Biological Warfare: Infectious Disease and Bioterrorism

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INTRODUCTION

The term *biological warfare* typically conjures images of medieval warriors tossing dead cattle over city walls or clandestine government agents secretly releasing mysterious microbes into enemy territory. Of course, biological warfare does encompass such activity, but the vast majority of what constitutes biological warfare is far more mundane. Ever since life evolved on Earth about 3.8 billion years ago, organisms have constantly devised new ways to kill each other. Any organism that makes use of toxins—from bacteria to snakes—is engaging in a form of biological warfare. Humans who engage in biological warfare do so by taking advantage of these toxin-producing organisms.

THE NATURAL HISTORY OF BIOLOGICAL WARFARE

An entire textbook could be filled with examples of organisms that employ toxins to kill other organisms. We therefore touch only briefly on the natural history of biological warfare.
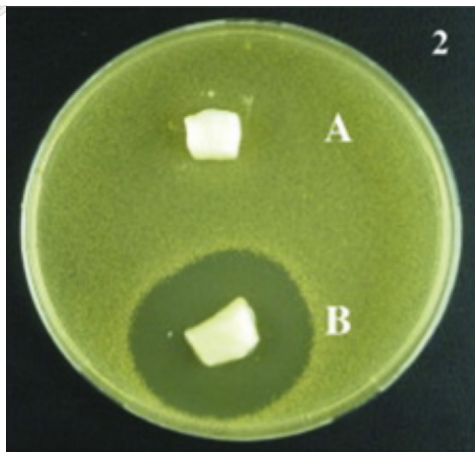
Bacteria are particularly adept at biological warfare. While humanity finds antibiotics incredibly useful in our battle against infectious disease, bacteria did not create them for our benefit. Instead, they make antibiotics to kill off other bacteria that are competing for the same habitat or resources. Similarly, bacteria synthesize toxic proteins known as **bacteriocins** to kill their relatives because closely related strains of bacteria are likelier to compete with each other. For example, many strains of *Escherichia coli* deploy a wide variety of bacteriocins (referred to as colicins) intended to kill other strains of *E. coli*. The genes for colicins are normally carried on plasmids, and many of these plasmids are commonly used in molecular biology and genetic engineering (see Chapter 3). *Yersinia pestis,* the plague bacterium, also makes bacteriocins (called pesticins in this case) designed to kill competing strains of its own species (Fig. 22.1).

A point of clarification: The distinction between *bacteriocin* and *toxin* has to do with the target. Bacteria deploy bacteriocins against their fellow—often closely related—bacteria with the deliberate intention of killing them. In contrast, proteins produced by bacteria that act against higher organisms are referred to as **toxins**. Perhaps counterintuitively, pathogenic bacteria do not usually "intend" to kill the organisms they infect. Rather, they want to manipulate them long enough to survive and reproduce. The longer the host stays alive, the longer it provides a home for the infecting bacteria. Just like antibiotics, some bacterial toxins are useful to humans. The bacterium *Bacillus thuringiensis* produces an insect-killing toxin that is harmless to vertebrates, and this "Bt toxin" has been used extensively in genetically modified crops. (See Chapter 15.)

FIGURE 22.1
Bacteriocins Inhibit Other Bacteria
A bacteriocin-producing strain of *Lactococcus* in a piece of cheese can inhibit the growth of a related microorganism. From Garde S, et al. (2011). Outgrowth inhibition of *Clostridium beijerinkii* spores by a bacteriocin-producing lactic culture in ovine milk cheese. *Int. J Food Microbiol* **150**, 59–65.

Lower eukaryotes also regularly engage in biological warfare. *Paramecium*, a ciliated protozoan, carries symbiotic bacteria *(Caedibacter)* known as **kappa particles** that grow and divide inside the larger eukaryotic cell (Fig. 22.2).

Strains of *Paramecium* with kappa particles are known as killers and, due to unknown genetic factors and resistance mechanisms, are naturally tolerant of them. Killer strains release kappa particles into the environment, and if a sensitive *Paramecium* (i.e., one lacking the ability to harbor kappa particles) eats and digests just a single kappa particle, a protein toxin is released and kills the *Paramecium.* Interestingly, the toxin is not encoded by a gene on the bacterial chromosome, but on a plasmid derived from a defective bacteriophage. So a toxin encoded by a virus infecting the kappa particle bacterium has been commandeered for the purpose of killing other strains of *Paramecium.*

This phenomenon is not at all unusual. Many toxins used by pathogenic bacteria that infect humans are actually encoded by foreign DNA of nonchromosomal origin, such as viruses,

plasmids, or transposons. These elements are often integrated into the chromosome of pathogenic strains of bacteria. For example, the only strains of *Corynebacterium diphtheriae*—the causative agent of diphtheria—that are dangerous to humans are the ones that carry a toxin-encoding virus.

Higher eukaryotes can either create their own toxins—such as the venom produced by snakes and scorpions—or expropriate toxins produced by other species. One species of caterpillar that feeds on tobacco plants can exhale noxious nicotine at spiders, chasing them away. Other insects rely on microbes to wage biological warfare. Certain parasitic wasps inject their eggs into the maggots (i.e., larvae) of plant-eating insects. After the eggs hatch, the newborn wasps eat the living maggots from the inside (Fig. 22.3).

The maggots are eventually killed, and a new generation of wasps is released. The secret to the wasp's success is the injection of an adenovirus along with the eggs. The virus targets the maggot's "fat body" (vaguely equivalent to the liver of higher animals) and cripples the maggot's developmental control system and immune system. The maggot loses its appetite for plants and is prevented from molting and turning into a pupa, the next stage in its life cycle.

KILLER *PARAMECIUM* CONTAINS *CAEDIBACTER*

A

KAPPA PARTICLES DIVIDE INSIDE *PARAMECIUM*

B

FIGURE 22.2
Killer *Paramecium* Uses a Bacterial Toxin
(A) The kappa particles are found in the cytoplasm of the *Paramecium*. (B) Kappa particles are symbiotic *Caedibacter* that are found in many strains of *Paramecium*, yet they have their own DNA and divide like typical bacteria.

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Many different kinds of organisms engage in biological warfare. Bacteria kill other bacteria with antibiotics or bacteriocins. They also make toxins that are targeted at higher organisms. Eukaryotes can either make their own toxins or commandeer those produced by lower organisms.

MICROBES VERSUS MAN: THE RISE OF ANTIBIOTIC RESISTANCE

Although we rarely perceive it this way, infectious disease is just another manifestation of biological warfare that is ubiquitous throughout life. The evolutionary relationship between hosts and pathogens is essentially a never-ending arms race. When a pathogen evolves a new toxin, the host evolves a response to it. Humanity has taken this arms race one step further by utilizing technology such as vaccines and industrial-scale manufacturing of antibiotics. However, the microbes are fighting back.

Perhaps the biggest problem plaguing medical microbiology today is the rise of antibiotic resistance. There are many reasons why bacteria have developed this resistance, but all of the explanations have one thing in common: the proliferation and misuse of antibiotics. For instance, medical doctors often prescribe antibiotics to patients who have an infection, even if it is unknown whether the disease is bacterial. Other times, the wrong antibiotic is prescribed. In many developing countries, antibiotics can be bought over the counter without a prescription. Compounding the dilemma, patients who receive antibiotics often do not comply with the recommended dose, ending treatment as soon as they feel better. This has the effect of selecting for the survival of the bacteria that have already developed a slight resistance to the drug. When the patient propagates the infection, he unintentionally passes on these toughened survivors. The widespread use of antibiotics in animal feed—which farmers use to fatten up livestock—is also a major contributor to the problem.
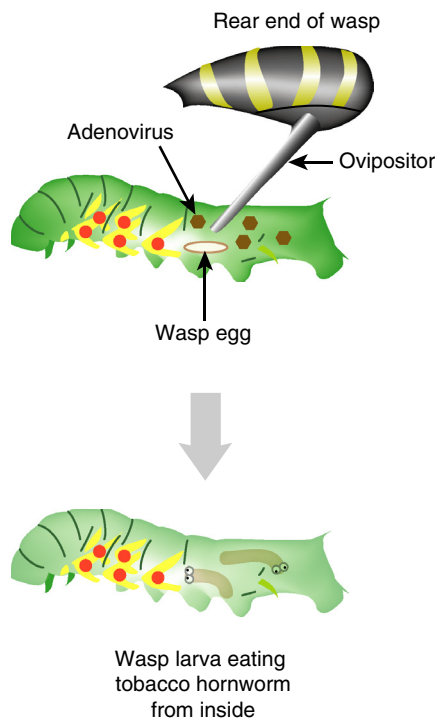
Today, many experts worry about "incurable" infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) gets a lot of media attention, but it is not the only worrisome microbe.
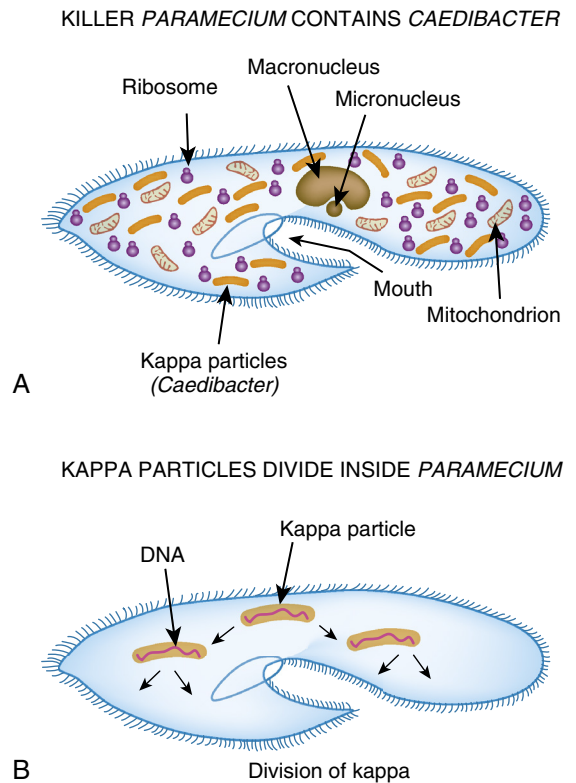
Rear end of wasp

Adenovirus

Ovipositor

Wasp egg

Wasp larva eating
tobacco hornworm
from inside

**FIGURE 22.3 Wasps
Use Viruses against
Maggots**

Certain types of wasps lay their eggs inside tobacco hornworm larvae. The wasp lands on the back of the larva and injects the eggs plus adenovirus into the maggot through the ovipositor. The adenovirus prevents the larva from eating and therefore developing into a pupa. When the eggs hatch, the young use the insides of the larva as a food source, to grow and develop into adult wasps.

There have been reports from around the world of totally drug-resistant tuberculosis, which as the name implies, appears to be resistant to all treatment. In a 2013 report, the Centers for Disease Control and Prevention (CDC) issued an urgent warning about infections from (1) *Clostridium difficile*, which causes diarrhea and is often acquired by patients in health-care settings who were treated with antibiotics for other infections; (2) Carbapenem-resistant Enterobacteriaceae (CRE), such as *Klebsiella* and *E. coli*, which also cause health-care-associated infections and may be resistant to all known antibiotics; and (3) *Neisseria gonorrhoeae*, the etiologic agent of gonorrhea, which is growing in resistance to several antibiotics.

While these developments are alarming, much research is being done to combat the rise of antibiotic resistance. Although microbes have responded to our antibiotic assault, we are developing some new weapons to regain the upper hand.

Novel Targets for Antibiotics

Although there has been speculation of an inevitable "post-antibiotic era," there are still plenty of opportunities for the development of novel antibiotics.

One strategy is to attack previously unexploited vulnerable spots in a bacterium's metabolism or life cycle, preferably those that bacteria cannot easily defend by acquiring resistance. For instance, bacteria use iron chelators, known as **siderophores**, to bind iron and extract it from host proteins. Siderophores are excreted, bind iron, and are then taken back into bacteria by specialized transport systems. Absence of high-potency siderophores largely abolishes virulence in both plague and tuberculosis. Because mammals do not make siderophores, their unique biosynthetic pathways provide an attractive target for development of novel antibiotics. Yersiniabactin, the siderophore of several pathogenic *Yersinia* species, is capped by a salicyl group (Fig. 22.4).

The intermediate in the pathway, produced when ATP activates salicylate, is salicyl-AMP. A chemically synthesized analog of salicyl-AMP, called salicyl-AMS, replaces the phosphate with a sulfamoyl group. The compound is highly active and specifically inhibits siderophore synthesis. This prevents the growth of *Yersinia* under iron-limiting conditions, such as encountered in the human body.

Another strategy is to screen novel microbes for antibiotics. As discussed earlier, bacteria produce antibiotics for the explicit purpose of killing other bacteria. Since most microbes that exist in nature have neither been cultured nor identified, it is likely that many natural antibiotics have yet to be discovered. In 2013, a new antibiotic, called anthracimycin, was isolated from an Actinomycete that lives in the ocean. The new antibiotic is active against *Bacillus anthracis* and MRSA, and modifying it with chlorine groups expanded its spectrum of activity.

Yet another strategy is to identify and clone potential antimicrobial biosynthetic pathways. For example, based on its DNA sequence, one research group cloned a biosynthetic gene cluster from an Actinomycete called *Saccharomonospora* that was predicted to produce an antimicrobial lipopeptide. Expressing the gene cluster resulted in the discovery of a new antibiotic, taromycin A. The major advantage of this technique is that it can be applied to microbes that are difficult to culture in the laboratory.

A different approach is to disrupt existing antibiotic resistance, rather than developing new antibiotics. For example bacteriophage, such as those that live in the human gut, can shuttle antibiotic resistance genes between bacteria. Consequently, developing drugs that kill or disable bacteriophage is an innovative way to combat the spread of antibiotic resistance.

