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ATTN: MCMR-CDT-N
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

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ATTN: MCMR-CDZ-I
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CHAPTER 31

Anthrax

COREY J. HILMAS, ALEXANDRE M. KATOS, PATRICK T. WILLIAMS, AND JAIME ANDERSON

I. INTRODUCTION

Anthrax is a virulent, contagious, and potentially fatal disease. The first accounts of anthrax infection were written by the Roman poet Vergil in early antiquity. While its lethal effects were ascribed to the actions of an exotoxin over a half century ago, the pathogenesis and mechanism of anthrax toxicity continue to be refined. Depending on the route of exposure, anthrax can cause a different disease, including inhalational, cutaneous, and oral/ingestional forms. Anthrax infection involves a complex set of steps in its pathogenesis from spore uptake by immune cells, germination, transport to local lymph nodes, production of deadly toxins, systemic spread, and ultimately death of the host. The details of each step are continually being debated. Even today debate ensues regarding the mechanisms of macrophage killing by the toxins and the importance of other cell types involved in toxin-induced fatality.

While anthrax infection causes high numbers of bacilli and overt septicemia, it is the exotoxins which are responsible for the intoxicating symptoms and death. Birth of the molecular age in modern research led to the identification and enzymatic characterization of three proteins that constitute two anthrax exotoxins. These are the protective antigen (PA), edema factor (EF), and lethal factor (LF). A better understanding of the precise mechanisms of toxicity initiated by anthrax toxins will uncover many of the unsolved mysteries surrounding *Bacillus anthracis* infection. Moreover, nature's clever engineering will be evident from the sometimes contradictory actions of the spore form, vegetative bacilli, and bacterial toxins.

II. HISTORY

Anthrax has plagued man and beast since early recorded history. Scholars have attributed several plagues in antiquity to anthrax. The Plague of Athens (430–427 BC) and two of the plagues of Egypt (the fifth – death of livestock – and sixth plagues – boils), during the time of the Israelites' captivity have both been ascribed to outbreaks of anthrax (McSherry and Kilpatrick, 1992). Publius Vergilius Maro or Vergil (70–19 BC) provided the earliest, definitive, and detailed descriptions of an anthrax epidemic in his four-volume *Georgics*, a narrative on agriculture and animal husbandry (Sternbach, 2003). Vergil described the same disease ravaging sheep, horses, cattle, dogs, and various other animals. In addition to signs of toxicity, he provided insight into knowledge of how the disease was transmitted, namely wool. He even noted the virulent and contagious nature of anthrax, as well as its ability to spread to humans (Dirckx, 1981).

> The pelts of diseased animals were useless, and neither water nor fire could cleanse the taint from their flesh. The sheepmen could not shear the fleece, which was riddled with disease and corruption, nor did they dare even to touch the rotting strands. If anyone wore garments made from tained wool, his limbs were soon attacked by inflamed papules and foul exudates. (Dirckx, 1981)

During the course of the next 1,500 years, Europe witnessed sporadic outbreaks of anthrax as they occurred in 14th century Germany and 17th century central Europe and Russia. The disease was classified as anthrax or charbon malin (Morens, 2003) in 1769 by the French physician Nicholas Fournier (Fournier, 1769; Morens, 2003). The name is derived from the black eschar lesions, the hallmark of cutaneous infection. Fournier also noted a link between those who worked with raw animal hair or wool and an increased susceptibility to disease. In the 18th century, an epidemic destroyed half of the sheep in Europe, possible evidence that anthrax was a major problem. Inhalation anthrax became known in the Victorian era as woolsorters' disease; however, infection was more often the result of contact with goat hair or alpaca than wool.

In 1850, Pierre-Francoise Olive Rayer (Rayer, 1850) and Casimir-Joseph Davaine (Davaine, 1863) reported the presence of "small filiform bodies" in the blood of anthrax-infected sheep (Carter, 1988). By 1855, Franz Aloys Antoine Pollender confirmed this discovery and implicated their role in producing anthrax disease (Pollender, 1855). In 1858, Freidrich August Brauell noted the "bodies" to be absent from healthy animals or animals infected with diseases other than anthrax. Brauell also noted their inability to be transmitted from pregnant sheep to fetus (Brauell, 1857).

In the 1870s, Robert Koch, a Prussian physician, isolated the anthrax bacillus and traced the complete life cycle using suspended-drop culture methods. Koch determined the bacillus could form spores which remained viable, even in hostile environments (Koch, 1876). Louis Pasteur provided demonstration of infectious disease transmission. He inoculated one cohort of cattle with live attenuated vaccine and a control cohort without vaccine. When all animals were injected with virulent anthrax, only the control cattle died.

A. Modern History: Weaponizing Anthrax and Terrorism

Research into the utilization of anthrax spores as a biological weapon began in the early 20th century. During World War I, German development of *B. anthracis* and other disease-causing organisms gave rise to covert programs intended to infect livestock and animal feed to be exported to the Allies. These plans included contaminating feed for horses and cattle to be exported from the USA to England, infecting sheep from Romania to be exported to Russia, and exporting contaminated livestock from Argentina to various Allied nations (Hugh-Jones, 1992; Merck, 1946).

