactive sulfur mustard through the skin (Logan et al., 1999), as well as an animal model for evaluating pretreatment regimens to protect against CWAs (Wetherell et al., 2006) and for assessing ChE activity responses (Haigh et al., 2005) to GD exposure and OP-induced seizure (Harrison et al., 2004).

For evaluating the decontamination of guinea pig skin, Clarkson et al. (2012) sedated and clipped guinea pigs. The animals were clipped with Oster brand clippers (model: Golden A5) with a #40 CryogenX blade instead of being shaved with shaving cream and a razor blade, as previous work showed that shaving caused razor burn and increased the rate and amount of agent that was absorbed (Snider et al., 2003). Guinea pigs were cutaneously exposed to neat nerve agents on their sides. A sponge wrapped around a pair of forceps was moved across the guinea pig's side 1 min after the exposure; then the forceps were rotated 180° so that the clean surface of the sponge was pointed at the animal. Three more passes were taken from the rear toward the front. An identical procedure was used when the protocol required an additional second sponge to decontaminate the animal. Similarly, guinea pigs were used for studies on the decontamination of sulfur mustard. In this case, 24 h after neat HD exposure and decontamination, animals were injected with trypan blue and then euthanized. The skin covering the backs of the animals was removed. In addition, skin punches were taken from each of the exposure sites (control, exposed, and decontaminated; Gordon et al., 1999; Gordon and Doctor, 2003).

Swine

Pig skin has long been a valuable model for human skin (Meyer et al., 1978; Riviere and Monteiro-Riviere, 1991) since it has a sparse hair covering, epidermis, and an arrangement of dermal collagen and elastic fibers similar to that of human skin. Again, many investigations have used the porcine skin model to study cutaneous toxicology of HD (Gold et al., 1994). Pig skin, because of its similarity to human skin with respect to hair covering, apocrine sweat glands, and other morphological features (Reifenrath et al., 1991), is an attractive model for cutaneous absorption and toxicology studies of OP CWAs (Hamilton et al., 2004). Cutaneous absorption studies show that pig skin permeability, compared to that of the rat and rabbit, most closely resembles that of human skin (Bartek et al., 1972) with a variety of test agents. Therefore, the pig is a good model with which to assess the effects of extraneous material or chemicals during early exposure. The downside is that pigs are large animals, are difficult to house, are more costly

to maintain than rodents, and require special cages to maintain them. Their size creates a serious caging issue when neat CWAs are applied to skin, and the animals may spend days in a chemical fume hood. This caging issue also occurs with nonhuman primates.

DECONTAMINATION REQUIREMENTS

Medical decontamination requires removal or neutralization of CWAs, which upon penetration of the skin produce vesication or, with OPs, enter the systemic circulation and inactivate ChEs. The most important process for the exposed soldier or civilian is to remove the chemical agent from the skin as quickly as possible. The soldier, under harsh conditions, must use the treatment product quickly to minimize transdermal penetration. A decontaminant that inactivates the chemical agent prevents its penetration through the skin and potentially protects a medical worker or colleague from suffering a secondhand exposure.

Another criterion for the decontaminating system and reagents is that they be as universal as possible and protect against the various classes of chemical agents (as well as radiochemicals and biological agents, although the latter compounds will not be discussed in this chapter). In other words, soldiers have a limited amount of space and can bear only a limited amount of weight, so they cannot carry multiple decontamination products. Furthermore, soldiers are unlikely to be able to determine the type of agent with which they are contaminated in the absence of symptoms.

In addition, the proven efficacy of a decontamination product would have to meet US Food and Drug Administration (FDA) guidelines and approval, assuring the safety of the product for soldiers. The product should be environmentally safe to use by itself and render the chemical or biological agent environmentally safe to prevent cross-contamination. Logistics would preclude decontamination products that require freezing or refrigeration since those services would not be available in the field. Finally, application of the product needs to be simple so that soldiers can easily use the product under stressful conditions, thereby reducing the probability of failure. Products like the M291 Skin Decontamination Kit (SDK), discussed next, are used by simply wiping the contaminated skin and do not require significant training. However, the M291 SDK has some drawbacks, such as a black offensive dust that is precluded from the eye. The ideal decontaminating product should be nonirritating and nonallergenic and should not have an offensive odor, as some potential mercapto compounds exhibit (Shi et al., 2008); otherwise, people will be reluctant to use it.