SALICYLATE

ATP

SALICYL-AMP

SALICYL-AMS
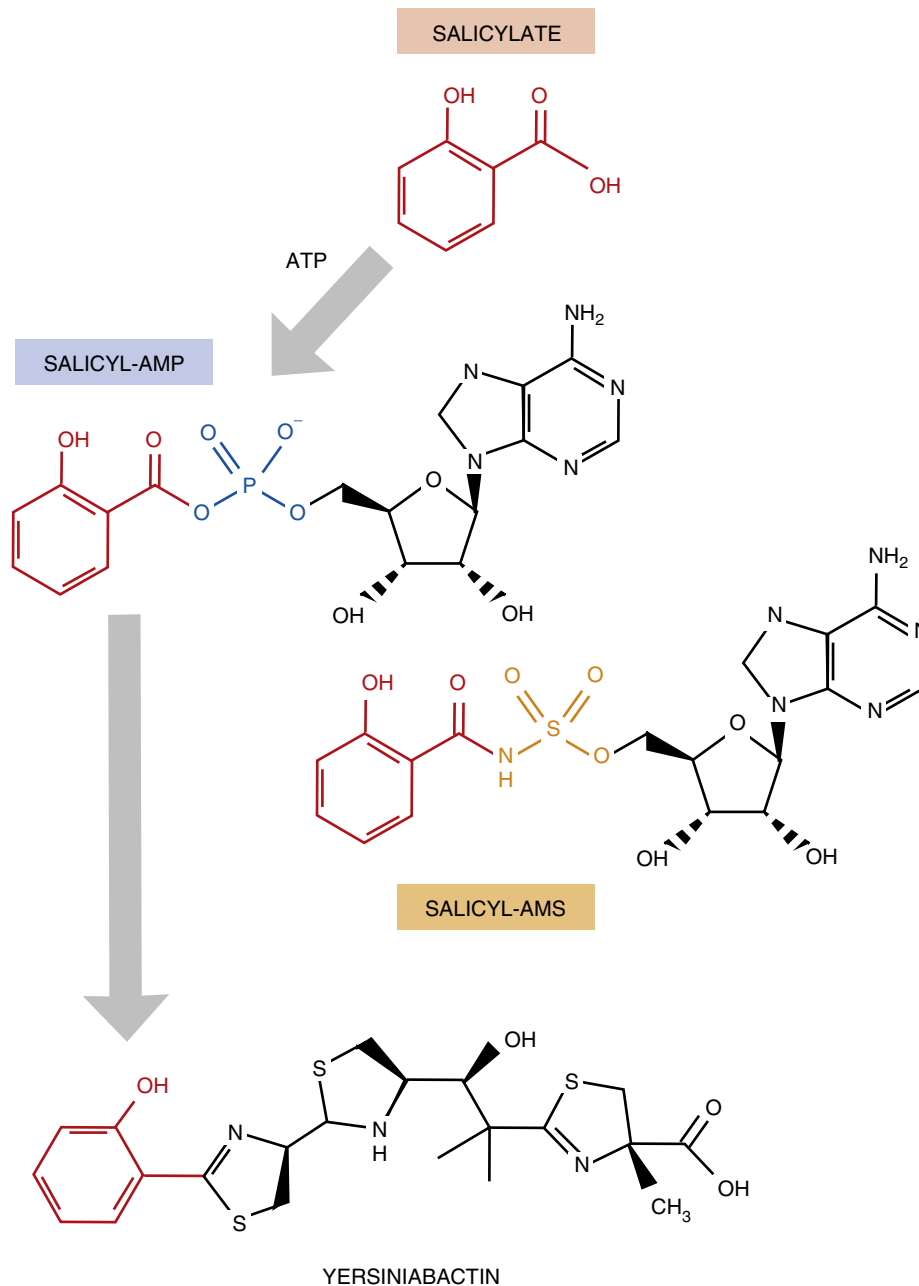
YERSINIABACTIN

FIGURE 22.4
Salicyl-AMS Inhibits the Production of Yersiniabactin
The structure of yersinia-bactin shows the salicyl group in red. The precursor, salicyl-AMP, is made by activating salicylate with ATP. The sulfamoyl analog, salicyl-AMS, inhibits the incorporation of the salicyl group into yersiniabactin.

Additionally, disrupting bacterial **quorum sensing** has been suggested. Bacteria use quorum sensing as a communication system in order to coordinate behavior (Fig. 22.5).

By releasing particular chemical compounds into the environment, bacteria can detect when a threshold population density, or "quorum," has been reached. Many pathogens construct antibiotic-resistant biofilms after the population has reached a particular density. Disrupting their communication system would cripple their ability to coordinate behavior and keep the bacteria more vulnerable to antibiotics.

Phage Therapy and Bacterial Predators

The history of **phage therapy**—that is, using bacteriophage (also called "phage") to treat bacterial infections—begins in France in 1921. That year, microbiologist Felix d'Hérelle used phage to treat patients suffering from dysentery (Fig. 22.6).

FIGURE 22.5
Quorum Sensing
Bacteria can coordinate behavior by detecting the presence of a signal molecule that indicates the density of the population. From Boyen F, et al. (2009). Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. *Vet. Microbiol* **135**, 187–195.

Low bacterial density
Low signal molecule concentration

High bacterial density
High signal molecule concentration
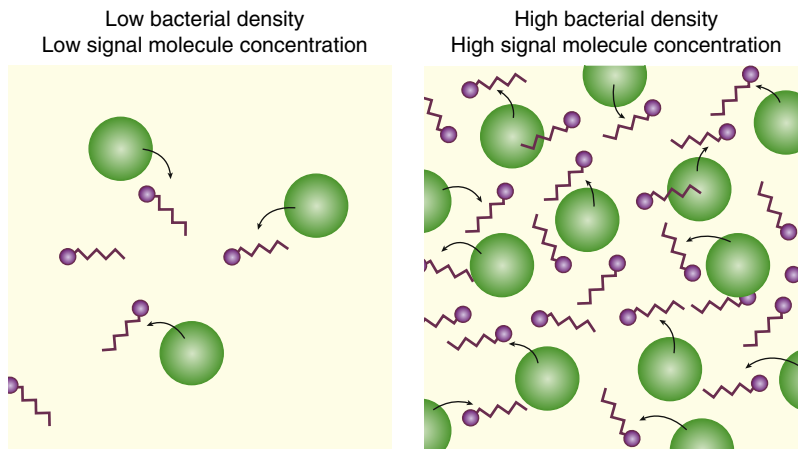
FIGURE 22.6
Felix d'Hérelle
Microbiologist Felix d'Hérelle helped pioneer phage therapy.

In 1927, he also used phage therapy to treat cholera victims in south Asia. Unfortunately, many other scientists in the United States and elsewhere were unable to replicate his work, and when the widespread production of antibiotics started in 1945, the scientific community mostly lost interest in phage therapy. The French, however, enthusiastically practiced phage therapy into the 1990s and, during those seven decades, there were reports of successful treatment of typhoid fever, colitis, septicemia, skin infections, and various other bacterial diseases. Other countries that embraced phage therapy include Poland, Russia, and Georgia. Today, patients there can receive phage therapy for chronic and antibiotic-resistant bacterial infections.

Since the 1990s, the Western scientific community has renewed its interest in phage therapy. One benefit of using phage, as opposed to antibiotics, is their specificity. Antibiotics kill many different types of bacteria—which is harmful if they destroy helpful gut bacteria—but individual phage species infect only a group of very closely related bacteria. Every bacterial infection could, in theory, be targeted by a highly specific phage.

As predicted, however, bacteria also can develop resistance to phage, mainly through thwarting viral attachment. Now, researchers are investigating the use of **lysins**, a class of toxins that phage use to dismantle bacterial cell walls as part of their lytic cycle (Fig. 22.7). Because lysins target conserved regions within peptidoglycan, it is believed that bacteria will be less able to develop resistance. Lysins work best against Gram-positive bacteria, but genetic engineering can expand the spectrum of activity to include Gram-negative bacteria also.

As an alternative to phage, it may be possible to deploy predatory bacteria against human pathogens. *Bdellovibrio*, which invades other bacteria rather like a virus, and *Micavibrio*, which attaches to bacterial cell surfaces, have been shown to kill antibiotic-resistant pathogenic bacteria *in vitro*.

Fighting Pathogens with Genetic Engineering

Because of a persistent fear that we will run out of novel antibiotics, many clever new technologies have been suggested to fight bacterial infections. Some of the most promising of these antibiotics utilize genetic engineering.

For example, many pathogenic *Escherichia coli* use the FimH adhesin to bind to mammalian cells via mannose residues on surface glycoproteins. Several alkyl- and aryl-mannose derivatives bind with extremely high affinity to the adhesin and block its attachment to the natural receptor. Such mannose derivatives, therefore, could serve as anti-adhesin drugs. However, manufacturing pharmaceuticals is quite expensive. It would be far cheaper to genetically

engineer nonpathogenic strains of *E. coli* to express the mannose derivatives on their cell surfaces. Pathogenic bacteria would then bind to these decoys instead of to mammalian cells. This would also avoid the need for continuous administration of sugar derivatives because the decoy strains of *E. coli* would multiply naturally in the intestine. Alternatively, nonpathogenic strains of *E. coli* could be engineered with genes for adhesins that would allow them to compete with pathogens for mammalian cell receptors. (Such engineered strains would also have the advantage of being able to deliver protein pharmaceuticals or large segments of DNA for gene therapy into mammalian cells.)
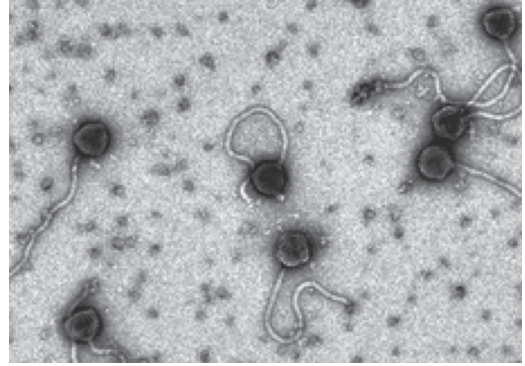
A different approach is to generate altered toxins that interfere with their natural analogs. Typical A-B bacterial toxins are made from a single "active" A subunit, which carries out a toxic enzymatic reaction inside a target cell, and often several "binding" B subunits, which serve as a delivery system by attaching to the cell surface. Because several properly functioning binding subunits are required to deliver the active subunit, one approach to antitoxin therapy relies on utilizing **dominant-negative mutations** in the binding subunit of the toxin. The mechanism involves the binding of a defective protein subunit to functional subunits resulting in a complex that is inactive overall. (The term *dominant-negative* refers to mutations in which an abnormal gene product sabotages the activity of the wild-type gene product. Consequently, most dominant-negative mutations affect proteins with multiple subunits.) Dominant-negative mutations have been deliberately isolated in the B protein (called the "protective antigen") of anthrax toxin. Mixing mutant subunits with wild-type ones resulted in the assembly of inactive heptamers that bind the A subunits (called "lethal factor" and "edema factor") of anthrax toxin. As a result, the toxic A subunits cannot be transported into target cells (Fig. 22.8). This technique has been shown to protect both cultured human cells and whole mice or rats from death by lethal levels of anthrax toxin.

FIGURE 22.7
Bacteriophage Tsamsa Kills *Bacillus anthracis*
The lysin isolated from the bacteriophage Tsamsa kills *Bacillus anthracis* and other closely related species. From Ganz HH, et al. (2014). Novel giant Siphovirus from *Bacillus anthracis* features unusual genome characteristics. *PLoS One* **9**(1), e85972.

Fighting Pathogens with Nanotechnology

Many of the advances in nanotechnology aimed at fighting pathogens involve the creation of bactericidal surfaces (see Chapter 7 for more on nanotechnology). Several metals are inherently antibacterial. For instance, silver ions kill bacteria through several mechanisms, such as generating reactive oxygen species and disrupting protein disulfide bonds. Surfaces coated with silver, selenium, and copper nanoparticles all show antimicrobial activity.

Metals are not the only option. A substance known as "black silicon" is made of tiny "nanopillars" that are able to physically destroy bacteria, including endospores, through mechanical stress (Fig. 22.9). Antimicrobial activity has also been demonstrated with stacked carbon nanotubes called nanocarpets (see Chapter 7). Additionally, polymers of esters and cyclic hydrocarbons reduce attachment of bacteria. Such discoveries could allow for improved sanitation in health-care settings and the manufacture of antimicrobial medical devices.

Antibiotic resistance is a growing concern, but contrary to popular reports, it is not necessarily an intractable problem. Novel targets for antibiotics, phage therapy, genetic engineering, and nanotechnology provide multiple possibilities for fighting antibiotic-resistant pathogens.

A BRIEF HISTORY OF HUMAN BIOLOGICAL WARFARE

Throughout history, humans have devised new and innovative ways to kill other humans. When technology was primitive, warriors used whatever nature provided. Burning crops was probably the easiest and earliest form of warfare aimed at undermining an enemy, as was poisoning a community's drinking water with dead or rotting animals.

FIGURE 22.8
Dominant-Negative Mutations

For anthrax, the B subunit (called PA63 protein or "protective antigen") binds the A subunits (called lethal factor, LF, and edema factor, EF) and transports them into the target cell cytoplasm via an endocytic vesicle. The dominant-negative inhibitory (DNI) mutant of the PA63 protein (purple) assembles together with normal PA63 monomers (pink) to give an inactive complex that cannot release the LF and EF toxins from the vesicle into the cytoplasm.
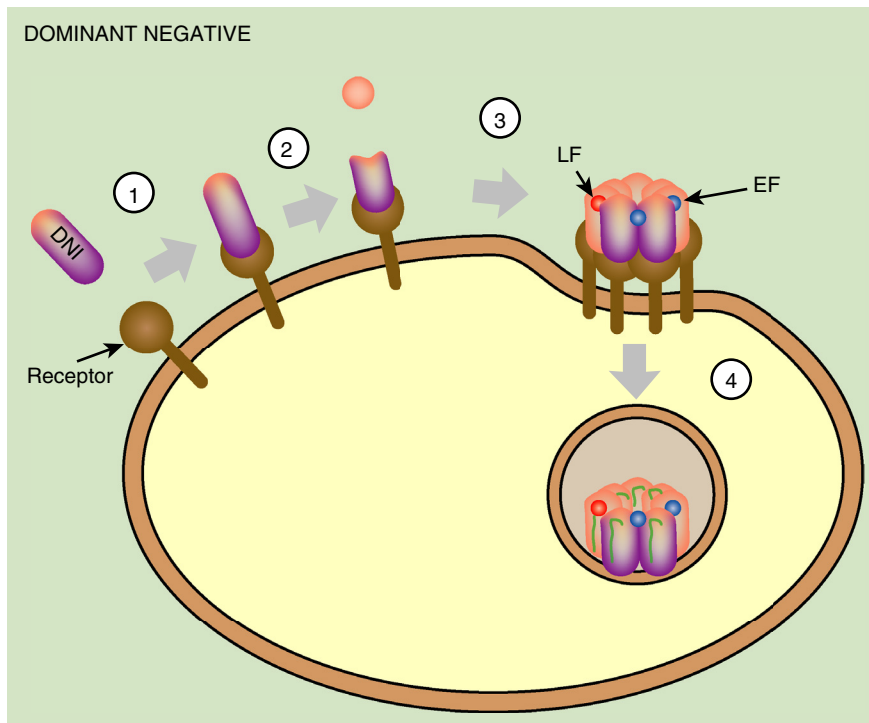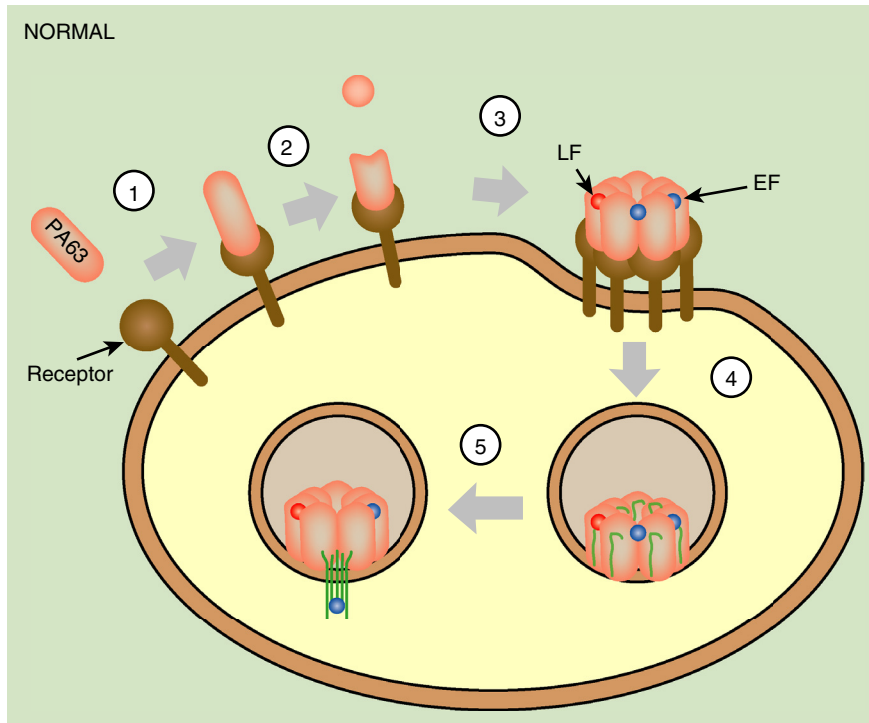
FIGURE 22.9
Nanostructures Can Kill Bacteria

Scanning electron micrograph of black silicon surface showing its hierarchical structures. (A) Periodically arranged micropillar arrays; (B) a micropillar with nanostructures; (C) nanostructures formed on the top of the micropillar. From He Y, et al. (2011). Superhydrophobic silicon surfaces with micro-nano hierarchical structures via deep reactive ion etching and galvanic etching. *J Colloid Interface Sci* **364**, 219–229.