During World War II, the pace of anthrax and biological weapons research in general accelerated. Imperial Japan had a large, active bioweapons program that included a substantial anthrax component. The central Japanese research facility was located in Manchuria, known as Unit 731. It is believed that in excess of 10,000 prisoners of war died either by direct experimental exposure to *B. anthracis*, among other pathogens, or by execution following exposure (Harris, 1992, 1994). Much of the Japanese bioweapons research transitioned to the battlefield. Operations such as Nomonhan in 1939, where Japanese troops entered the Soviet Union to infect Russian herds, met with only partial success. As it turned out, Japanese troops were unprepared to operate in a biological weapons environment. Their actions resulted in many inadvertent friendly casualties (Harris, 1992).

The Allies were pursuing biological weapons programs at the same time as the Axis powers. In 1942, the United Kingdom conducted anthrax experiments off the coast of Scotland at Gruinard Island. British scientists working at the biological weapons laboratory at Porton Down had demonstrated the lethality and military utility of the bacillus. Spores persisted and remained theoretically capable of infection for decades afterwards. A subsequent decontamination effort took nearly 10 years to clean up the island (Carter, 1992). The USA began developing anthrax as a biological weapon in 1943. A civilian agency, the War Reserve Service, constructed a research facility at Camp Detrick (later Fort Detrick in 1956), and conducted research into a number of pathogens, including *B. anthracis*.

In April and May of 1979 an anthrax epidemic occurred in Sverdlovsk, a city of then 1.2 million people, 1400 km east of Moscow. The Soviet medical community reported an outbreak in livestock south of the city, and human exposures by ingestion of infected meat and contact with diseased animals led to cases of gastrointestinal and cutaneous anthrax. According to Soviet medical reports, 96 cases of human anthrax were identified, of these 79 were said to be gastrointestional and 17 cutaneous. These cases resulted in 64 deaths, all reported to be gastrointestional exposures (Meselson *et al.*, 1994). In 1986, the Soviet Union invited a group of American scientists to visit Russia and investigate the outbreak. In collaboration with the Russian clinicians who treated the victims, the panel concluded that the outbreak was the result of inhalation exposure due to the accidental release of an estimated 10 kg of military-grade anthrax from the Soviet military microbiological facility in Sverdlovsk. This event remains the largest documented outbreak of inhalation anthrax (Sternbach, 2003).

In 2001, the first case of intentional anthrax release in the USA occurred. In October and November of that year, 11 confirmed cases of inhalation anthrax and 11 confirmed or suspected cases of cutaneous anthrax were reported in postal workers and others who handled mail that had been deliberately contaminated with anthrax spores (Abalakin *et al.*, 1990). These contaminated letters were mailed anonymously to several news media and Federal government offices. The letters contained handwritten threats as well as cryptic references to the terrorist attacks on September 11 of that year. The anthrax spores were analyzed and determined to be of the "Ames" variety, the strain which originated in the USA and had been acquired by Army research institutes for vaccine development.

Anthrax remains both a serious public health hazard and a very real biological weapon threat. A deliberate release of an anthrax weapon in a populated area could have catastrophic implications. An economic model developed by the Centers for Disease Control and Prevention (CDC) suggested a cost of $26.2 billion to treat 100,000 people exposed to anthrax (Kaufmann *et al.*, 1997). A risk assessment, provided at the end of this chapter, will serve to highlight the dangers of a realistic scenario involving anthrax spores.

III. EPIDEMIOLOGY

A. Persistence

Bacillus anthracis can remain for extended periods of time in soil. The mechanism responsible for its persistence is unclear. Therefore, persistence may involve multiplication cycles and sporulation. It may involve multiplication of the organism in the soil or bacterial amplification in infected animals prior to soil contamination by the carcass. Spores may germinate simply upon application of water to soil (Hanna and Ireland, 1999; Oncul *et al.*, 2002).

B. Infection

Infection typically results from herbivores grazing on soil or feed contaminated with spores. Oral consumption may

produce oropharyngeal or gastrointestinal infection, an invariably fatal condition. Terminally ill herbivores generally bleed from the nose, mouth, and bowel, resulting in further contamination of the soil or drinking source (Shafazand *et al.*, 1999). While the actual number of cells or spores shed by an infected animal is unknown, studies have shown that counts of 10^4 to 10^6 spores/g of soil can be found near infected carcasses (Turnbull *et al.*, 1998).

C. Dissemination

Dissemination of anthrax spores may result from biting flies or vultures (de Vos, 1990; Hugh-Jones and De Vos, 2002; Davies, 1983; Turell and Knudson, 1987). Flies and mosquitoes contaminated with the vegetative cells, as a result of feeding on blood, can remain infectious for hours. The infectious material is deposited onto leaves through defecation, leading to contamination of herbivore species such as cattle, sheep, horses, and goats. Vultures often feed on contaminated carcasses and disseminate the organism to other birds or common drinking sources shared by various animals.