Methods for decontamination, neutralization, and removal of chemicals, such as OP and organosulfur compounds, herbicides, and insecticides, are known in literature (Hurst, 1997; Baker, 2004; Houston and Hendrickson, 2005; Rosenberg, 2005). The compositions and devices utilized for medical purposes are markedly different from nonmedical devices; the latter are not compatible with the skin or other sensitive tissues because they can be corrosive, flammable, toxic, difficult to make and store, composed of two component systems, and have a limited shelf life. For example, DS2, a standard decontamination agent, is comprised of 70% diethylenetriamine, 28% ethylene glycol monomethyl ether, and 2% NaOH by weight (Modec, 2003). Although DS2 is effective, it is corrosive upon exposure to air. DS2 and any matter resulting from its use is classified and regulated as hazardous material. After an application, the DS2 must stand for 30 min before rinsing the treated area with water. Additionally, DS2 comprises a teratogen. Clearly, this is not a better method for neutralizing, detoxifying, decontaminating and cleaning personnel exposed to CWAs.

DECONTAMINATION SCHEMES

OP nerve agents are a serious threat to military and civilian personnel, but not just from exposure during warfare or a terrorist event. With these agents, the possibility exists that exposed individuals could cross-contaminate the medical personnel treating them. Several medical decontamination schemes, each exhibiting advantages and disadvantages, are described next. While the ideal candidate does not exist, the final product must be fieldable for the individual—that is, for personal use in a rapid, and deployable manner. Simple materials, such as bleach, and complex products, such as OP-degrading immobilized enzyme sponges, are described. Inexpensive and readily available household materials were tested for pesticide decontamination of fabric materials over 20 years ago (Easter and DeJonge, 1985). The household products provided only marginal decontamination efficacy.

Classical Liquid: Sodium Hypochlorite (Bleach)

Decontamination methods employing hypochlorite formulations have some corrosive and toxic side effects. A Clorox (hypochlorite) solution is composed of household bleach, which is about 5% sodium hypochlorite. Thus, for a 0.5% solution, bleach is mixed with 9 parts water, although even this diluted solution is contraindicated for use in or on a number of anatomical areas including the eye.

Undiluted bleach is not used because it is toxic to the skin and may create more damage than no decontaminant. Hairless guinea pigs were exposed to sulfur

mustard in wounds and the surrounding intact skin, and then decontaminated with water, 0.5% or 2.5% bleach. No significant differences were observed among wounds decontaminated with the three solutions. Unexpectedly, the skin surrounding nondecontaminated (but exposed) control animals showed the least visual pathology. The lesions observed after decontamination might be due to the mechanical flushing of sulfur mustard onto the perilesional skin, by chemical damage of the skin induced by the solution, enhanced penetration of the agent, or interaction of sulfur mustard with the decontaminating solutions (Gold et al., 1994). In a study evaluating decontamination of GB from skin, rabbits that received GB but were not decontaminated were observed to neither convulse nor die. In contrast, when decontaminated with 5% bleach, symptoms and death increased, suggesting that 5% bleach perturbed the protective barrier of the skin or facilitated GB transport through the skin (Kondritzer et al., 1959). Since diluted bleach (0.5%) is a nonirritant to human skin, it is preferred (Racioppi et al., 1994).