NORMAL

DOMINANT NEGATIVE

A   X1,000   10μm
B   X4,000   5μm
C   X20,000   1μm
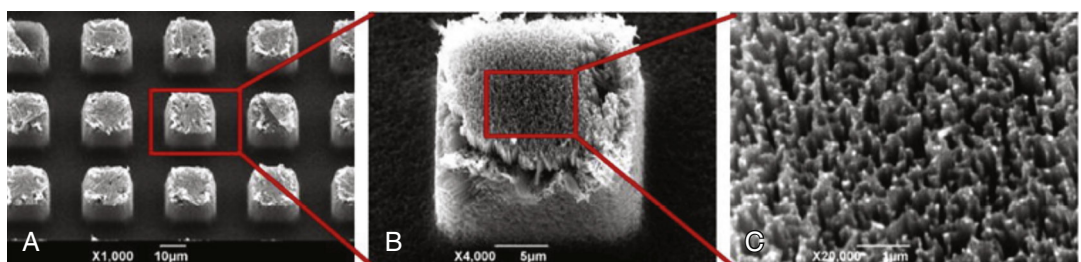
Early Human Biological Warfare

Slightly more advanced forms of biological warfare emerged when soldiers began dipping spears in feces and throwing poisonous snakes. During the **Black Death** epidemic of the mid-1300s, the Tartars catapulted plague-ridden corpses over the walls into cities held by their European enemies. Although this is sometimes credited with spreading the plague, rats and their fleas were far more effective at spreading **bubonic plague** than contact with corpses (Fig. 22.10).

Given the state of hygiene in most medieval towns or castles, there was little need to provide an outside source of infection. With plague, typhoid, smallpox, dysentery, and diphtheria already around, all that was usually necessary was to let nature take its course. Similarly, a widespread myth exists that European settlers purposefully infected Native Americans with smallpox. While it is true that the British military attempted this strategy during the French and Indian War in the mid-1700s, the vast majority of Native American deaths—perhaps as much as 95% of the population—were due to inadvertent infection with smallpox and other diseases.

The truth is, until very recently, humans were not particularly hygienic. Consider, for instance, that antiseptic surgery—invented by Joseph Lister and now considered a mainstay of modern medicine—wasn't widely adopted until the late 1870s. Before then, armies and civilian populations were so dirty and disease-ridden that practicing germ warfare was like throwing mud on a pig. It is only in our modern hygienic age that biological warfare has become a more meaningful threat.

Modern Human Biological Warfare and Bioterrorism

Modern biological warfare began during World War I. Although the Germans refused to use biological agents against people, they did use them against animals, infecting Allied horses with glanders (*Burkholderia mallei*) and anthrax. The French also employed glanders against German horses. During World War II, the infamous Japanese Unit 731 experimentally infected Chinese prisoners of war with horrifying diseases, such as cholera, epidemic hemorrhagic fever, and venereal disease. It was also responsible for dropping plague-infected "flea bombs" on cities in China, although this likely had little effect partly because plague was already endemic to the region (Fig. 22.11).

After World War II, particularly during the Korean War, the United States ratcheted up its biological weapons program. Perhaps the most controversial aspect of the program was the purposeful release of biological agents, such as the relatively harmless *Serratia marcescens*, over American cities to study weapons dispersal. The military unintentionally infected 11 civilians, one of whom died. By 1969, the U.S. had weaponized anthrax and tularemia. However, in 1975, the U.S. renounced all biological weapons by signing the Biological Weapons Convention (BWC).

The Soviet Union also signed the BWC but then deceitfully enlarged its efforts. The scope of the Soviet program was astonishing. The Soviets manufactured several hundred tons of anthrax, and an accidental release in 1979 killed 66 people. The former USSR also made thousands of pounds of smallpox and plague, and in 1989, they supposedly managed to weaponize Marburg virus, which causes a deadly hemorrhagic fever similar to Ebola. These allegations remain unconfirmed. Finally, under President Boris Yeltsin in 1992, Russia ended its biological weapons program, but the fate of the weapons stockpiles remains unclear.

FIGURE 22.10
Bubonic Plague
This painting by Arnold Böcklin, simply titled *Plague*, depicts the fear that bubonic plague provoked in antiquity. From ET Rietschal, et al. (2004). How the mighty have fallen: fatal infectious diseases of divine composers. *Infect Dis Clin North Am* **18**, 311–339.

Today, biological warfare is feared less from nations and more from terrorist groups or "lone wolves." But there is disagreement over just how much of a threat this poses. Many believe that terrorists would be incapable of carrying out an effective, large-scale biological attack. For instance, in 1984, the Rajneesh cult gave food poisoning to about 750 citizens of a small Oregon town for political purposes by adding *Salmonella* to salad bars. Aum Shinrikyo, a Japanese cult that perpetrated a sarin gas attack in the Tokyo subway in 1995, experimented with biological weapons, but to no avail. The 2001 U.S. anthrax attack (discussed in more detail in the following section) killed only 5 people. Skeptics point to incidents like these as evidence that bioterrorists are incapable of inflicting widespread damage. Other analysts disagree (Fig. 22.12).

Some biological agents, such as anthrax, require little expertise to grow or weaponize. With microbiological information universally available on the Internet, some experts believe that it is just a matter of time before a large bioterrorist attack occurs. A small crop duster airplane loaded with anthrax and flown over a major city could potentially kill hundreds of thousands if not millions of people. Exacerbating the problem is the fact that a 2010 federal commission found the United States to be completely unprepared in the event of a bioterrorist attack.

Psychological Impact and Cost

During the Vietnam War, the Viet-Cong guerillas dug camouflaged pits as booby traps. Inside, they often positioned sharpened bamboo stakes or splinters smeared with human waste. Although it was possible to contract a nasty infection from these, the main purpose was psychological. The tactic worked. The response of American troops was to alter their movements in a way that was disproportionate to the actual threat. An analogous scenario played out following the 2001 anthrax attack in the United States in which there was a colossal disruption of postal services and massive new expenses. Yet, only 5 people died in the attack. (Compare that to the roughly 62,000 Americans who died from influenza and pneumonia that same year.)

FIGURE 22.11
Unit 731
Japanese military Unit 731 killed thousands of Chinese people with experimental infections and biological warfare. Source: Figure 6 from: López-Muñoz F, et al. (2007). Psychiatry and political-institutional abuse from the historical perspective: the ethical lessons of the Nuremberg Trial on their 60th anniversary. *Prog Neuropsychopharmacol Biol Psychiatry* **31**, 791–780.

FIGURE 22.12
Bioterrorism
Some experts believe that a large-scale bioterrorist attack will occur in the not-too-distant future, but others say bioterrorism is an ineffective tactic. Attack methods include contamination of food and water supplies (A), bombs (B), using the mail (C), contamination of water (F), spraying aerosolized agents (E, G), direct injection (D), or the infiltration of "suicide infectees" (H). From Osterbauer PJ, Dobbs MR (2005). Neurobiological weapons. Neurol Clin **23**, 599–621.

Day 4   Day 6   Day 8   Day 10   Day 12   Day 14   Day 16   Day 18   Day 20
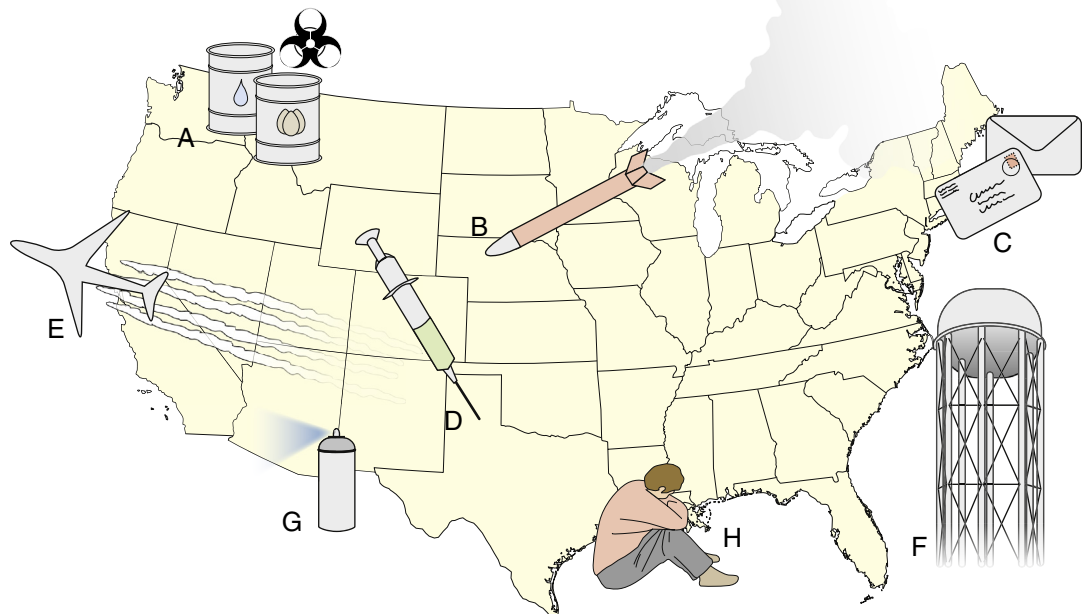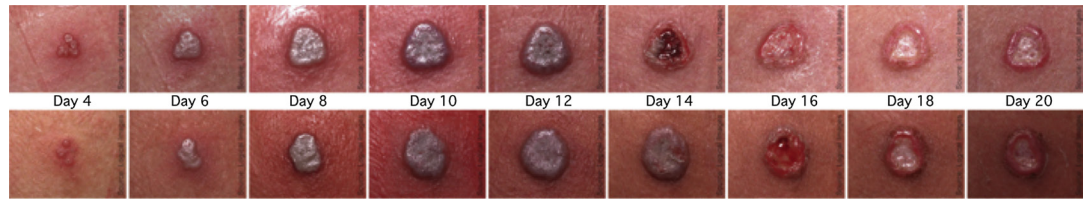
FIGURE 22.13
Smallpox Vaccine
How the normal skin reaction to smallpox vaccination progresses in two patients. Source: Centers for Disease Control and Prevention.

Both of these examples serve to underscore two important points: First, biological warfare will almost certainly have a far greater psychological impact than direct impact; and second, protective measures against biological attacks are costly and inconvenient. For instance, giving soldiers vaccines against all possible biological agents would be impractical and possibly dangerous if they have been developed under emergency conditions without thorough testing. Also, vaccines have side effects. Consider the anthrax vaccine used by the U.S. army that was approved in 1971. Vaccination requires six inoculations plus annual boosters. It produces swelling and irritation at the site of injection in 5% to 8% and severe local reactions in about 1% of those inoculated, although major systemic reactions are rare. Although it works against "natural" exposure, it is uncertain whether it would protect against a concentrated aerosol of anthrax spores.

Or consider the smallpox vaccine (Fig. 22.13). For every 1 million people vaccinated, the CDC estimates that 1,000 people will have serious side effects, 14 to 52 people will have life-threatening side effects, and 1 or 2 people will die. Is it worth vaccinating an entire army or nation—knowing ahead of time that many will die or become sick—to protect them against an unlikely threat? From an epidemiological standpoint, the answer is clearly no, which explains why citizens do not receive smallpox vaccinations. The general rule in public health is to vaccinate only if the risk of the disease is greater than the risk of vaccination.

Even if widespread vaccination is forgone in favor of other measures, such as protective clothing or respirators, there is still the financial cost. A nation that invests heavily in bioterrorism preparedness could have spent that money in more productive ways. Dressing troops in special clothing and equipment could promote heat stress or make them easier targets for conventional weaponry. Additionally, medications taken prophylactically to prevent infectious diseases are expensive, rarely 100% effective, and may have long-term negative health consequences.

Biological warfare has been practised since ancient times. However, it has only rarely been effective. Naturally occurring infectious diseases have killed far more people. Still, bioterrorism may pose a serious threat today. Even if an attack kills relatively few people, the psychological impact could be enormous.

IDENTIFYING SUITABLE BIOLOGICAL WARFARE AGENTS

Biological warfare is used to kill, injure, and psychologically intimidate enemies. Many naturally occurring diseases are effective agents, although it might be possible to "improve" them with genetic engineering, as discussed later.

What makes for an effective biological agent? Five major factors need to be considered.

Preparation. Some pathogenic microorganisms are relatively easy to grow in culture, whereas others are extremely difficult or expensive to manufacture in sizeable quantities. Viruses, for instance, can grow only inside host cells, and culturing animal cells is more complex than growing bacteria. Similarly, pathogenic eukaryotes such as *Plasmodium* (malaria) or *Entamoeba* (amoebic dysentery) are difficult to culture on a large scale, although some pathogenic fungi can be grown relatively easily. Bacteria are generally the easiest to manufacture

FIGURE 22.14
Nebulizer
A medical nebulizer could be used to aerosolize a biological agent for an indoor attack. Two general types of nebulizer are in use: the jet nebulizer that uses pressurized gas and the ultrasonic nebulizer that relies on ultrasonic vibrations.