D. Forms of Anthrax Disease

Anthrax in humans is associated with agricultural, horticultural, or industrial exposure to infected animals or animal products. Three forms of anthrax can be diagnosed in humans: cutaneous, gastrointestinal, and inhalational. Cutaneous anthrax infection can occur through handling of contaminated hides, wool, bones, and carcasses. Cutaneous anthrax is the most common form of natural human infection, consisting of >95% of anthrax cases, and is treatable if recognized early. Infection occurs as a result of direct contact with infected animals or animal products. The skin is typically damaged or abraded prior to establishment of successful skin lesions by the bacteria. The persons most at risk of a natural cutaneous exposure are industrial or agricultural workers, such as herders, butchers, slaughterhouse workers, or processing mill workers (Pile *et al.*, 1998). Infection can also occur through an insect bite (Spencer, 2003). While cutaneous anthrax is rarely fatal (mortality <1% in treated cases; Anon, 2000), it can progress to a systemic infection with a mortality rate of 5–20% in untreated cases (Pile *et al.*, 1998). In general, cutaneous anthrax is not as life threatening as the inhalational form, which results in much higher mortality. However, it is still important to study dermal pathogenesis models, particularly because cutaneous anthrax cases may result from an aerosol release (Inglesby *et al.*, 1999), a method most likely to be used by bioterrorists.

Inhalational anthrax may occur after inhaling aerosols of spores, formed from processing contaminated animal products (wool) or as the result of direct bioweaponization. Inhalational anthrax contributes to only 5% of all reported cases but is by far the most lethal form. The estimated mortality is approximately 90% in untreated patients (Atlas, 2002; Bales *et al.*, 2002; Dixon *et al.*, 1999; Friedlander, 1999, 2000; Meselson *et al.*, 1994; Oncu *et al.*, 2003). Inhalational anthrax is typically reported in industrial settings where animal products are handled in enclosed spaces, allowing for exposure to aerosolized spores. Individuals passing by these industrial facilities have been stricken with inhalational anthrax. In one study of 27 anthrax cases involving textile mills, 21 cases were cutaneous and six were inhalational. All but one of the inhalational cases was fatal. One case of inhalational anthrax occurred in a secretary at a goat hair-processing facility.

Gastrointestinal anthrax is far less common than inhalational anthrax, but the mortality rate is extremely high, from 50 to 75%, even with early treatment (Mansour-Ghanaei *et al.*, 2002). Oropharyngeal or gastrointestinal forms of anthrax can result from ingestion of contaminated meat (Atlas, 2002; Bales *et al.*, 2002; Dixon *et al.*, 1999; Friedlander, 1999). Gastrointestinal anthrax has never been confirmed in the USA, but this may be the result of cases being unreported in rural communities, where physicians may not be aware of this form (Atlas, 2002; Bales *et al.*, 2002; Dixon *et al.*, 1999; Friedlander, 1999; Oncu *et al.*, 2003; Pile *et al.*, 1998; Shafazand *et al.*, 1999). Two out of 53 persons in a 1998 Kazakhstan outbreak developed the gastrointestinal form, resulting from the consumption of contaminated raw meat. In Minnesota, several family members consumed steer meat and fell ill with gastrointestinal symptoms (Bales *et al.*, 2002). The meat was later confirmed to contain *B. anthracis*, but the bacteria could not be cultured to confirm the presence of the gastrointestinal pathogen. According to one case of a 15-year-old male who was infected after ingesting half-cooked sheep meat, the incubation period for gastrointestinal anthrax varies from 2 to 5 days (Mansour-Ghanaei *et al.*, 2002).

Worldwide, the annual incidence of human anthrax infection is estimated between 20,000 and 100,000 (Oncu *et al.*, 2003; Pile *et al.*, 1998). The vast majority are cutaneous anthrax. In the USA, less than one case is diagnosed per year, as compared to 127 cases/year diagnosed in the early 20th century (Oncu *et al.*, 2003; Pile *et al.*, 1998; Shafazand *et al.*, 1999). Occasionally, outbreaks of anthrax will occur as a result of breakdown in public health standards and practices or lack of public health services.

IV. PATHOGENESIS

A. Overview

Themes common among all anthrax infections are the following: (1) uptake by macrophages and other immune cells, (2) germination to the vegetative form at or near the site of inoculation prior to transit to target tissues, (3) time course of transport to target organs, (4) organs targeted for toxicity, (5) overwhelming septicemia, and (6) release of

soluble factors responsible for death. Two types of *B. anthracis* have been identified: a spore form and a vegetative form. Unless stated otherwise, the vegetative bacillus will be referred to as *B. anthracis* because this is the form that produces the deadly toxins; however, both forms will be discussed at length. The spore form is essential for uptake by host cells.