In contrast, the effectiveness of diluted bleach has been demonstrated. This study measured the rate of sulfur mustard disappearance from the skin after topical application of the vesicant, which rapidly penetrates the skin because of its hydrophobicity. Three swabbing treatments of undiluted HD-exposed skin with gauze pads soaked in 0.5% hypochlorite caused 68% reduction in skin HD content and a 64% reduction when hypochlorite was replaced by water (Wormser et al., 2002). The effectiveness of 0.5% hypochlorite with water for decontaminating sulfur mustard on guinea pigs was also evaluated. However, the gauze pads soaked with the bleach contained microgram quantities of HD when water was used, but no detectable HD levels when 0.5% bleach was used. Thus, the neutralizing effect of 0.5% bleach occurred after the agent was removed from the skin and away from the lipophilic structures of the skin, where the 0.5% hypochlorite could react with and reduce the levels of the agent.

Similar to bleach's oxidative properties, topical povidone-iodine at 15 and 30 min post-exposure to sulfur mustard exhibited protective effects. Severity of the dermal parameters, acute inflammation, and dermal necrosis were significantly reduced, and reduced skin damage was observed in areas adjacent to treated sites (Brodsky and Wormser, 2007).

The impact of using 0.5% bleach as a CWA decontaminant has been significant. US Navy warships are equipped to operate in a chemical, biological, or radiological warfare environment where their exterior surfaces may become contaminated while the interior is kept clean. Most of these ships have decontamination facilities accessible from the ship's deck. Thus, in a contaminated environment, sailors or other personnel would be brought to the ship's facilities by personnel wearing protective gear. Next, contaminated clothing would be removed and contaminated skin washed with 0.5% bleach. This bleach solution is made from Navy High Test Hypochlorite (65–70%). After washing with 0.5%, the patients are rinsed with warm water and monitored to determine if it is safe to bring them into the ship for treatment (National Academy of Sciences, 2004).

Powder Decontamination Material: M291 SDK

In the 1990s, the product provided to the US soldier for field use was the M291 SDK (Figure 76.1). For 15 years, the US military fielded the M291 SDK, but in the late 2000s, the United States began to replace it with the Reactive Skin Decontamination Lotion (RSDL). However, throughout the world, many decontamination facilities continue to use the M291 SDK; therefore, it is important to discuss it in detail here. In this product, three main components are incorporated into individual pouches: a fiber pad (six to a pouch), an absorbent activated charcoal, and a reactive resin (Ambergard XE-555). Each component serves a unique purpose. The cotton pad provides structural integrity for use on a finger; the carbon incorporated into the pad absorbs organic material such as OPs and HD; and the ion-exchange resin binds chemical agents and very slowly detoxifies them. The soldier takes the M291 SDK pad and rubs the area that needs to be decontaminated. The goal is to rapidly bind chemical agents still on the surface of the skin and prevent their penetration through the stratum corneum. The M291 SDK is precluded from use in the eye because its particulate nature is irritating, but otherwise, it can be used on the face and anywhere else on the body. Much of the black powder from the M291 SDK remains on the

FIGURE 76.1 The M291 SDK, a powdered decontamination material.

skin, which has been a deterrent to its use. The presence of the black powder is an indication to the soldier that the area has been decontaminated. However, since ion-exchange resin binds chemical agents and very slowly detoxifies them, for maximum effectiveness it is best to brush off excess black powder, which may contain agents that have not been inactivated.

Efficacy of the M291 SDK has been evaluated in a number of animal models for OP poisoning (DO49 Technical Report, 1987). In an early report, rabbit skin was shaved (to mimic human skin without fur) and then exposed to GD and VX for 2 min. Decontamination with the M291 SDK yielded higher LD_{50} values (of more than 10-fold and 20-fold, respectively (Hobson et al., 1985)), in comparison to control animals that were not decontaminated. In another study, with rabbits under similar conditions, the penetration of the OP VX was measured by red blood cell (RBC) AChE inhibition. The M291 SDK increased the amount of VX required to inhibit AChE by 50% (Joiner et al., 1988). Thus, the M291 SDK neutralized or removed VX so that less of it penetrated the skin, as indicated by RBC AChE inhibition.