JET NEBULIZER

ULTRASONIC NEBULIZER

Air flow

Aerosol

Baffle

Ultrasonic vibrations

Liquid containing sample or culture

Pressurized gas

Aerosol

Liquid containing sample or culture

Piezoelectric transducer

Power source
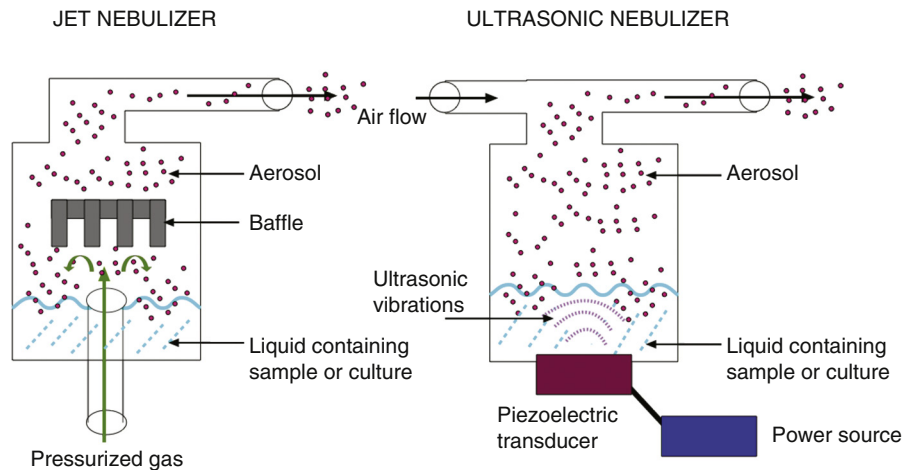
on a large scale, but most bacterial infections can be cured with antibiotics. Viruses, though more difficult to grow, have the advantage of being largely incurable despite a small and growing range of specific antiviral agents.

Another factor is **weaponization**. The disease agent must be prepared in a manner that facilitates storage and dispersal. Because bacterial cells and spores tend to clump together spontaneously, they must be weaponized to allow effective delivery.

Dispersal. Dispersal is a particular challenge for biological weapons. The most likely option would be some form of airborne delivery. However, if applied outdoors, this tactic would be vulnerable to the whims of the weather. Not only is a pleasant breeze required, but also the wind needs to blow in the right direction! During the 1950s, the British government conducted field tests with harmless bacteria. When the wind blew them over farmland, many of the airborne bacteria survived the trip and reached the ground alive. In contrast, when the wind blew the bacteria over industrial areas, especially oil refineries or similar installations, the airborne bacteria were almost all killed. Ironically, air pollution may help protect an urban population from a bioterrorist attack. To aerosolize a biological agent for an indoor attack, a building's ventilation system or a medical nebulizer could be used (Fig. 22.14).

Persistence. Persistence may be the most difficult factor to consider. On the one hand, the biological agent should be able to persist in storage until it is ready to be deployed, and it must survive long enough in the environment to infect the enemy. On the other hand, it should not persist so long that the victor is unable to invade and conquer enemy territory.

FIGURE 22.15
Spores of *Bacillus anthracis*
Anthrax spores, which are seen here forming inside bacterial cells, are difficult to destroy and last a very long time. From Ringertz SH, et al. (2000). Injectional anthrax in a heroin skin-popper. *Lancet* **356**, 1574–1575.

Many infectious agents are sensitive to desiccation and become inactive if exposed to air for significant periods of time. Moreover, natural UV radiation from the sun also inactivates many bacteria and viruses. Thus, most biological warfare agents must be protected from this "open air factor" before use and then dispersed as rapidly as possible. For instance, many viruses last only a few days, if even that, outside their animal or human hosts. (However, infections due to these agents may persist among the local population.)

Anthrax is often chosen as a biological weapon because of its ability to persist for long periods of time. The bacterium *Bacillus anthracis*, which causes the disease, spreads by forming spores that are tough and difficult to destroy (Fig. 22.15). When

suitable conditions return, for example, inside the lungs of a human, the spores germinate and resume growth as normal bacterial cells, releasing life-threatening toxins.

Incubation time. A problem unique to biological warfare, compared to conventional weapons, is that death or incapacitation from infectious disease is a relatively slow process. Even the most virulent pathogens, such as Ebola virus or pneumonic plague, can take a few days to kill. An infected enemy would therefore still be capable of fighting for a significant period. Yet, a biological agent that kills too quickly may not have time to spread among the enemy population.

High-containment laboratories. High-containment laboratories are needed for research and development of infectious biological agents. Biological containment is rated on a scale with four levels. Biosafety level 1 (BSL-1) microbes are mostly harmless, such as nonpathogenic *E. coli*. BSL-2 organisms are human pathogens, but not easily transmitted in the laboratory, such as *Salmonella*. BSL-3 organisms are dangerous and often can be transmitted via aerosol, such as tuberculosis and SARS. BSL-4 laboratories are for extremely dangerous and easily transmissible microbes, such as Ebola.

The whole BSL-4 laboratory is sealed off and kept at a little under normal atmospheric pressure. In case of a leak, outside air will flow into the laboratory, helping ensure contaminated air will remain there instead of seeping out. Operations are conducted inside safety cabinets with glove ports. To enter a BSL-4 lab, a researcher must use an air lock and exchange outside clothes for a separate set of lab clothes, including a special "spacesuit" that is equipped with its own air supply (Fig. 22.16).
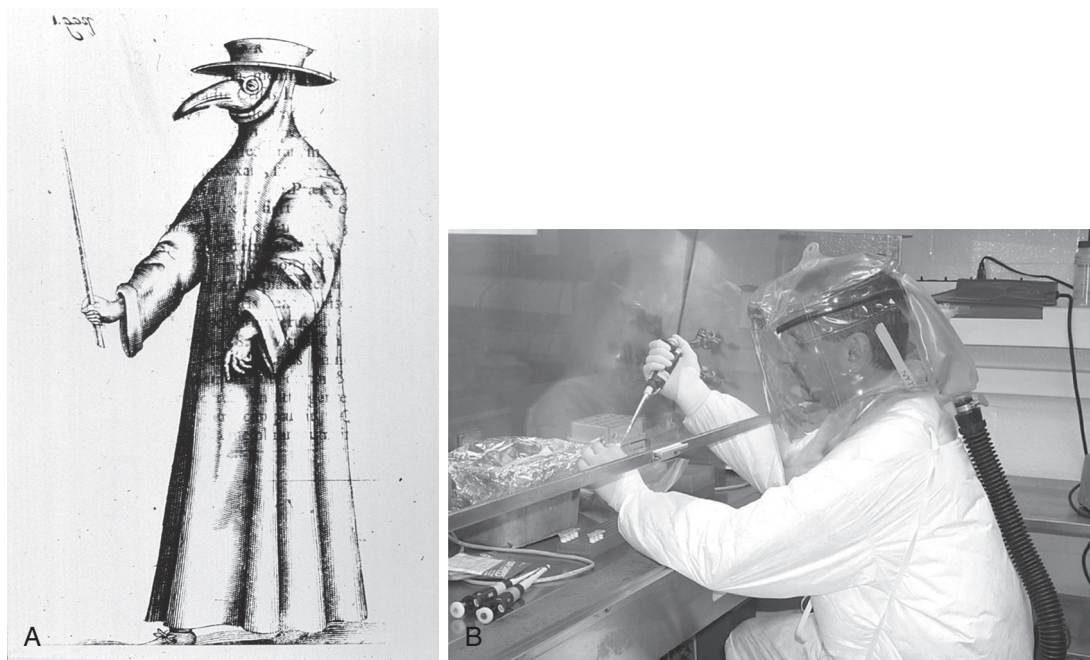
FIGURE 22.16

Biohazard Clothing, Then and Now

(A) Even during the bubonic plague, doctors wore protective clothing to prevent exposure to the deadly pathogens. The large beak was often stuffed with flowers and herbs to create a pleasant scent that was thought to keep away the plague, as illustrated in Bartholin, Thomas Hafniae, 1654–1661: *Historiarum anatomicarum.* Courtesy U.S. National Library of Medicine. (B) Today's suits are more scientific and streamlined, but serve the same purpose. Laboratory worker wearing BSL-4 protective gear. Courtesy of USAMRIID, DoD, and the NIAID Biodefense Image Library.

When finished, a scientist leaves behind his lab clothes and uses an exit equipped with disinfectant showers and ultraviolet lights. Some high-containment labs are designed so that the only exit is via total submersion in a pool of disinfectant. Ultraviolet lights are used to sterilize both the laboratories themselves and the air locks, especially when working with viruses.

Using high-containment facilities for research is expensive and time consuming. For manufacturing biological weapons on an industrial scale, the inconveniences are correspondingly worse. However, terrorist groups or rogue nations may only care about secrecy and may be willing to forgo biosafety considerations. The U.S. Army's criteria for a biowarfare agent are given in Box 22.1.

Five major factors that influence the use of a biological warfare agent include preparation, dispersal, persistence, incubation time, and the necessity of high-containment laboratories. A variety of viruses, bacteria, and toxins have been proposed as effective agents. These are classified into three categories by the Centers for Disease Control and Prevention (CDC) according to their level of risk.

Box 22.1 Requirements for Biological Warfare Agents

According to the U.S. Army, a biological warfare agent should fulfill the following requirements:

1. It should consistently produce death, disability, or damage.
2. It should be capable of being produced economically and in militarily adequate quantities from available materials.
3. It should be stable under production and storage conditions, in munitions, and in transportation.
4. It should be capable of being disseminated efficiently by existing techniques, equipment, or munitions.
5. It should be stable after dissemination from a military munition.

A CLOSER LOOK AT SELECT BIOLOGICAL WARFARE AGENTS

The Centers for Disease Control and Prevention (CDC) has classified biological warfare agents into three categories based on the potential level of threat they pose to society. These categories are summarized in Table 22.1. Anthrax was used in the 2001 bioterror attack in the USA (see Box 22.2).

Anthrax and Other Bacterial Agents

Anthrax is a virulent disease of cattle that infects humans quite easily. It is caused by the bacterium *Bacillus anthracis,* which is relatively easy to culture and forms spores, which can survive harsh conditions that would kill most bacteria. The spores may lie dormant in the soil for years and then germinate on contact with a suitable animal victim.

Three main forms of anthrax occur. Cutaneous anthrax, that is, infection of the skin, is rarely dangerous. Gastrointestinal anthrax occurs mostly in grazing animals and is relatively rare among humans, although it can occur via ingestion of bacteria or spores from contaminated meat. Inhalational anthrax, in which the spores enter via the lungs, gives a high death rate. In many ways, anthrax is the ideal biological weapon—lethal, highly infectious, and cheap to produce, with spores that store well.

The problem with anthrax, however, is that the spores are so tough and long-lived that getting rid of them after hostilities are over is nearly impossible. During World War II, the British tested anthrax (using sheep as the targets) on the tiny island of Gruinard, which lies off the coast of Scotland. Although it was firebombed and disinfected, the island remained uninhabitable for nearly 50 years because of anthrax spores still surviving in the soil. Finally, in 1990, the island was declared safe after it was treated with a solution of formaldehyde and seawater. The indestructibility of anthrax spores would thus be problematic for a military occupation, but it could be useful as a defense

Table 22.1 CDC–Listed Agents Relevant to Biological Warfare

CATEGORY A AGENTS INCLUDE ORGANISMS THAT POSE A RISK BECAUSE:

- They can be easily disseminated or transmitted person-to-person
- They cause high mortality
- They might cause public panic and social disruption
- They require special action to protect public health

Bacteria	
Anthrax	*Bacillus anthracis*
Plague	*Yersinia pestis*
Tularemia	*Francisella tularensis*
Viruses	
Smallpox	*Variola major*
Filoviruses	Ebola hemorrhagic fever
	Marburg hemorrhagic fever
Arenaviruses	Lassa fever
	Junin virus (Argentine hemorrhagic fever)
Toxins	
Botulinum toxin from *Clostridium botulinum*	

CATEGORY B AGENTS INCLUDE THOSE THAT:

- Are moderately easy to disseminate
- Cause moderate morbidity and low mortality
- Require improved diagnostic capacity and enhanced surveillance

Bacteria	
Brucellosis	*Brucella* (several species)
Glanders	*Burkholderia mallei*
Melioidosis	*Burkholderia pseudomallei*
Q fever	*Coxiella burnetti*
Several food- or waterborne enteric diseases, including	*Salmonella, Shigella dysenteriae, Vibrio cholerae*
Viruses	
Alphaviruses	Venezuelan encephalomyelitis
	Eastern and Western equine encephalomyelitis
Toxins	
Ricin toxin from *Ricinus communis* (castor bean)	
Epsilon toxin from *Clostridium perfringens*	
Enterotoxin B from *Staphylococcus*	

CATEGORY C AGENTS:

Emerging pathogens that could possibly be engineered for mass dissemination in the future, such as Nipah virus, hantaviruses, flaviviruses (yellow fever, dengue fever), multidrug-resistant tuberculosis

## Box 22.2 The 2001 Anthrax Attack in the United States

Shortly after the terrorist attack on the World Trade Center in September 2001, anthrax spores were distributed via the U.S. Postal Service. The anthrax attack was notable in two respects. First, it killed only a small handful of victims, supporting the contention that biological warfare is not usually very effective in practice. Second, it generated a vastly disproportionate reaction, illustrating the importance of the psychological aspects of bioterrorism. Undoubtedly, governmental overreaction and public panic did far more damage than the anthrax attack itself.

An insider in America's own biodefense research establishment perpetrated the attack. Detectives believe the culprit was Bruce Ivins, an army scientist and anthrax expert, but he committed suicide in 2008 without ever being charged. The FBI officially closed the case in 2010. However, doubts surrounding the evidence against Ivins have led some observers to call for a new investigation.

The attacker used the Ames strain of *Bacillus anthracis,* which is widely used in laboratories across the United States. A major problem with tracing the origin of anthrax outbreaks is that all the various strains of *Bacillus anthracis* are closely related and difficult to tell apart. No differences in either 16S rRNA or 23S rRNA sequence occur between different strains. In practice, analysis is done using single-nucleotide polymorphisms (SNPs) or variable number tandem repeats (VNTRs; see Chapter 23). For example, the *vrrA* gene of *Bacillus anthracis* contains from two to six copies of the sequence CAATATCAACAA within the coding region for a protein of unknown function (Fig. A).