B. Uptake of Spores

Anthrax infection typically results from entry of *B. anthracis* spores into the host through a minor abrasion, insect bite, ingestion of contaminated meat, or inhalation of airborne spores. These routes of exposure lead to cutaneous, intestinal/oropharyngeal, and inhalational anthrax diseases (Figure 31.1). While *B. anthracis* is not categorized as an intracellular pathogen, it uses tissue macrophages as a sanctuary where the engulfed spores can germinate during the early phase of infection (Guidi-Rontani and Mock, 2002). Successful infection and eventual disease requires uptake of spores. While spores are resistant to phagosomal superoxide (Baillie *et al.*, 2005), they have evolved to recognize receptors contained on host phagocytic cells through their pathogen-associated molecular patterns (PAMPs). While most studies have shown uptake of the bacterium by macrophages, one study showed that human dendritic cells can be triggered to internalize *B. anthracis* spores (Brittingham *et al.*, 2005). This led to the hypothesis that these cells may take part in bacterial transport to the lymph nodes, similar to macrophages. Spore PAMPs are antigenic determinants containing highly conserved molecules which interact with cell-surface Toll-like receptors (TLRs) on tissue macrophages, dendritic cells, polymorphonuclear leukocytes (PMNs), and other cell types (Janeway and Medzhitov, 2002). PAMPs typically include lipopolysaccaride (LPS) from Gram-negative bacteria and lipoteichoic acid and peptidoglycan from the cell wall of Gram-positive bacteria such as *B. anthracis*. Anthrax spores can trigger a strong inflammatory response by activating TLR4 on antigen presenting cells of the immune system at the entry site into the host (Hsu *et al.*, 2004). In addition, anthrolysin O, a protein secreted by *B. anthracis*, is a potent agonist for TLR4 (Park *et al.*, 2004).

C. Uptake Via Lungs

Anthrax spores are approximately 1 to 2 μm in diameter, optimal for inhalation and deposition in the alveolar spaces (Brachman *et al.*, 1966; Penn and Klotz, 1997; Brachman, 1970, 1980). In the case of inhalational anthrax, inhaled spores reach the respiratory bronchioles and alveoli (Figure 31.2). While most spores are internalized rapidly into phagolysosomes by resident macrophages in the alveolar space (Guidi-Rontani *et al.*, 1999b; Ross, 1957), the exosporium layer of anthrax, discovered by Flügge (1886) prevents its degradation. It should be noted that the exosporium is not present on vegetative forms of anthrax bacteria and therefore only spores contain the antigenic

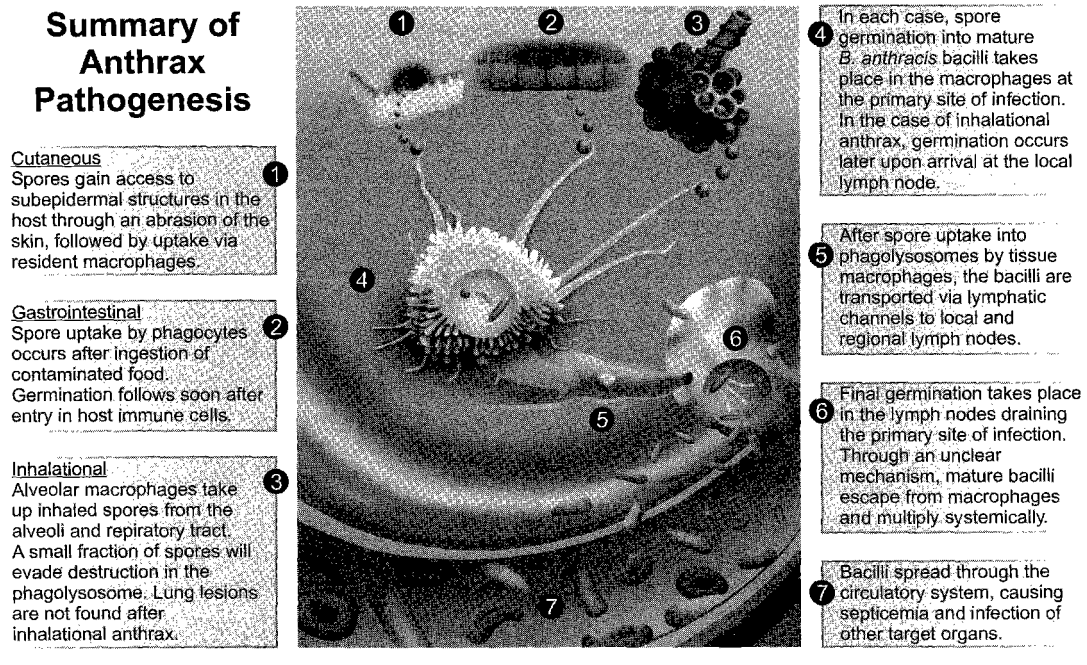


FIGURE 31.1. Overview of anthrax disease and pathogenesis. The steps of anthrax intoxication including spore uptake by macrophages, germination to the vegetative form, migration to lymph nodes, bacillus multiplication, release into the circulatory system, and septicemia are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