Three recent reports examined the effectiveness of the M291 SDK in treating GD and VX on animal models of anesthetized, clipped guinea pigs (Braue et al., 2011a,b; Clarkson et al., 2012); the LD_{50} was determined to be 24 h. Clarkson et al. (2012) reported that decontamination with the M291 SDK 1 min after neat (undiluted) exposure to GD increased the LD_{50} from 11.6 to 76.9 mg/kg, yielding a protective ratio of 6.6. Decontamination with the M291 SDK 1 min after neat exposure to VX increased the LD_{50} from 0.10 to 0.87 mg/kg, yielding a protective ratio of 8.7. In contrast, Braue et al. (2011a,b) reported that decontamination with the M291 SDK 2 min after neat exposure to GD increased the LD_{50} from 11.0 to 30.0 mg/kg, yielding a protective ratio of 2.7, and decontamination after neat exposure to VX increased the LD_{50} from 0.13 to 0.14 mg/kg, yielding a protective ratio of 1.1. The untreated LD_{50} in the studies discussed here were similar, while the difference in protective ratio for GD can be explained by the time difference in decontamination (1 versus 2 min). GD penetrates skin faster, so the additional time delay can have a significant impact on systemic dissemination of the nerve agent compared to the slower-penetrating VX, which is reflected in the decreased protective ratio. The difference in protective ratios for VX is more difficult to explain, as VX does not penetrate the systemic circulation as rapidly as GD.

The M291 SDK is also efficacious in rabbits against the vesicating agents HD and L. Thus, clipped dorsal skin of rabbits was exposed to neat HD and then decontaminated after 1 min. Vesicant-induced histopathology determined after tissue staining was reduced over 20-fold compared to nondecontaminated control rabbits.

Liquid Decontamination Material: Sandia Foam

Sandia National Laboratories decontaminating foam (licensed to Modec Inc., Denver, CO; Figure 76.2) is a solution (MDF-100) composed of two parts. The first is a solution of 6.6% *N,N,N,N′,N′*-penta-methyl,-*N′*-tallow alkyl 1,3-propanamine diammonium, 2.6% tallow pentamethyl propane quaternary ammonium compounds, benzyl-C12-18 alkyl dimethyl, and 1% isopropyl alcohol. The second is a solution of 8% hydrogen peroxide. Mixing the two parts results in a foamlike product that lasts for up to 30 min. The mixture was tested in the guinea pig model, where fur was clipped on the side of the animal 1 day before exposing the skin of the anesthetized animals to neat GD or VX (Lukey et al., 2004; Clarkson et al., 2012). The animals were decontaminated 1 min later with sterile gauze soaked in the combined solution in a defined manner: that is, the contaminated side was wiped across the exposure site in the direction of the shaved fur once and then rotated so that a clean surface of the gauze could be used to wipe the skin three additional times. Next, the OP-exposed area was similarly dried with a second piece of gauze. The exposed area was wiped a total of eight times. Then survival was determined 24 h later. Control nondecontaminated animals yielded an LD_{50} of 11.6 mg/kg for GD, while animals decontaminated with the mixture yielded an LD_{50} of 412 mg/kg, a 36-fold protective ratio. For VX, cutaneous neat exposure and decontamination with Sandia foam yielded an LD_{50} of 10.4 mg/kg compared to the control animals' LD_{50} of 0.10 mg/kg, a 104-fold protective ratio.

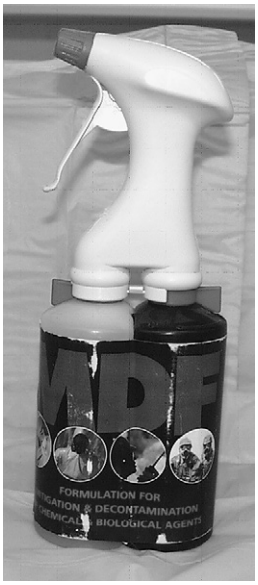

FIGURE 76.2 Sandia decontamination foam for mitigation and decontamination of CWA.

Despite its efficacy, Sandia foam has a number of drawbacks for field use by the soldier. First, it must be stored as its separate components, which would require a rapid, personal, on-site mixing chamber for combining the two solutions. Second, the presence of hydrogen peroxide, a strong oxidizing agent, precludes its use near the eye and would create much discomfort if used in a wound (Watt et al., 2004). To address these concerns, at least in part, Sandia developed the formulation DF-200, which contains less hydrogen peroxide and surfactant.