These repeats were probably originally generated by slippage of DNA polymerase during replication. The repeats do not alter the reading frame, but result in corresponding repeats of the four-amino-acid sequence Gln-Tyr-Gln-Gln within the encoded protein. Several other VNTRs are also now used, including some on the pOX1 virulence plasmid. The greatest diversity of *Bacillus anthracis* strains, as assessed by multiple VNTR analysis, comes from southern Africa, which is therefore regarded as the probable homeland of anthrax.
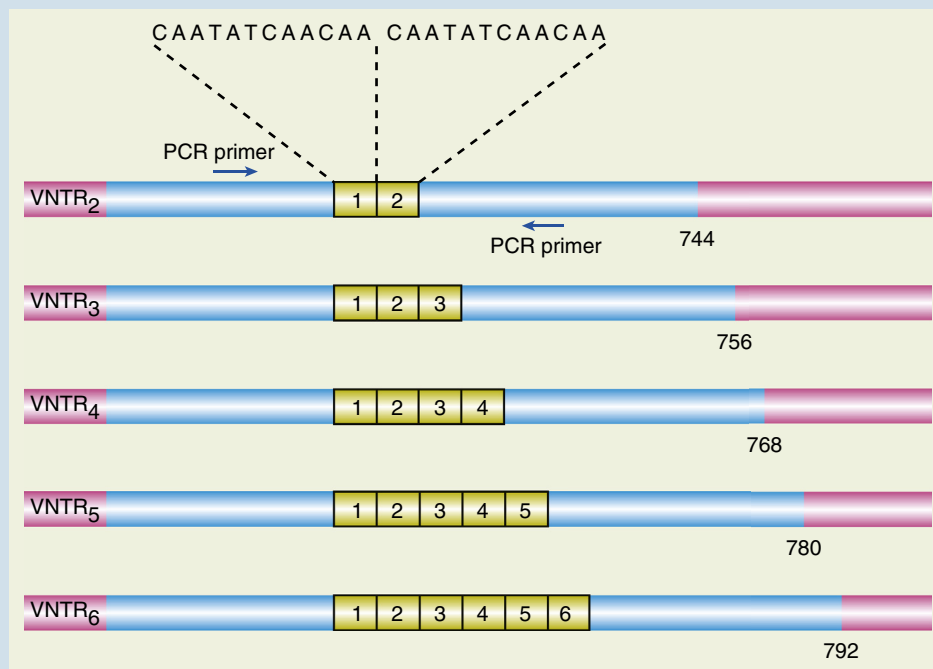
FIGURE A The *vrrA* VNTR of *Bacillus anthracis*
The *vrrA* gene of anthrax (blue) has a stretch of repeats in the coding region. Different strains of anthrax have different numbers of repeats (green) due to polymerase slippage and can therefore be traced by comparing the number of repeats. PCR is used to amplify the region containing the repeats. The length of the PCR product reveals the number of repeats.

mechanism. Anthrax spores seeded into the soil of sparsely populated land could serve to protect against foreign invaders.

Yersinia pestis, the causative agent of bubonic plague, was responsible for the notorious Black Death epidemics of the Middle Ages and is a current bioterrorism threat. Plague is typically spread by flea bites. However, aerosolized bacteria can cause the pneumonic form of plague. This

form of the disease is highly infectious and has a mortality rate close to 100% if untreated. Besides Japan's use of plague as a biological weapon during World War II (discussed previously), the British biological warfare center at Porton Down maintained large-scale plague cultures for several years following the war. In the 1960s, the United States experimented with spreading plague among rodents in Vietnam, Laos, and Cambodia to little practical effect. Because it can be obtained relatively easily from nature, plague may be an attractive weapon for bioterrorists (Fig. 22.17).

An unconfirmed report in 2009 indicated that 40 al-Qaeda terrorists in Algeria accidentally became infected with plague and died, presumably from a biological weapons experiment gone awry.

Other potential bacterial agents include the following:

- *Brucella.* **Brucellosis** is a disease of cattle, camels, goats, and related animals. Brucellosis was developed as a biological weapon by the United States from 1954 to 1969. In humans, it behaves erratically, both in the time for symptoms to emerge and the course of the disease. Although human victims often fall severely ill for several weeks, it is rarely fatal, even if untreated. It could be used as an incapacitating agent.
- *Francisella tularensis.* **Tularemia** is a disease of rodents or birds that has a death rate of 5% to 10% in humans if untreated. It is highly infectious and generally regarded as an incapacitating agent.
- *Burkholderia pseudomallei.* **Melioidosis** is related to glanders *(Burkholderia mallei)*, a disease of horses. Melioidosis is a rare disease of rodents from the Far East that is spread by rat fleas. Melioidosis is more virulent than glanders and, untreated, is fatal some 95% of the time in humans.

In many ways, anthrax is one of the best biological weapons. It is lethal, highly infectious, and easy to produce, and it has long-lasting spores. Plague is also lethal, and its pneumonic form can spread from person to person. Potential incapacitating agents include brucellosis and tularemia.

Smallpox and Other Viral Agents

Variola, the viral etiologic agent of **smallpox**, is a member of the poxvirus family. These large viruses contain double-stranded (ds) DNA (Fig. 22.18).

Poxviruses are the most complex animal viruses and are so large they may be seen with a light microscope. They measure approximately 0.4 by 0.2 microns, compared to 1.0 by 0.5 microns for bacteria such as *E. coli.* Unlike other animal DNA viruses, which replicate inside the cell nucleus, poxviruses replicate their dsDNA in the cytoplasm of the host cell. They build subcellular factories known as inclusion bodies, inside which virus particles are manufactured. Poxviruses have 185,000 nucleotides encoding 150 to 200 genes, about the same number as the T4 family of complex bacterial viruses.

Variola virus infects only humans, which allowed its eradication by the World Health Organization, a task completed by 1980. Smallpox is highly infectious and exists as two variants: *Variola major* with a fatality rate of 30% to 40% and *Variola minor* with a fatality rate of around 1% (Fig. 22.19). Their genome sequences differ by approximately 2%. *Vaccinia* virus is a related, mild poxvirus of unknown origin that is used as

FIGURE 22.17
Plague Reservoir
Rodents, such as squirrels and prairie dogs, serve as a natural reservoir for plague. A California ground squirrel is shown here. From Hayward P. (2013). Rare zoonoses in the USA. *Lancet Infect Dis* **13**, 740–741.

FIGURE 22.18
Variola Virus
Poxviruses, including smallpox, are closely related in structure and DNA sequence. They have genomes of dsDNA surrounded by two envelope layers. A protein layer, known as the palisade, is embedded within the core envelope. Premade viral enzymes are also packaged with the genome to allow replication immediately on infection. Poxviruses infect animals, and the outermost viral membrane is derived from the membrane of the previous host cell.

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Outer membrane · Palisade · Core envelope · Virion enzymes · DNA genome
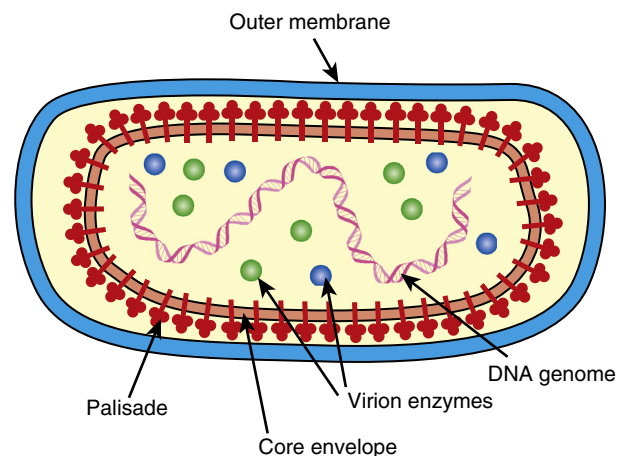
FIGURE 22.19
Smallpox
A person with smallpox develops a characteristic rash. Source: Centers for Disease Control and Prevention.

a live vaccine. Immunity is conferred against several closely related poxviruses including smallpox and monkeypox.

For governments, the preparation of large amounts of a virus whose particles are fairly stable and long-lived, such as smallpox, is feasible. It is believed that the former Soviet Union had done so, as discussed earlier. The virus could be delivered using a medical nebulizer (see Fig. 22.14) or through the use of suicidal volunteers who would deliberately infect themselves and then travel to densely populated target areas. They would mingle with as many people as possible, by attending large events and utilizing mass transit. However, transmission requires close contact, and when a person is most contagious, he may be feeling far too ill to actually walk around in public.

Though many viruses are difficult to culture in large amounts and are unstable during storage, several others have been considered as possible biological warfare agents:

■ **Filovirus.** Ebola and Marburg viruses make up a family of negative single-stranded (ss) RNA viruses, known as the Filoviruses, which form long, thin filaments (Fig. 22.20). Patients vomit and ooze blood from various orifices, including their eyes and ears. Ebola outbreaks in Sudan and Zaire have had 80% to 90% fatality, but closely related strains exist that are not as virulent. For example, in 1989, an Ebola outbreak occurred among long-tailed macaques in a research facility in Reston, Virginia. However, this strain was not lethal to humans. Transmission generally requires substantial exposure to infected body fluids, so filoviruses are difficult to acquire by casual exposure. In practice, this makes them a relatively poor choice for use as a biological warfare agent.

FIGURE 22.20
Ebola Virus
This electron micrograph depicts Ebola virus. From Moran GJ, Talan DA, Abrahamian FM (2008). Biological terrorism. *Infect Dis Clin North Am* **22**, 145–187. Courtesy of Centers for Disease Control and Prevention/Cynthia Goldsmith.

■ **Flavivirus.** Dengue fever and yellow fever are both caused by members of the Flavivirus family. Yellow fever is frequently lethal, whereas dengue is rarely fatal, but it is very painful and incapacitates its victims for several days. However, both are spread by insect bites, which would make their use as biological weapons difficult.

■ **Arenavirus.** An Arenavirus that appeared in the Lassa River region of Nigeria in the late 1960s causes Lassa hemorrhagic fever, which symptomatically resembles an Ebola infection. This segmented ssRNA virus has extremely high mortality and is typically spread by rodents.

Smallpox is the most likely virus to be used as a biological warfare agent. It is highly infectious, and the death rate may reach 30% to 40%. Other viruses with higher mortality rates may be prohibitively difficult to distribute.

Rust and Other Fungal Agents

Fungal agents perhaps may be most effectively used against staple crops, for instance, cereals and potatoes, which are an important part of the food supply. A wide variety of fungi exist that destroy these crops, such as rusts, smuts, and molds. Their spores are often highly infectious and easily dispersed by wind or rain, and in many cases there is no effective treatment.

Soybean rust and wheat stem rust are examples of pathogenic fungi that could destroy major crops. In addition to destruction of the crop, certain fungi may produce toxins. For example, when **ergot** grows on rye or other cereals, it produces a mixture of toxins that cause a syndrome referred to as ergotism, which can lead to convulsions, hallucinations, and even death

(Fig. 22.21). Some researchers believe that community ergot poisoning may have been responsible for the hysteria that led to the Salem witch trials.

Another potential fungal agent is *Aspergillus flavus*, which infects cereals and legumes and produces the carcinogenic **aflatoxin**. Acute aflatoxin poisoning can cause liver damage and death, and chronic exposure can cause cancer. Herculean efforts are made to keep the food supply free of aflatoxin.

From the perspective of biological warfare, there are many advantages to using a fungal agent against crops. First, an entire crop might have to be screened even if only a small part was infected, causing major disruptions and economic losses. Second, dispersal could be rather easily accomplished by spraying fungal spores with a crop duster airplane over farmland. Alternatively, seeds could be infected, especially since many of them are imported to the United States and may be more easily accessed for contamination. Third, modern agriculture is particularly vulnerable to infection because large acres of genetically identical cultivars are often planted in high density. This lack of genetic variability could allow for an infection to spread rapidly. Finally, fungal agents that attack crops pose little danger to those using them.

FIGURE 22.21
Ergot on Quackgrass
Fungi such as *Claviceps purpurea* infect various grains such as wheat or rye as well as grasses such as quackgrass, shown here. The mature fungus forms purple to black bodies, called ergot bodies or sclerotia, where the grain would normally be positioned. Courtesy of David Barker, Department of Horticulture and Crop Science, Ohio State University, Columbus, OH.

> The spores of highly infectious fungi could be used as biological warfare agents to target staple crops.

Purified Toxins

Another approach to biological warfare is to use purified toxins rather than a living infectious agent. A variety of toxins are known that may be purified in substantial quantities. Bacteria, primitive eukaryotes such as algae or fungi, higher plants, and animals all make toxins (Table 22.2).

Botulinum toxin. The most toxic substance known is **botulinum toxin**. It is made by the anaerobic bacterium, *Clostridium botulinum*, and is the cause of botulism, a severe form of

Table 22.2 Toxins Relevant to Biowarfare

Toxin	LD50 (µg/kg)	Producer Organism
Botulinum toxin A	0.001	Bacterium (*Clostridium botulinum*)
Enterotoxin B	0.02	Bacterium (*Staphylococcus*)
Ciguatoxin P-CTX-1	0.2	Marine dinoflagellate
Batrachotoxin	2	Poison arrow frog
Ricin	3	Castor bean (*Ricinus communis*)
Tetrodotoxin	8	Pufferfish
VX	15	Synthetic nerve agent
Anthrax lethal toxin	50	Bacterium (*Bacillus anthracis*)
Aconitine	100	Plant (monkshood, a.k.a. wolf's bane)
Mycotoxin T-2	1200	Fungus (*Fusarium*)