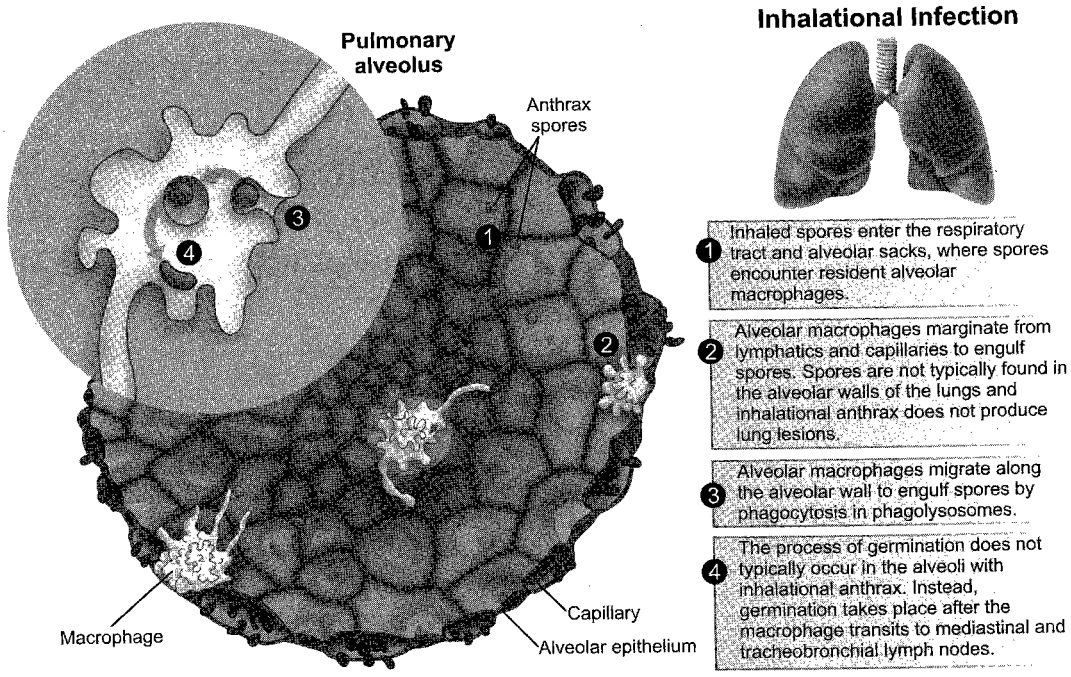


FIGURE 31.2. Spore uptake in inhalational anthrax disease. The steps involving spore deposition into a pulmonary alveolus, margination of alveolar macrophages out of capillaries into the alveolar space, spore uptake, and entry into lymphatic channels are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

determinants recognized by TLR4 receptors of immune cells. In any case, spores will escape destruction due to their resistance to superoxide and enzymes of the phagolysosome. Spore-bearing alveolar macrophages migrate along lymphatic channels to mediastinal, peribronchial, and tracheobronchial lymph nodes (Ross, 1957; Lincoln *et al.*, 1965), while germinating en route.

D. Uptake Via Skin

The cutaneous infection process initiates as a result of anthrax spores colonizing an abrasion in the skin (Figure 31.3). A small eruption or lesion develops into a painless, black eschar. Eschars usually develop within 2–5 days following exposure. During this stage of infection, low-level spore germination can occur at the primary site of infection, leading to localized edema and necrosis. While this infection often remains localized, some patients experience systemic symptoms.

Systemic disease, a rare secondary occurrence of cutaneous anthrax, is likely due to phagocytosis by macrophages in the dermis that marginate out of lymphatic channels and blood capillaries (Figure 31.3). In cutaneous anthrax, germination typically occurs immediately inside the host macrophage. Macrophages carrying *B. anthracis* cells migrate back into lymphatic ducts *en route* to regional lymph nodes draining the primary site of infection (Dixon *et al.*, 1999). The anthrax bacilli spread through the blood and lymph and proliferate to high concentrations, creating acute septicemia.

E. Uptake Via Gastrointestinal Route

The infection process of gastrointestinal anthrax starts with ingestion of spore-contaminated food/drink or ingestion of inhaled spores. In general, gastrointestinal anthrax is similar to cutaneous anthrax, but gastrointestinal anthrax occurs in the intestinal mucosa. As in cutaneous anthrax, the organisms probably invade the mucosa through a preexisting wound or lesion or possibly through interaction with a cell-surface receptor on epithelial cells. Presumably, the mucosal lining is the entry point for the endospores (Figure 31.4), but the exact germination location is yet unknown in cases of gastrointestinal anthrax infection. The bacteria may spread from the mucosal lesion to the lymphatic system by way of macrophages migrating to the primary site of infection. Germination can occur inside host macrophages after uptake prior to macrophage migration back to lymphatic channels draining to lymph nodes (Figure 31.4). Ulcer formation is a typical symptom during gastrointestinal anthrax, and ulcers may be present at various locations along the gastrointestinal tract from the oral cavity to the cecum. However, it is not known whether ulceration occurs only at sites of bacterial infection, or if it is caused by the anthrax toxins and therefore has a less restricted occurrence (Dixon *et al.*, 1999).