Liquid Decontamination Material: Diphoterine

Diphoterine is a product for chemical spatters on the eye and skin. Prevor Laboratory in France manufactures this odorless, colorless liquid dispensed as an eye wash or skin decontamination spray. It is composed of an aqueous solution to wash many chemical families and pull hydrophilic chemical agents away from the surface of tissues, an amphoteric solution that acts on acids and bases and restores the tissue physiological pH, and a hypertonic solution that stops penetration of corrosive chemicals into tissues. The pH is slightly alkaline (pH 7.2–7.7) and is sterile. Although not classified as a medical device in the United States, it is classified as such in Europe, Canada, Australia, and Brazil (www.prevor.com).

Diphoterine's action on more than 600 chemical compounds was reviewed by Hall et al. (2002). These chemicals included acids, alkalis, oxidizing and reducing agents, irritants, lacrimators, solvents, alkylating agents, and radionuclides. In the literature, there is one abstract describing the decontamination of sulfur mustard (Gerasimo et al., 2000). In this report, radiolabeled sulfur mustard was placed on human skin for 5 min *in vitro*. The skin was then treated with Diphoterine, water and soap, or saline at different time periods after sulfur mustard exposure, and Diphoterine was reported to be significantly better at removing sulfur mustard. No reports could be found in the literature for OP decontamination, although Hall et al. (2002) also state that Diphoterine is suitable for the decontamination of OP pesticides.

Evaluation of Diphoterine has occurred mainly in the European workplace, where it has been reported to be nonirritating to normal human eyes or skin. No adverse effects have been observed in ongoing post-marketing surveillance in European industrial facilities (Hall et al., 2009). It is nontoxic in guinea pigs and does not sensitize their skin (Mathieu et al., 2007). The product has prevented or decreased the severity of chemical eye and skin burns from 96% sulfuric acid, 100% acrylic acid, 50% acrylamide, solid sodium hydroxide flakes, and dimethylethylamine; 24 workers in a German metallurgy facility who were exposed to weak or strong acids and bases and obtained immediate Diphoterine decontamination did not require further medical or surgical burn treatment, nor did eye or skin burns develop (Nehles et al., 2006). Clearly, Diphoterine's potential is intriguing and needs to be critically evaluated for the purpose of decontaminating and detoxifying CWAs.

Liquid and Sponges: RSDL

RSDL (Figure 76.3) was developed for the cutaneous decontamination of CWAs. It was developed for topical use by the Defence Research Establishment Canada in Suffield, with broad spectrum decontamination properties for chemical agent cutaneous threats. The RSDL solution is composed of 1.25 M potassium 2,3-butanedione monoximate in polyethylene glycol monoethyl ethers with an average molecular weight of 550 daltons ($MPEG_{550}$) with 10% w/w water (pH 10.6). The pads consist of a spongelike plastic foam, Opcell, which is lightweight and easy to store. It is used instead of a cotton pad, and the Opcell holds more of the decontaminating solution for spreading on the skin.

The toxicological profile of this formulation was determined by the US military prior to FDA clearance (Tonucci et al., 2004). The army tested the product's safety by conducting skin irritation, sensitization, and photoirritation studies in more than 300 people. Its effectiveness was also tested by treating animals that had been exposed to chemical agents. On March 28, 2003, the FDA approved the product to remove or neutralize CWAs and T-2 fungal toxin (but not other biological threat agents or radiological contaminants) from the skin.

The efficacy of RSDL has been demonstrated. It was selected as the Joint Service Personnel Decontamination

FIGURE 76.3 RSDL used to decontaminate skin exposed to CWA.

System in March 2007 for use in the US Army, Navy and Air Force. It is also an approved medical device for nerve agent decontamination in the European Union, Australia, and Canada. The efficacy of RSDL to remove and decontaminate CWAs was demonstrated *in vivo* with guinea pigs and *in vitro* with chick embryos.