food poisoning. It has been proposed as a biological warfare agent but has actually found its most frequent application in cosmetics, under the name Botox. It is also used to treat a few clinical conditions in which a muscle relaxant is needed.
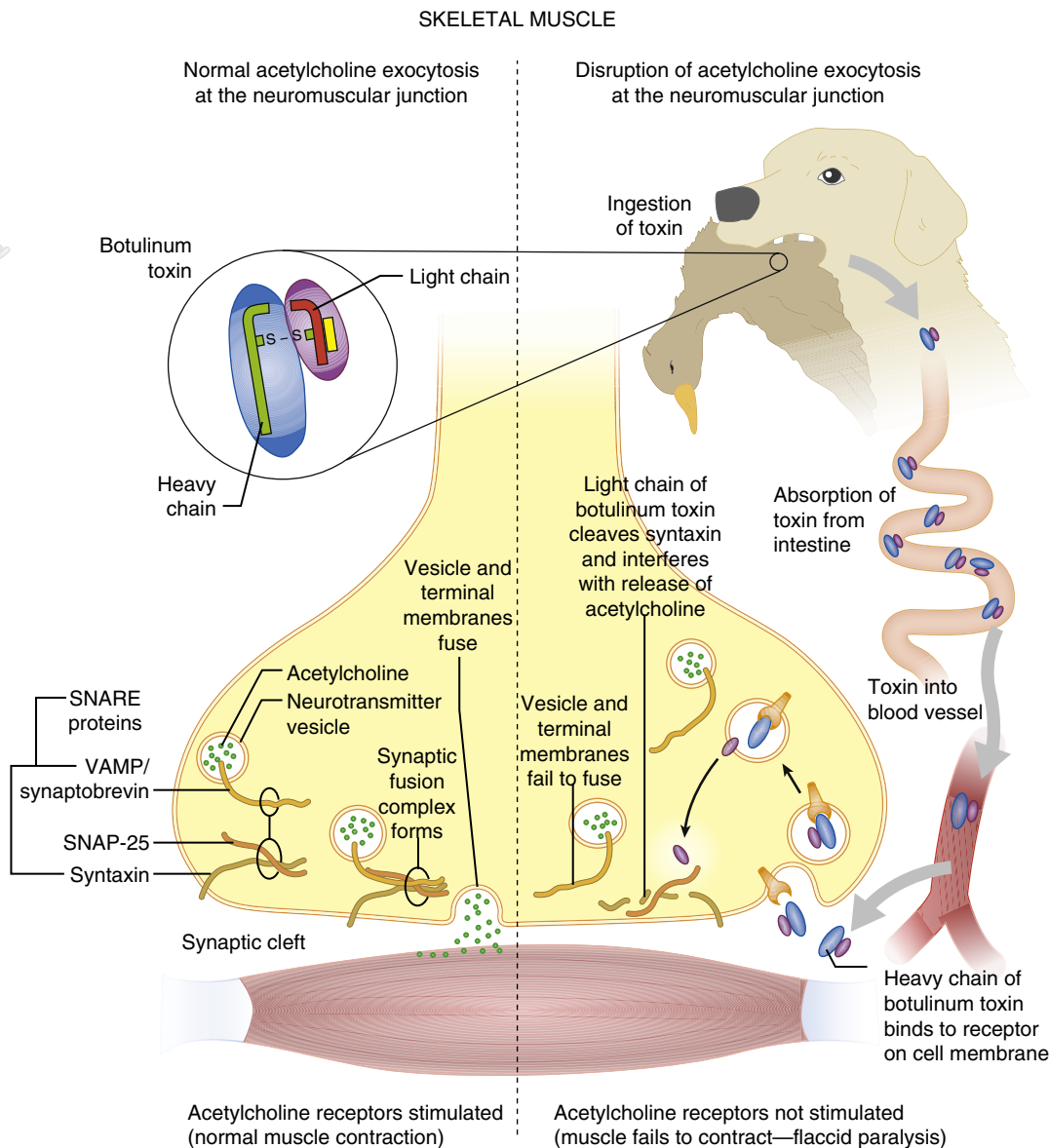
Botulinum toxin is a neurotoxin that blocks transmission of signals from nerves to muscles, thus causing muscular paralysis. The incredible potency of botulinum toxin is due to its enzymatic activity. It is a zinc protease that cleaves SNARE proteins in the neuromuscular junction that are required for release of the neurotransmitter acetylcholine. Death is generally due to paralysis of the lungs and respiratory failure (Fig. 22.22).

C. botulinum almost never causes infections but will grow in improperly canned food. Proper canning uses a pressure cooker to destroy the hardy spores produced by *Clostridium*. If the spores are not destroyed, they can germinate. After the bacteria die, they release botulinum toxin, which accumulates in the food. Merely 50 ng of botulinum toxin is enough to kill the average human. The toxin can, however, be destroyed by heating.

Terrorists of the Japanese cult Aum Shinrikyo, discussed previously, have attempted to use botulinum toxin. Aerosols were dispersed at various sites in Tokyo and at U.S. military installations in Japan on several occasions between 1990 and 1995. The attacks failed, mainly because

FIGURE 22.22
Mechanism of Botulinum Toxin
Botulinum toxin disrupts the normal functioning of a neuromuscular junction by inhibiting the release of acetylcholine. From Sykes JE (2014). Tetanus and botulism. In *Canine and Feline Infectious Diseases*, Ch. 54, pp. 520–530. Saunders/Elsevier, Philadelphia, PA, USA.

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SKELETAL MUSCLE

Normal acetylcholine exocytosis at the neuromuscular junction

Disruption of acetylcholine exocytosis at the neuromuscular junction

Botulinum toxin

Light chain

Heavy chain

Ingestion of toxin

Absorption of toxin from intestine

Light chain of botulinum toxin cleaves syntaxin and interferes with release of acetylcholine

Vesicle and terminal membranes fuse

Toxin into blood vessel

SNARE proteins

Acetylcholine

Neurotransmitter vesicle

Vesicle and terminal membranes fail to fuse

VAMP/synaptobrevin

Synaptic fusion complex forms

SNAP-25

Syntaxin

Synaptic cleft

Heavy chain of botulinum toxin binds to receptor on cell membrane

Acetylcholine receptors stimulated (normal muscle contraction)

Acetylcholine receptors not stimulated (muscle fails to contract—flaccid paralysis)

the cult used strains of *C. botulinum* that failed to produce toxin. On the other hand, millions of people have willingly had extremely dilute preparations of botulinum toxin (Botox) injected into their face to eliminate wrinkles. The procedure works because botulinum toxin inhibits the muscle contraction responsible for causing them.

Ricin. Many higher plants make **ribosome-inactivating proteins (RIPs)**. These enzymes split the N-glycosidic bond between adenine and ribose from a specific sequence in the large-subunit ribosomal RNA. Clipping adenine from the rRNA totally inactivates the ribosome. A single RIP molecule is sufficient to inactivate all the ribosomes and kill a whole cell. Because RIPs are synthesized as precursor proteins that are fully processed only after exiting the plant cell's cytoplasm, the toxin does not kill the plant. Intact ribosomes from different types of organisms differ greatly in their sensitivity to RIPs. Mammalian ribosomes (which contain 28S rRNA) are by far the most sensitive. On the other hand, the activity of many RIPs against bacterial ribosomes (which contain 23S rRNA) is low or negligible, and this has allowed the genes for some RIPs to be cloned and expressed in *E. coli*.

FIGURE 22.23
Seeds of the Castor Bean, *Ricinus communis*
Courtesy of Dan Nickrent, Department of Plant Biology, Southern Illinois University, Carbondale, IL.

Like many bacterial toxins, **ricin** is a typical A-B toxin in which the A chain exhibits toxic enzymatic activity and the B chain mediates entry into the target cell. Ricin is lethal at around 3 μg/kg body weight, meaning that 300 μg should kill a large human. Ricin is extracted from the seeds of the castor bean plant, *Ricinus communis* (Fig. 22.23). This plant is widely grown, both for ornamentation and on a large scale for castor oil production. Because of its widespread availability, high toxicity, stability, and lack of any antidote, there are several examples of the use of ricin as a biological weapon.

Ricin achieved international notoriety in 1978 when the Bulgarian defector Georgi Markov was assassinated in a London street by ricin. The communist assassin wielded a modified umbrella that injected a hollow 0.6-mm-diameter metal sphere, filled with ricin, into Markov's leg. In 1991, four members of the Patriots Council, an extremist group in Minnesota with an antigovernment and antitax ideology, purified ricin in a home laboratory. They were arrested for plotting to kill IRS and law enforcement agents with ricin. In late 2013, actress Shannon Richardson pleaded guilty for mailing letters containing ricin to President Barack Obama and New York City Mayor Michael Bloomberg in a scheme to frame her estranged husband. In a completely separate incident, in January 2014, James Dutschke also pleaded guilty to sending ricin to President Obama and other government officials with the intention of framing an Elvis Presley impersonator with whom he had a personal feud.

Abrin. Though less well known, **abrin**, which is also a ribosome-inactivating protein, is four times more toxic than ricin. Abrin is derived from the seeds of *Abrus precatorius*, commonly known as jequirity or rosary pea (Fig. 22.24).

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FIGURE 22.24
Seeds of the Rosary Pea, *Abrus precatorius*
The beautiful seeds of the rosary pea are highly toxic when the coat is damaged. Courtesy of Kenneth R. Robertson, University of Illinois.

The beautiful seeds are widely used in jewelry, particularly rosary beads. However, the seeds are so toxic that, if broken or damaged, a small prick in the skin is sufficient to absorb a lethal dose of abrin. There have been reports of abrin poisoning in jewelry makers, as well as in individuals who ingested seeds, but there are no known instances of abrin being used as a biological weapon.

Conotoxin. Cone snails are predators that use a venom cocktail containing at least 100 different conotoxins to paralyze and kill their prey. The most dangerous cone snail to humans, *Conus*

geographus, stabs fish with a venom-filled "harpoon" located in its proboscis (Fig. 22.25).

Death from the sting of a cone snail is largely due to α-conotoxins, which cause muscle paralysis leading to respiratory arrest. Other toxins may trigger cardiovascular collapse. Symptomatically, α-conotoxins resemble botulinum toxin, although the mechanism of action is different. Because most conotoxins are short peptides 10–30 amino acids in length, the concern from a biological warfare perspective is not that a government or terrorist would harvest venom from cone snails but that the toxins would be chemically synthesized.

Purified toxins are possible biological warfare agents. Natural toxins can be isolated from bacteria, plants, and animals, but other toxins could be chemically synthesized. Botulinum toxin disrupts the neuromuscular junction and is the most potent toxin known. Ricin and abrin are ribosome-inactivating proteins that are made by certain plants.

ENHANCING BIOLOGICAL WARFARE AGENTS WITH BIOTECHNOLOGY

It is often suggested that genetic engineering could be used to create more dangerous versions of infectious agents. Although there is some truth to this assertion, consider the following:

Suppose a bioterrorist tries to genetically modify a harmless laboratory bacterium, such as *E. coli*. The bacteria could be engineered to go "under cover" when they enter the human body, hiding from the immune system. Additionally, the bacteria could be programmed to rebuff immune cells by injecting them with toxins, and other genes could be added for ripping vital supplies of iron away from blood cells. Finally, the bacteria could be modified to be highly infectious. Such a biological agent would make for a fearsome weapon.

Unfortunately, this bacterium already exists. It is called *Yersinia pestis*. It is the agent of bubonic plague and is still endemic in many parts of the world, including China, India, Madagascar, and the United States. Instead of devoting years to genetically engineer a lethal biological weapon, a bioterrorist could simply isolate one of Mother Nature's very own products. The "improvement" of infectious diseases by genetic engineering, therefore, is probably a minor threat.

Still, genetic engineering of biological warfare agents is theoretically possible, so we briefly consider the issue here.

Engineering Pathogens to Be More Lethal

The Soviet germ warfare facility is known to have modified smallpox virus and generated a variety of artificial mutants and hybrids. The details are largely unavailable. However, recent experiments with mousepox *(Ectromelia* virus) have given disturbing results. Mousepox is related to smallpox, but it only infects mice. Its virulence varies greatly depending on the strain of mouse. Genetically resistant mice rely on cell-mediated immunity, rather than antibodies. Natural killer (NK) cells and cytotoxic T cells destroy cells infected with mousepox virus, thus clearing the virus from the body.

Researchers modified mousepox virus by inserting the human gene for the cytokine interleukin-4 (IL-4). IL-4 is known to stimulate the division of B cells, which synthesize antibodies. The rationale for engineering the virus was that IL-4 would stimulate the production of

antibodies and lead to an improved and more balanced immune response. What actually happened was the opposite of what was expected: the creation of a virus with vastly greater virulence. Not only did it kill all of the genetically resistant mice, but it also killed 50% of mice that had been vaccinated against mousepox. The expression of excess IL-4 suppressed the NK cells and cytotoxic T cells. Furthermore, it failed to increase the antibody response. The reasons are not fully understood, but they do serve as a reminder that the immune system is under extremely complex control.

Similar results have been seen with strains of *Vaccinia* virus, which is used for vaccination against smallpox. Whether insertion of IL-4 or other immune regulators into smallpox itself would lead to increased virulence by undermining the immune response is unknown. poxviruses already possess genes designed to protect the virus by interfering with the action of NK cells and cytotoxic T cells (Fig. 22.26). These are the cytokine response modifier (*crm*) genes, and they vary in effectiveness among different poxviruses. One reason smallpox is so virulent may be that it already subverts the body's cell-mediated immune response. In this case, adding IL-4 would not be expected to increase virulence.

Creating Camouflaged Viruses

With genetic engineering, it is also possible to hide a potentially dangerous virus inside a harmless bacterium. This strategy is already used in nature when bacteriophages insert their genomes into bacterial chromosomes or plasmids and later re-emerge to infect other hosts.
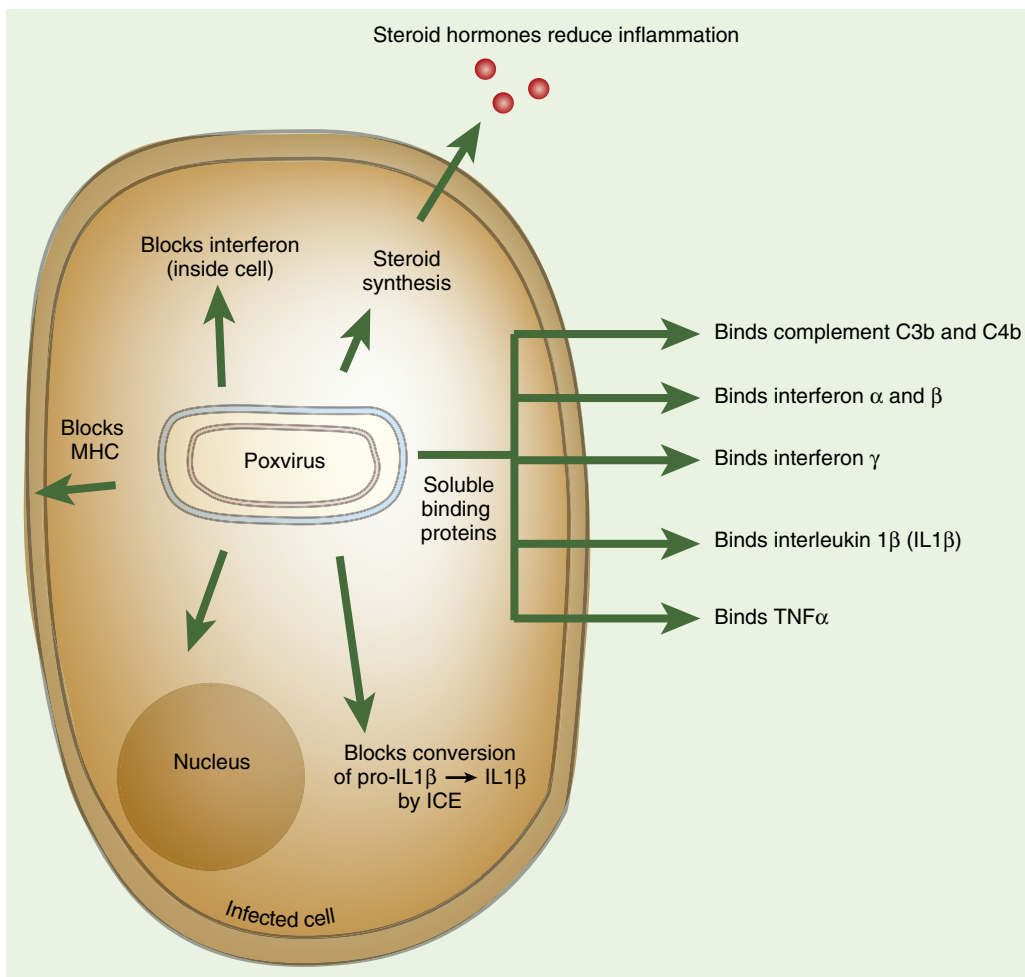
FIGURE 22.26 **Poxvirus Immune Evasion**
Poxvirus deploys many different proteins to prevent the infected cell from being attacked by the host's immune system.