F. Spore Function

Several studies have examined the molecular mechanisms by which *B. anthracis* spores undergo phagocytosis,

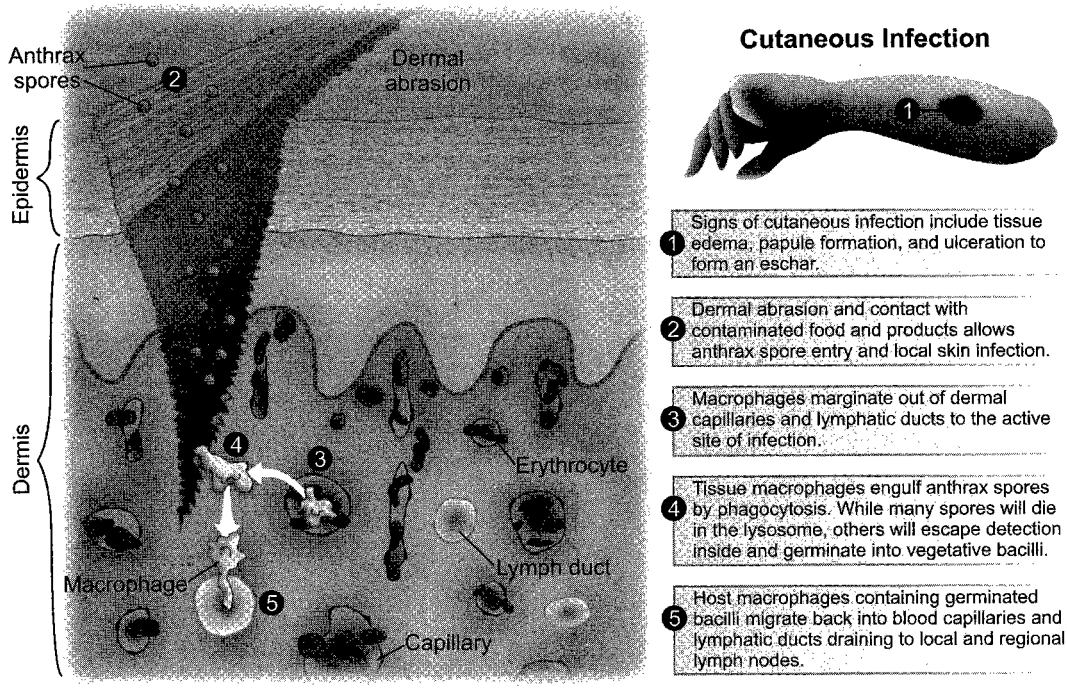


FIGURE 31.3. Spore uptake in cutaneous anthrax disease. The steps involving spore uptake at the primary site of local infection on the skin, germination inside the macrophage, and migration back into lymphatic ducts are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