The *in vitro* work by Sawyer et al. (1991) involved primary cultures of chick embryo neurons to test the efficacy of the RSDL. By relating the anticholinesterase (anti-AChE) activity in these cultures of the OP/RSDL mixture to that of pure OP standards, a sensitive measure of the value of the RSD in inactivating tabun, sarin, soman, and VX was obtained. Data from experiments with all four nerve agents in this *in vitro* system correlated well with the *in vivo* data, and also indicated that the inactivation process was time- and agent-dependent and was related to the ratio of OP to RSDL. The authors showed that RSDL is also effective in decontaminating GD and VX in cutaneously exposed guinea pigs (Table 76.2; also see Gordon, unpublished observations). The protective ratios reported in Table 76.2 are in agreement with the protective ratios reported by Braue et al. (2011a,b).

The product was also compared to Fuller's earth in a pig model. The potency of the RSDL sponge was statistically better than Fuller's earth against skin injury induced by sulfur mustard, according to observations made 3 days post-exposure. In addition, RSDL was more efficient than Fuller's earth in reducing the formation of perinuclear vacuoles and inflammation processes in the epidermis and dermis. The potencies of the RSDL sponge and Fuller's earth were similar to severe inhibition of plasma ChEs induced by VX poisoning. Both systems completely prevented ChE inhibition, which indirectly indicates a prevention of toxic absorption through the skin (Taysse et al., 2007).

TABLE 76.2 Decontamination of Organophosphate-Exposed Guinea Pigs

Agent/Treatment	LD_{50} (mg/kg)	PR[a]
SOMAN		
M291 SDK	17.7	1.8
RSDL	240	24
Sponge	290	29
None	9.9	n/a
VX		
M291 SDK	0.14	n/a
RSDL	19.1	137
Sponge	21.0	150
None	0.14	n/a

[a]*Protective ratio.*

However, there are some caveats to RSDL use. The application of RSDL directly to open wounds impaired wound strength and decreased collagen content in the early phases of wound healing. This may have clinical implications for the treatment and outcomes of chemical casualty combat trauma (Walters et al., 2007). RSDL is also reported to be flammable.

Polyurethane Sponge

At the Walter Reed Army Institute of Research in Silver Spring, MD, an enzyme-immobilized polyurethane foam sponge (Figure 76.4) for decontaminating the skin of CWAs in a wide variety of environmental conditions is being developed (Munnecke, 1979; Wood et al., 1982; Havens and Rase, 1993). A porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers has been combined with enzymes such as ChEs to produce immobilized enzyme sponges (Ember, 1997; Medlin, 1998; Gordon et al., 1999; Gordon and Doctor, 2003). In this method, the enzyme becomes an integral part of the solid support. Some of the advantages of this technique include retention of similar kinetic characteristics as the soluble form of the enzyme. Most important, the immobilized enzyme retains high activity after prolonged storage and is resistant to the detrimental effects of low and high temperatures and to prolonged exposure to the environment. In addition, the enzymes are covalently attached to the polyurethane, so they will not leach from this polymer support.

To increase the OP/enzyme stoichiometry, polyurethane-immobilized enzymes were combined with oximes (enzyme reactivators such as HI-6; Peter et al., 2007) so that the catalytic activity of OP-inhibited AChE (or BChE) is rapidly and continuously restored before irreversible aging of the enzyme–OP complex can occur. Thus, the OP is continuously detoxified. A reusable immobilized-enzyme sponge of ChEs and oximes for OP decontamination is the envisioned product. Next, it

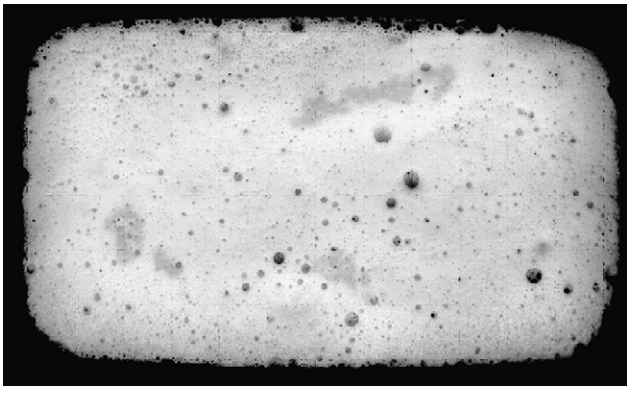

FIGURE 76.4 An enzyme-immobilized polyurethane foam sponge used to remove and decontaminate skin of CWA material.