Theoretically, cloning the entire genome of a small animal or plant virus into a bacterial plasmid could create a biological weapon. Larger viruses could be accommodated with bacterial or yeast artificial chromosomes. In the case of RNA viruses, a cDNA copy of the virus genome must first be generated by reverse transcriptase before cloning it into a bacterial vector. Any virus containing a **poison sequence**, a base sequence that is not stably maintained on bacterial plasmids, could perhaps be cloned as separate fragments. Such a strategy works for yellow fever virus, but a complete, functional cDNA requires ligation of the fragments *in vitro*.

Many cell types, both bacterial and eukaryotic, can take up DNA or RNA under certain circumstances by transformation. Consequently, the naked nucleic acid genomes of many viruses, both DNA and RNA, are infectious even in the absence of their protein capsids or envelopes. Thus, once a viral genome is cloned, the DNA molecule containing it may itself be infectious. Alternatively, the cDNA version of some RNA viruses can successfully infect host cells and give rise to a new crop of RNA-containing virus particles. This has been demonstrated for RNA viruses such as poliovirus, influenza, and coronavirus.

The cleverest strategy for generating an RNA virus is to clone the cDNA version of its genome onto a bacterial plasmid downstream of a strong promoter (Fig. 22.27). The natural RNA version of the viral genome will be generated by transcription. When induced, the bacterial cell would generate a large number of infectious viral particles. A dangerous human RNA virus loaded into a harmless intestinal bacterium under the control of a promoter designed to respond to conditions inside the intestine could pose a formidable threat.

> Genetically engineering biological warfare agents to make them deadlier is a minor threat since many naturally occurring microbes are already very dangerous. However, certain poxviruses have been modified to become more virulent. Inserting viral DNA into plasmids carried by harmless bacteria could create camouflaged viruses.

DETECTION OF BIOLOGICAL WARFARE AGENTS

In the laboratory, some pathogenic bacteria grow slowly or not at all. This may be because the microbe has fastidious nutrient requirements or is otherwise difficult to culture outside its host organism. However, thanks to advances in biotechnology, infectious microbes can be identified using a variety of different techniques.
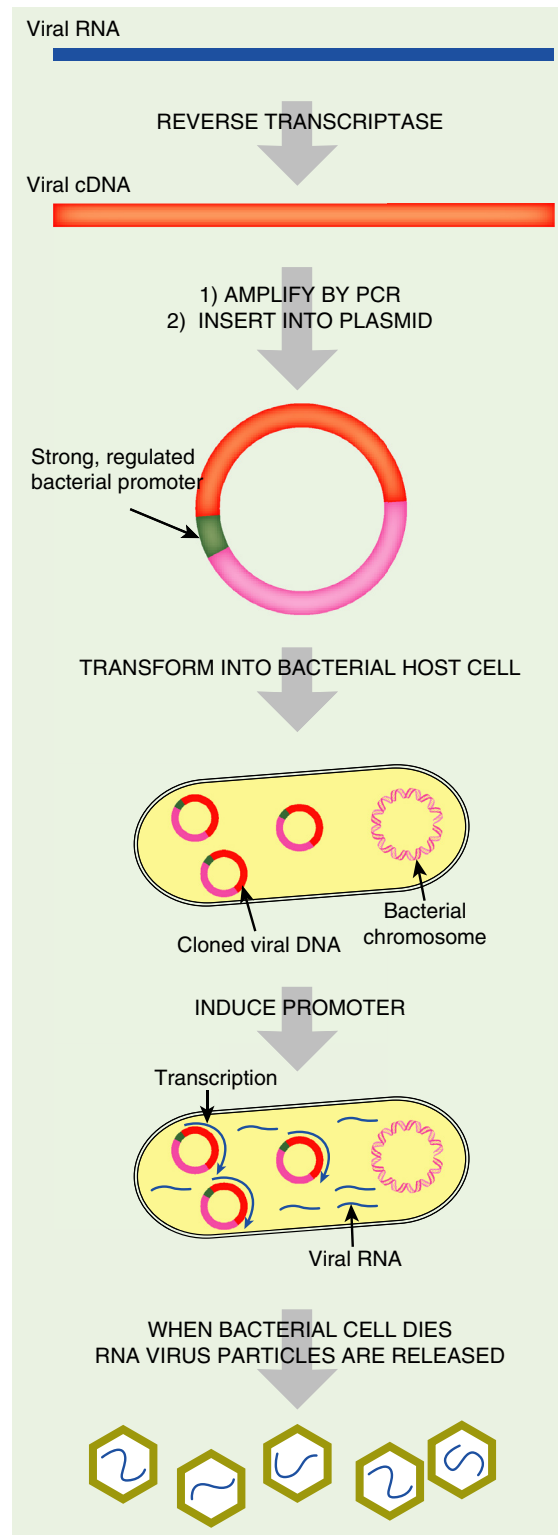
Molecular Diagnostics

Rather than attempting to grow and identify disease-causing agents using classical microbiological techniques, molecular diagnostics analyzes molecules; typically DNA, but RNA, proteins, and volatile organic compounds can also be used. (Other diagnostic methods involve the use of antibody technology and are discussed in Chapter 6.) Molecular techniques have the advantage of being quicker, more accurate, and more sensitive.

One diagnostic method is called fluorescent *in situ* hybridization (FISH; for details see Chapter 3). Biopsies or other patient samples are directly probed with fluorescent DNA oligonucleotides specific to a pathogen of interest. If the pathogen is present, the probe binds to the complementary DNA in its chromosome and the fluorescence can be visualized under a microscope. A new innovation, called peptide nucleic acid (PNA), replaces the negatively charged sugar-phosphate backbone of DNA with a neutral peptide backbone. Probes made of PNA bind complementary DNA more tightly and enter bacterial cells more easily (Fig. 22.28).

Most other methods based on DNA detection involve extracting DNA from a sample followed by amplification via PCR. Because primers can be designed to amplify DNA sequences unique

Viral RNA

REVERSE TRANSCRIPTASE

Viral cDNA

1) AMPLIFY BY PCR
2) INSERT INTO PLASMID

Strong, regulated
bacterial promoter

TRANSFORM INTO BACTERIAL HOST CELL

Cloned viral DNA

Bacterial
chromosome

INDUCE PROMOTER

Transcription

Viral RNA

WHEN BACTERIAL CELL DIES
RNA VIRUS PARTICLES ARE RELEASED

FIGURE 22.27
Expression of Cloned RNA Virus
Cloning an RNA virus requires making a double-stranded DNA copy using reverse transcriptase. The cDNA is inserted into an appropriate bacterial plasmid and transformed into bacterial cells. To control the expression of the viral DNA, a strong promoter is placed upstream of the viral cDNA. If the promoter is inducible, when the bacteria are given the appropriate stimulus, the viral cDNA will be expressed, resulting in production of viral particles that could infect many people.
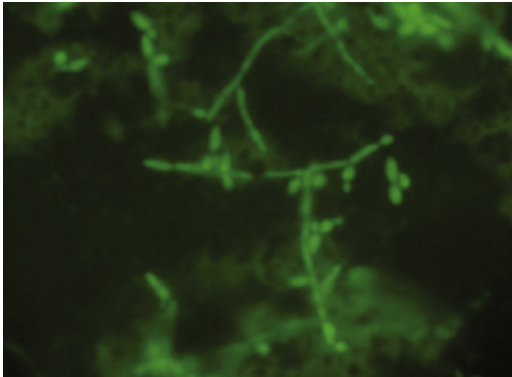
FIGURE 22.28
PNA FISH Probe
The yeast *Candida albicans* is detected in a blood culture using fluorescent *in situ* hybridization (FISH) with a peptide nucleic acid (PNA) probe. Fluorescence microscopy; original magnification 500x. From Bravo LT, Procop GW (2009). Recent advances in diagnostic microbiology. *Semin Hematol* **46**, 248–258.

to a particular pathogen, PCR itself can serve as a diagnostic tool. The advantages of PCR are that it theoretically requires only a single molecule of target DNA and works on microbes that cannot be cultured in the laboratory. The downside is that PCR is susceptible to contamination and false positives. A variant of PCR (see Chapter 4), called randomly amplified polymorphic DNA (RAPD), can be used to distinguish different strains of the same bacterial species. This capability is useful in epidemiology for tracking the spread of infectious disease.

Additionally, every species of microorganism has a different **small-subunit ribosomal RNA** (SSU rRNA) sequence (16S rRNA in bacteria and 18S rRNA in eukaryotes). Thus, if a patient has an unknown infection, clinicians can use PCR to amplify the gene encoding the microbe's SSU rRNA. Primers that recognize the conserved region of SSU rRNA are used to amplify the gene. The PCR fragment is then sequenced and compared with a database of known DNA sequences.

Another technique that generally relies on SSU rRNA is **checkerboard hybridization**. This allows multiple bacteria to be detected and identified simultaneously in a single sample. A series of probes corresponding to different bacteria are applied in horizontal lines across a hybridization membrane (Fig. 22.29). PCR is used to amplify a portion of the SSU rRNA gene from clinical samples, which may contain a mixture of pathogens. The PCR fragments are then labeled with a fluorescent dye and applied vertically to the membrane. After denaturation and annealing to allow hybridization, the membrane is washed to remove unbound DNA. Those samples that hybridize to the probes appear as bright fluorescent spots.

A potentially revolutionary technology called PLEX-ID has been developed by Abbott Laboratories. It combines traditional PCR with mass spectrometry to identify unknown microbes in patient samples. DNA is extracted and many different sets of primers are used to amplify various target sequences. The fragments are then analyzed with a mass spectrometer to
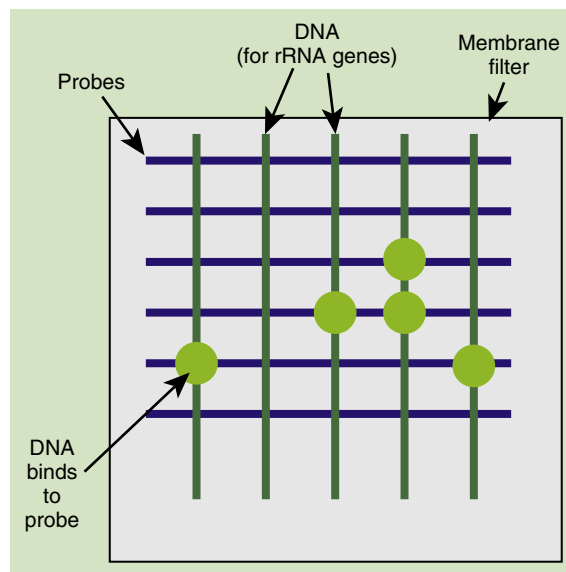
**FIGURE 22.29 Checkerboard Hybridization**
Probes corresponding to 16S rRNA for each candidate bacterium are attached to a membrane filter in long horizontal stripes (one candidate per stripe). DNA from patient samples is extracted and amplified by PCR using primers for 16S rRNA. The PCR fragments are tagged with a fluorescent dye and applied in vertical stripes. Each sample is thus exposed to each probe. Wherever a 16S PCR fragment matches a 16S probe, the two bind, forming a strong fluorescent signal where the two stripes intersect.

determine their mass. From this information, the DNA sequence can be deduced and the pathogen identified. PLEX-ID can make a diagnosis in 8 hours.

In the future, it may be possible to diagnose disease using an "electronic nose." As the name implies, the device detects volatile organic compounds that are released by pathogens or by the body in certain diseased conditions.

Biosensors

Biosensors are devices for the detection and measurement of reactions that rely on a biological mechanism (Fig. 22.30). Biosensors have been traditionally used in medical diagnostics and in food and environmental analysis. By far the biggest use has been the clinical monitoring
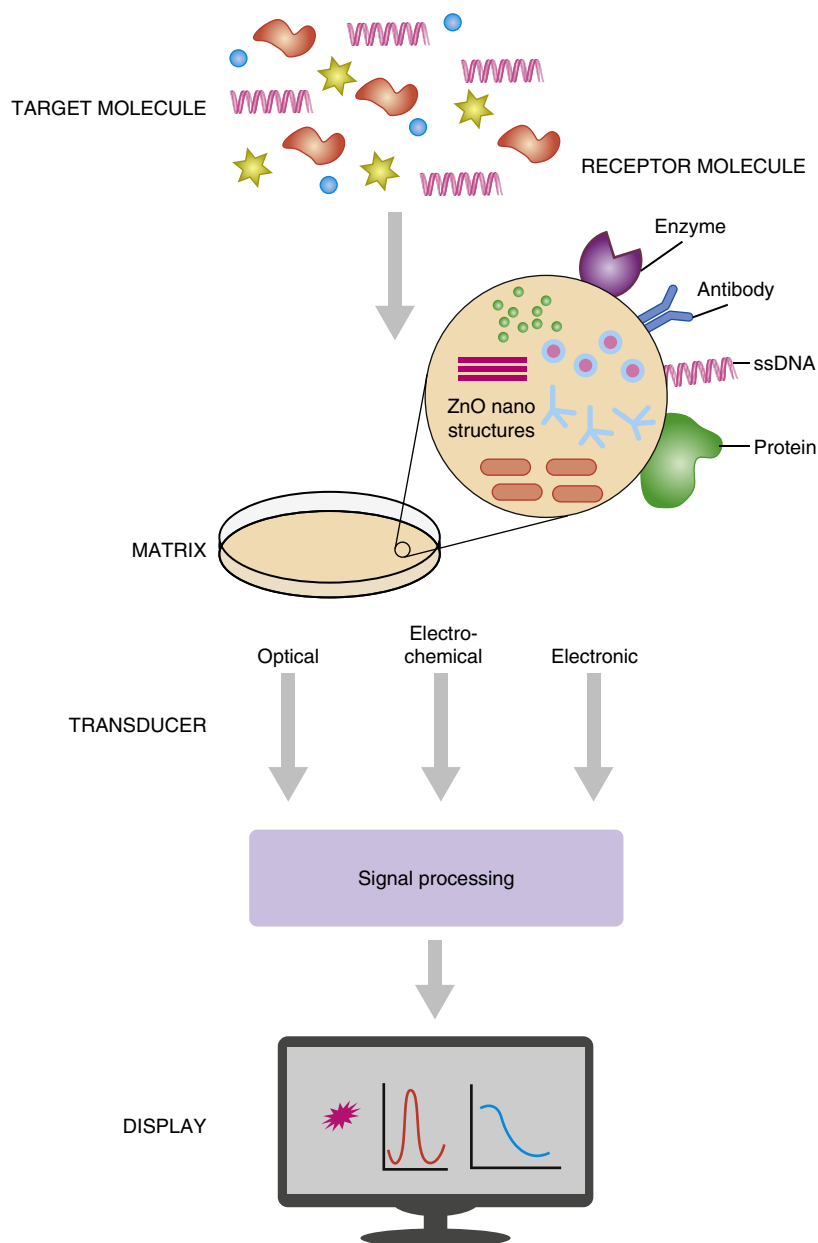
FIGURE 22.30 Biosensors
Biosensors, in general, share a common design. A highly specific biological receptor molecule detects or interacts with a target molecule of interest, for instance, a biological warfare agent. A signal is generated, processed, and displayed for the user. Modified from Arya SK, et al. (2012). Recent advances in ZnO nanostructures and thin films for biosensor applications: review. *Anal Chim Acta* **737**, 1–21.