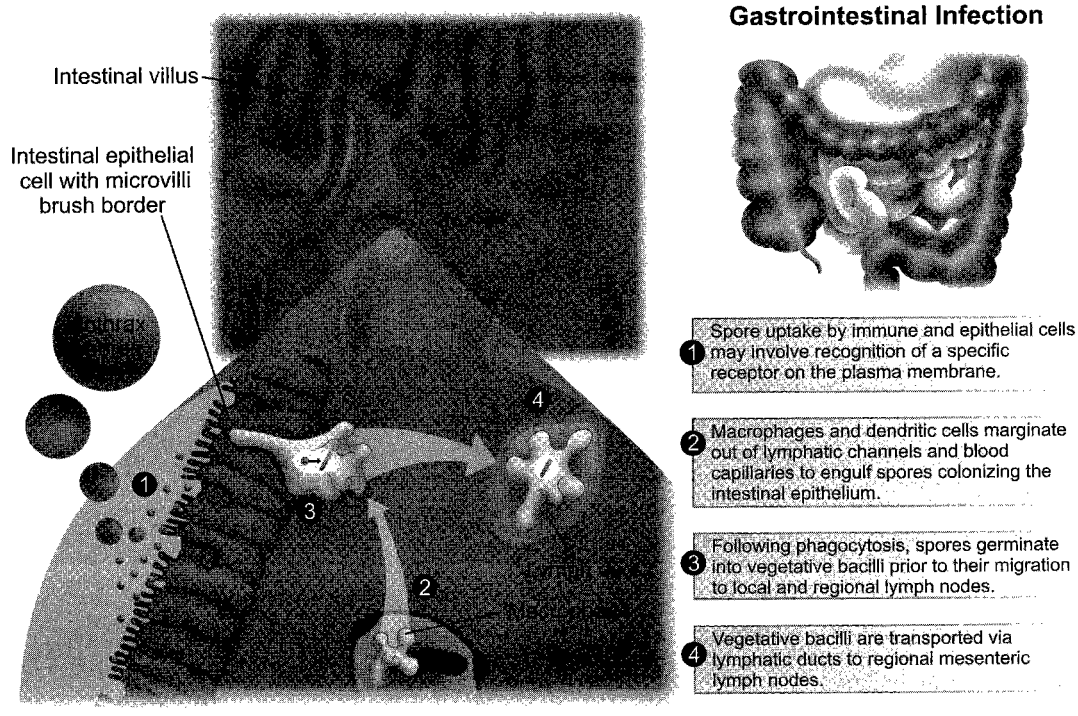


FIGURE 31.4. Spore uptake in gastrointestinal anthrax disease. The steps involving spore colonization of the gastrointestinal mucosa, uptake by phagocytes, germination, and lymphatic spread are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

germination, and subsequent escape to mediate systemic infection. Time-lapse microscopy suggested that the number of spores per macrophage can affect whether any spores are able to outlast the macrophage's inhibitory action of the bacteria. Macrophages that have engulfed a larger number of spores are more likely to have a few that survive to germinate and become vegetative bacilli (Ruthel *et al.*, 2004). Inside the macrophage, the spores must first avoid cellular nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation. This enzyme, which is present in the phagosomal membrane, initiates the reduction of oxygen to superoxide anion through NADPH oxidation. The superoxide anion is further converted to other reactive oxygen species (ROS), including hydrogen peroxide. To date, no *B. anthracis* enzymes have been discovered that are involved in the removal of ROS (Guidi-Rontani and Mock, 2002). After surviving the initial membrane oxidative burst, the anthrax spores must contend with the acid environment of the phagolysosome to further germinate and multiply. Several pathogens have shown an ability to evade phagolysosomal activity; however, the mechanism by which anthrax spores avoid hydrolysis in the phagolysosome is unclear (Guidi-Rontani and Mock, 2002).

Anthrax spores promote expression of interleukins (ILs) and other pro-inflammatory cytokines in macrophages and dendritic cells (Pickering and Merkel, 2004; Pickering *et al.*, 2004). Macrophages are important mediators of the inflammatory response, and produce tumor necrosis factor alpha (TNFα), interleukin-1β, and IL-6 in response to infection. While some spores will be killed by immune cells, many will evade the host cell detection only to initiate germination. The purpose of promoting inflammation here by anthrax spores is to increase their chance for internalization by tissue macrophages and dendritic cells, in order to germinate into the vegetative form of the bacteria (Welkos *et al.*, 1989).

An intact mitogen-activated protein kinase (MAPK) cascade and pro-inflammatory response by macrophages is also necessary for host cell migration to regional lymph nodes and recruitment of additional macrophages to the primary site of infection. Without functional immune cells, the toxin would not be able to enter lymphatics or the general circulation. In fact, dendritic cells which engulf *B. anthracis* spores change their pattern of chemokine-receptor expression. Specifically, they lose tissue-retaining receptors (CCR2 and CCR5) and up-regulate lymph node homing receptors (CCR7 and CD11c) (Brittingham *et al.*, 2005).

G. Time Course of Spore Germination

In cutaneous and gastrointestinal anthrax, spore germination takes place at or near the inoculation site following uptake by the macrophage or dendritic cell. It is the vegetative form, not the spore, which produces the deadly factors. After transformation to the vegetative bacilli, these bacteria are free to mediate toxicity at the inoculation site in skin and gastrointestinal tract (Beatty *et al.*, 2003). In inhalational anthrax, germination does not occur until the spores have been transported to the local lymphatics and mediastinal lymph nodes (Riedel, 2005). In the case of inhalational anthrax, spores are not believed to germinate at the site of infection in the alveoli or bronchioles. Instead, they germinate en route or after migrating to local nodes of the lymphatic system.

H. Spore Germination

While the anthrax endospore has no measurable metabolism, spore germination is a predictor of productive anthrax infection. Similar to endospores from other species, anthrax spores seem to represent a biologically inert organism with the ability to transform into one of the most lethal organisms on earth. Germination inside the host immune cell is the key step toward this transformation from benign bystander to active infection. The spores have little or no water, no ATP production, no macromolecular synthesis, and no active enzymes. Thus, endospores can remain stable in the environment under adverse conditions for decades. Once inside a host cell, spores start to germinate and initiate early synchronous *de novo* expression of genes vital for infection, as well as expression of genes necessary for vegetative growth (Hanna and Ireland, 1999; Oncul *et al.*, 2002).

Several putative germination proteins have been identified (Guidi-Rontani *et al.*, 1999a, b; Huang *et al.*, 2004), but their roles in spore germination have yet to be clarified. L-alanine appears to be essential in initiating germination (Foster and Johnstone, 1990), but the mechanism that leads to spore germination is unclear. A germinant receptor locus (*ger S*) essential to *B. anthracis* germination has been identified; without it, the organism could not germinate in macrophages (Ireland and Hanna, 2002). Once the spore has germinated, the vegetative bacilli are free to synthesize deadly bacterial toxins; however, the precise manner in which vegetative bacilli or spores break free from immune cells is still poorly understood. A recently proposed model suggests that an interaction between anthrax toxin from newly germinated spores inside the phagolysosome of macrophages and anthrax toxin receptor 2 promotes escape of the bacilli from the cells (Banks *et al.*, 2005).