was demonstrated that the OPs diisopropyl fluorophosphate and 7-(methyl-ethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) inhibited the activity of ChE sponges, as was observed for nonimmobilized ChE in solution. The oxime HI-6 restored activity of the AChE sponge until the molar concentration of MEPQ reached approximately 1,000 times that of the ChE active site. However, the AChE-sponge can be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original ChE activity would be restored to the sponge. Therefore, the bioscavenger approach could be used externally: the sponge would soak up OP, decontaminating the contaminated skin (Caranto et al., 1994). Then the ChE sponge and oxime would detoxify the OP in the sponge. We found that the ability of the immobilized enzymes and HI-6 to detoxify the OP MEPQ depended upon the efficiency of the sponge to decontaminate particular surfaces, including plastic and guinea pig skin.

Characteristics of polyurethane immobilized enzymes include the following: The longevity of sponges composed of immobilized ChEs is greater than 5 years at room temperature (according to unpublished observations). The immobilized enzymes are also very stable in aqueous environments. One significant difference (and advantage) that immobilized enzymes have compared to soluble ChEs is that immobilized enzymes do not dissociate (leach) from the sponge. Therefore, they can be left in liquid or other environments. For instance, the AChE activity in the immobilized sponge was stable for more than 60 days of continuous immersion in aqueous samples, including Allegheny River fresh water and brackish water (Gordon et al., 2002). Since the results were identical for autoclaved and untreated water, the immobilized enzymes were also resistant to microbial-induced proteolytic degradation. Also, note that the same sponge was assayed multiple times over many days, so it is evident that the immobilization process confers dramatic stability to covalently coupled ChEs, and that the enzymes do not leach from the polyurethane matrix.

The capacity of sponges to remove GD or OPs from guinea pig skin was determined using a back-titration method, where removed OP in the sponge was added to a known amount of ChE. Inhibition of the exogenously added ChE permits quantitation of the OP concentration. From these studies, it was determined that other components added to the sponge improved its efficacy by leaching out the CWAs. In this case, we settled on tetraglyme, which has a propensity to dissolve hydrophobiclike materials, including OPs (and sulfur mustard, as discussed later in this chapter). In part, the inability of tetraglyme sponge to remove all the GD likely reflects the rapid penetration of GD into skin and the inability of tetraglyme to extract this fraction of the poison. This further highlights the importance of decontaminating as rapidly as possible. These results clearly demonstrate that the sponge not only removed OP from the skin of the guinea pigs, but also, in the presence of oxime, effectively and completely detoxified the OPs within hours. Thus, these sponges would not pose any additional cross-contamination hazard.

It proved to be impossible to modify the prepolymer since there is currently no formulation with an increased hydrophobic nature that might be expected to absorb the OPs more effectively. Instead, we utilized the additives described previously to provide the additional ability to remove soman from the skin, protecting guinea pigs significantly better than the M291 SDK (Table 76.2). A comparison with RSDL is also shown in this table. Compared to the LD_{50} values of 9.9 and 17.7 mg/kg for untreated (not decontaminated) animals and the M291 SDK, respectively, the sponge provided an LD_{50} of 290. This combination is also effective against VX-contaminated guinea pigs: the sponge increased the LD_{50} from 0.14 to 21 mg/kg, yielding a protective ratio of 150 (comparable to that provided by RSDL).