FIGURE 22.31
Light Emission by Aequorin

Aequorin, from *Aequorea victoria,* emits blue light when provided with its substrate, coelenterazine, plus oxygen and calcium. The enzyme binds to aequorin via the oxygen; and when calcium is present, the complex emits blue light, degrades the substrate to coelenteramide, and releases carbon dioxide.
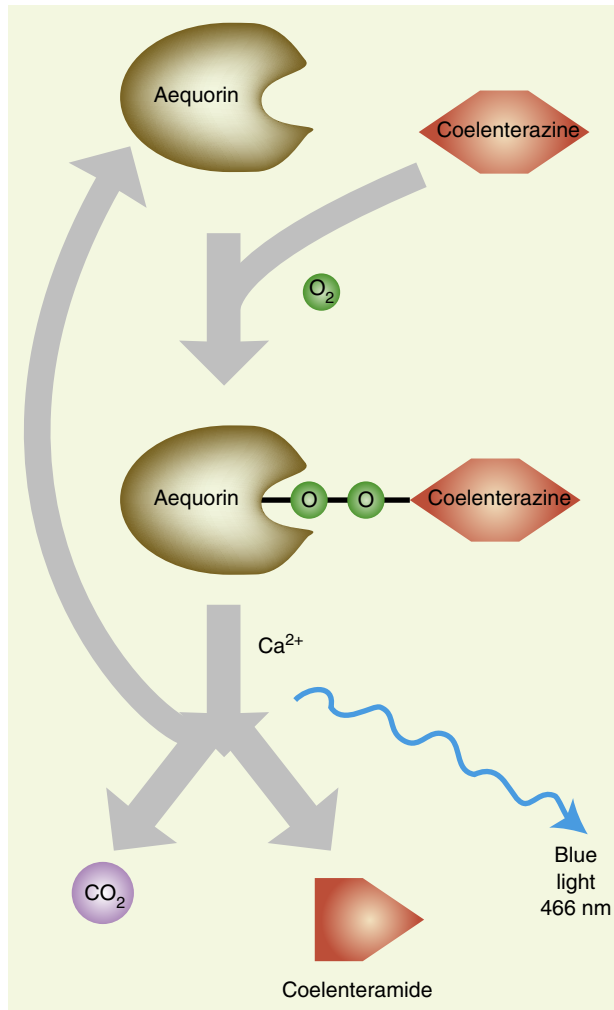
of glucose levels in diabetics using the enzyme glucose oxidase.

There is growing interest today in using biosensors to detect biological warfare agents. Placing biosensors in high trafficked areas, such as in malls or subway stations, could allow for continuous surveillance. Additionally, handheld devices giving a rapid response at the site of a possible attack would be highly useful. Several proposals exist that would use specific antibodies or antibody fragments as detectors for biological warfare agents (see Chapter 6 for antibody engineering).

B cells carry antibodies specific for one antigen, so one proposal is to use whole B cells in a biosensor. When an antigen binds to the antibody on the surface of a B cell, it triggers a signal cascade. Engineered B cells have been made that express **aequorin**, a light-emitting protein from the luminescent jellyfish *Aequorea victoria.* Aequorin emits blue light when triggered by calcium ions (Fig. 22.31). Living jellyfish actually produce flashes of blue light, which are transduced to green by the famous green fluorescent protein (GFP).

In a biosensor, when a B cell detected a disease agent (or any specific antigen), calcium ions would flood into the cell due to activation of a signal cascade (Fig. 22.32). This in turn triggers light emission by aequorin. The light emitted is detected by a sensitive charge-coupled device (CCD) detector. This approach could detect 5 to 10 particles of a biological warfare agent. Approximately 10,000 B cells specific to different pathogens could be assembled in array fashion onto a chip placed inside the biosensor.

Another scheme developed by the Ambri Corporation of Australia uses antibody fragments mounted on an artificial biological membrane, which is attached to a solid support covered by a gold electrode layer. Channels for sodium ions are incorporated into the membrane. When the ion channels are open, sodium ions flow across the membrane and a current is generated in the gold electrode. The ion channels consist of two modules, each spanning half the membrane. When top and bottom modules are united, the ion channel is open. When the top module is pulled away, the ion channel cannot operate. Binding of biological warfare agents by the antibody fragments separates the two halves of the channels, which in turn affects the electrical signal (Fig. 22.33).

Diagnosing pathogenic bacteria with molecular techniques, particularly using the genes encoding ribosomal RNA sequences, is faster and more sensitive than traditional microbiological methods. Biosensors use biological components themselves to monitor for suspicious biomolecules.
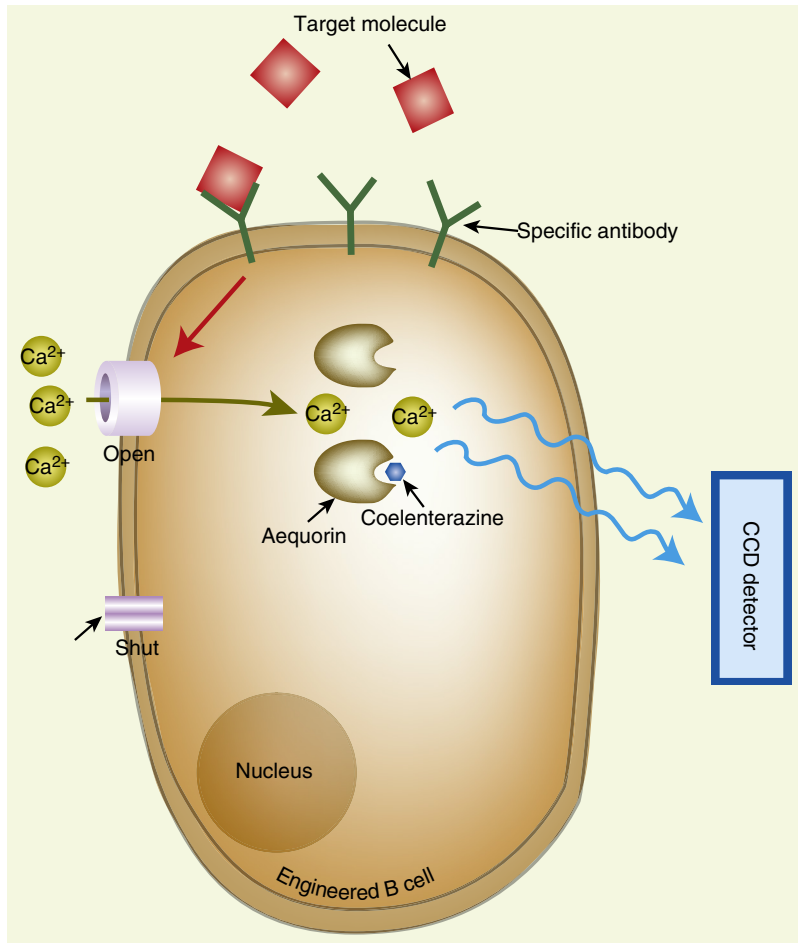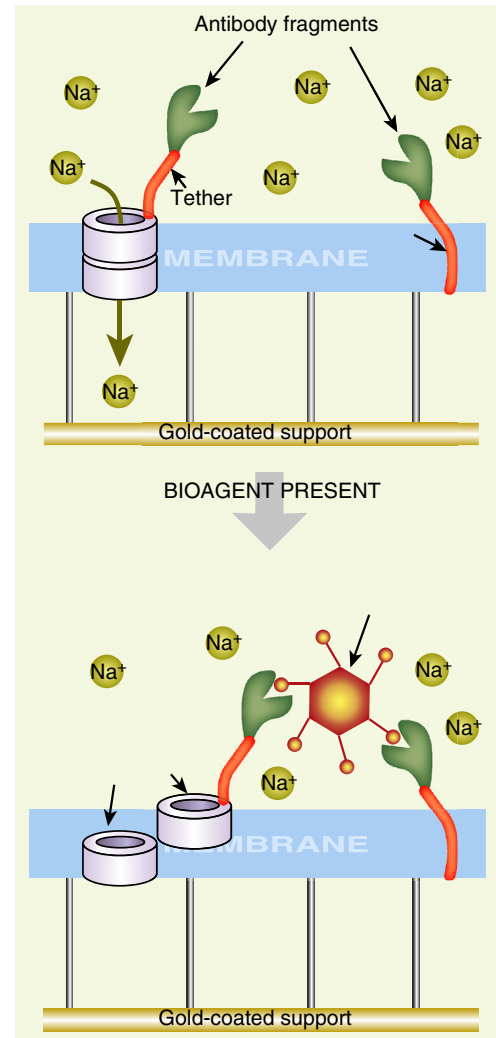
FIGURE 22.32
B-Cell Optical Biosensor
Expressing aequorin in a B cell would provide a detection system for B-cell activation. When a trigger molecule, such as a biological warfare agent, binds to receptors on the B cell, the calcium channels are opened and calcium floods the cell. The high calcium levels would activate aequorin to emit blue light. A charge-coupled device (CCD) would measure the photon emissions and warn the user of a biological agent.

FIGURE 22.33
Antibody Ion-Channel Biosensor
Antibody fragments that bind specific biological agents can be engineered and tethered to a fixed location on an artificial membrane. Another molecule of the same antibody fragment is tethered to a sodium channel. The artificial membrane is carried on a gold-coated solid support that acts as an electrode. This detects sodium ions that pass through the ion channel. When a biological agent is present, the antibody fragments bind it, pulling the top half of the sodium channel out of alignment with the bottom half. Sodium ions no longer pass to the gold electrode, decreasing the signal.

Summary

Biological warfare has been around since life first evolved. Humans are most concerned about the biological warfare directed at us, including infectious diseases and the ability of microbes to evolve resistance to antibacterial agents. Although this development is worrisome, new strategies and technologies are being developed to fight back against the growing problem of antibiotic resistance.

Humans have often attempted to use biological agents in warfare, although with little overall success so far. Several highly virulent infectious agents including anthrax, plague, and smallpox, as well as certain biological toxins such as ricin and abrin, are regarded as likely biological warfare agents. Whether or not genetic engineering can create "improved" bioweapons is as yet uncertain. Developing quicker ways to detect and diagnose microbes is an active area of research.

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End-of-Chapter Questions

1. What can bacterial toxins kill?
   a. insect cells
   b. human cells
   c. other bacterial cells
   d. protozoa
   e. all of the above

2. Which statement is true regarding novel antimicrobial strategies?
   a. Siderophores are a good target because they do not exist in humans, and thus the side effects would be diminished.
   b. Disruption of quorum sensing causes bacteria to produce more antibiotic-resistant biofilms.
   c. Alkyl- and aryl-mannose derivatives bind to FimH adhesins and enhance attachment to the natural receptor.
   d. Phage therapy increases the virulence of the bacteria due to transduction.
   e. Production of aflatoxin inhibits fungal agents from growing on cereals.

3. Which of the following is an important consideration of germ warfare?
   a. dispersal
   b. persistence of the agent
   c. incubation time
   d. storage and preparation of the agent
   e. all of the above

4. According to the U.S. Army, which of the following is a requirement for biological weapons?
   a. It should be able to be produced economically.
   b. It should consistently produce death, disability, or damage.
   c. It should be stable from production through delivery.
   d. It should be easy to disseminate quickly and effectively.
   e. All of the above are requirements for biological agents.

5. Which one of the following has rarely been considered as a possible biological warfare agent?
   a. viruses
   b. bacteria
   c. pathogenic eukaryotes
   d. pathogenic fungi
   e. none of the above

6. According to the text, which of the following is one of the best biological weapons?
   a. anthrax
   b. malaria
   c. amoebic dysentery
   d. smallpox
   e. none of the above

7. Since *B. anthracis* strains are closely related, how is it possible to differentiate between the strains?

    a. 16S rRNA sequencing

    b. VNTRs

    c. 23s rRNA sequencing

    d. gene expression profiles

    e. none of the above

8. How does bubonic plague spread?

    a. ticks

    b. person-to-person

    c. fleas

    d. rodents

    e. birds

9. Which of the following is used as a live vaccine for smallpox?

    a. *Variola* major

    b. monkeypox

    c. *Variola* minor

    d. *Vaccinia* virus

    e. none of the above

10. To what virus family do dengue fever and yellow fever belong?

    a. flaviviruses

    b. poxviruses

    c. filoviruses

    d. variola viruses

    e. arenaviruses

11. Which of these is a siderophore?

    a. aflatoxin

    b. PA63

    c. yersiniabactin

    d. anthracimycin

    e. lysin

12. What is the mode of action for ricin?

    a. inactivation of transcription

    b. inactivation of rRNA

    c. activation of the apoptosis pathway

    d. inactivation of the immune system

    e. creation of pores in cell walls

13. Which of the following could be used as a biological agent against agriculture crops?

    a. viruses

    b. bacteria

    c. pathogenic fungi spores

    d. prions

    e. none of the above

(*Continued*)

14. Which of the following gained virulence upon introduction of the IL-4 gene?
    a. monkeypox
    b. smallpox
    c. chickenpox
    d. mousepox
    e. camelpox

15. How are pathogens detected by using biosensors?
    a. by antibodies that are connected to components to give electrical signals or trigger light emission
    b. by isolating the pathogen directly from the sample
    c. biosensors detect antibodies against specific pathogens, similar to a Western blot
    d. by using PCR to amplify variable regions of the pathogen's genome
    e. none of the above

16. Which of the following has improved sanitation in healthcare settings by preventing the attachment of bacteria to surfaces?
    a. black silicon
    b. nanopillars
    c. nanocarpets
    d. polymers of esters and cyclic hydrocarbons
    e. all of the above

17. What is the mechanism of action for botulinum toxin?
    a. inhibits the release of acetylcholine
    b. activates the release of acetylcholine
    c. causes muscle contraction
    d. mimics the action of acetylcholine
    e. stimulates acetylcholine receptors

18. Which of the following techniques uses both PCR and mass spectrometry to identify a pathogen within eight hours?
    a. FISH
    b. PNA
    c. VNTR
    d. SNP analysis
    e. PLEX-ID

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