In sulfur mustard decontamination and formulation, the sponge was used to wipe guinea pig skin contaminated with neat sulfur mustard. The following day, the animals were injected with trypan blue. Those areas representing vesicant injury take up the dye, hence becoming clearly visible. It was observed that the neat HD-exposed tissue (positive control) had a significant dye uptake, while the area decontaminated by the sponge had only a slight uptake of dye. The control, meanwhile, had no dye uptake. In addition, the amount of HD taken up and removed by the sponge was measured over time. While neat mustard remained after 15 min in water, the corresponding amounts were destroyed in the matrix of the sponge and additives. Histopathology of the HD-exposed skin specimens after 24 h demonstrated microvesicles, coagulation at the dermal interface, and, in the most severe cases, dermal coagulation. Overall, HD-exposed areas decontaminated with the sponge exhibited characteristics associated with reduced exposure (microvesicles). Thus, these sponges could reduce the damage that HD produces. ChEs may provide sinks as alkylation sites for the sulfur mustard and account for some of the reduced toxicity of the agent upon sponge decontamination. Another feature demonstrated was that the tetraglyme leaches from skin not only exposed to OPs, but also the hydrophobiclike sulfur mustard, thereby reducing its ability to alkylate skin proteins. Finally, the formulation of the sponge was modified to include nucleophilic additives acting as a reactive moiety for the sulfur mustard in the place of skin proteins. Taken together, the polyurethane sponge was shown to decontaminate and detoxify guinea pig skin exposed to two classes of CWAs: OPs, such as GD; and alkylating compounds, such as HD.

Immobilized Enzyme Badges

The sponge can incorporate a detection system for OPs and alkylating agents—a unique attribute not present in current decontamination methods (Gordon et al., 2002). The immobilized enzymes provide a detector and a rapid field system capable of identifying the type of OP. In addition to OP detection, a coupled enzyme reaction provides a rapid colorimetric or electrochemical indication of mustard. With the constant threat of chemical warfare or terrorist acts, the development of alternative means of protecting and decontaminating individuals from exposure to CWAs is critical.

Like Diphoterine, which has been evaluated only for sulfur mustard decontamination, the polyurethane enzyme-immobilized sponge is a novel technology that has demonstrated efficacy for OP and sulfur mustard cutaneous poisoning, but it is only now being evaluated for decontamination of biological warfare agents (such as T-2 mycotoxin and botulinum toxin) and radionucleotides (Gordon et al., 2006). Another advantage of ChEs over general reacting additives is that the ChEs are the direct target for current or future warfare agents, so countermeasures that incorporate ChEs should not require major reformulation. These new technologies likely will provide more efficacious solutions for the soldier in the future.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

OP nerve agents pose a serious threat to military and civilian personnel. The recent use of sarin by Syria against rebels was confirmed by the United Nations in 2013. These chemicals are some of the most potent toxic agents and are specific inhibitors of ChEs. OP nerve agents can be inhaled as a vapor, absorbed by the skin as a liquid, or ingested if food or water is contaminated. Vesicating agents such as sulfur mustard cause irreversible cell damage as a result of rapid alkylation, and they were terroristic weapons used during World War I and more recently in Iran and Iraq. Another serious problem that may be encountered while treating personnel contaminated with CWAs is the possibility of cross-contamination to the medical providers. In addition, during combat or terrorist acts, individuals might be exposed to chemical toxins before they don protective gear. Decontamination post-exposure has the potential to be an important, integral, and therefore necessary step for medical countermeasures against CWAs. The products described in this chapter must meet several criteria to be effective personal decontaminants and detoxifiers of CWAs for soldiers, although there is room for improvement; thus, novel technologies have been discussed. However, any product must be lightweight for individual use and remain shelf-stable under the environmental conditions found in the field. Decontamination and detoxification products must be readily available and work quickly because of the rapidity with which CWAs cause damage: for instance, OPs penetrate skin in less than 5 min, and mustard produces irreversible cell damage as a result of alkylation equally rapidly. The product should also be environmentally friendly. In the future, one product should incorporate chemical, biological, and radiological decontamination (and, when possible, detoxification). With the constant threat of chemical warfare, terrorist acts, and spilling of pesticides, the development of alternative means of protecting and decontaminating individuals from exposure is critical.

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