I. Vegetative Anthrax and its Capsule

The role of the vegetative bacillus is in stark contrast to that of the spore form (Table 31.1). The vegetative form functions mainly to produce the deadly toxins lethal toxin (LT) and edema toxin (ET). Germination of *B. anthracis* within macrophages is closely followed by expression of the toxin genes (Guidi-Rontani *et al.*, 1999a). *Bacillus anthracis* contains no capsule *in vitro*, but capsule synthesis begins upon host infection (Preisz, 1909). Host signals, including carbon dioxide concentrations above 5%, are thought to induce transcription of capsule and toxin genes (Uchida *et al.*, 1997; Koehler *et al.*, 1994; Sirard *et al.*, 1994).

TABLE 31.1. Differences between spore and vegetative forms of *Bacillus anthracis*

Spore form	Vegetative form
• Contains an exosporium which promotes immune host detection and uptake	• Contains poly-D-glutamic acid capsule, essential for virulence and avoidance of immune detection
• Exosporium promotes internalization by macrophages	• Capsule avoids phagocytosis by macrophages
• Requires phagocytosis, internalization by macrophages, and germination to become pathogenic	• Survives as extracellular pathogen within the body of the host
• Does not produce anthrax toxins	• Produces lethal and edema toxins to kill the target host cell
• Requires target host cells to remain intact so they can circulate to local lymph nodes	• Kills the host cell
• Promotes TNF-α and cytokine production	• Inhibits TNF-α and cytokines
• Requires target host cells to remain intact so they can circulate to local lymph nodes	• Kills the host cell
• Enhances host inflammatory response	• Suppresses the immune system
• Can remain dormant for months	• Unable to survive outside the host for any appreciable length of time

In vitro studies by Ezzell and Abshire (1995) suggest that encapsulated vegetative cells appear within 30 min after germination *in vitro*. The capsule enables vegetative forms to survive as extracellular pathogens and avoid phagocytosis by macrophages (Guidi-Rontani and Mock, 2002). Indeed, *B. anthracis* isolates lacking capsules are significantly less virulent. Vegetative cells move through the bloodstream and lymphatics to cause systemic infection.

J. Systemic Infection and Septicemia

Significant numbers of bacilli in the blood are characteristic of the late stages of infection (Atlas, 2002; Shafazand *et al.*, 1999; Oncu *et al.*, 2003; Riedel, 2005). Without an activated immune response due to the release of soluble anthrax toxins which suppress immune function, *B. anthracis* is free to multiply to high concentrations. It is currently unclear whether *B. anthracis* multiplies within the macrophage as some studies suggest (Shafa *et al.*, 1966) or the bloodstream. Cultured blood from infected rabbits and guinea pigs suggest a continuous rise in bacterial counts until the final hours of life; numbers increase sharply to 10^7 and 10^9 organisms per milliliter of blood for rabbits (Bloom *et al.*, 1947) and guinea pigs (Smith and Keppie, 1954; Keppie *et al.*, 1955), respectively. Death was shown to be dependent on bacteremia, but death was not shown to be a consequence of mechanical obstruction by large bacterial counts. Regardless of the route of infection, systemic spread results in similar colonization. The lungs and gastrointestinal tract are particular targets of system infection by mature bacilli, with the spleen, brain, liver, and almost any other organ being colonized within hours or days (Riedel, 2005). *Bacillus anthracis* has been isolated from stool specimens of both animals and humans, reflecting its presence in the bowel (Beatty *et al.*, 2003). While septicemia is important, death is the direct result of soluble factors (discussed below) secreted by the vegetative bacilli which weaken the immune response and initiate cell death.

K. Anthrax Infection Cycle

An infection cycle for *B. anthracis* has been proposed by Hanna and colleagues (Hanna and Ireland, 1999). Phagocytosis of spores by macrophages, germination, vegetative cell proliferation, and toxin release occur within hours after exposure to *B. anthracis* in a productive infection. During the middle stages of infection, the bacilli grow extracellularly and express toxins and other virulence factors. During the final stages of infection, the bacilli, having depleted nutrients from the host, form endospores, which return to the environment for the next cycle of infection (Hanna and Ireland, 1999; Oncul *et al.*, 2002). The precise sequence of events leading to successful establishment of infection is not completely understood. After germination, anthrax bacilli multiply in the lymph nodes, causing an immediate lymphadenitis, hemorrhagic mediastinitis, and spread throughout the body via the circulatory system (Dutz and Kohout, 1971; Albrink, 1961).

L. Release of Soluble Factors

Bacillus anthracis possesses four known virulence factors, including an antiphagocytic capsule of the vegetative form, lethal factor (LF, 90 kDa), edema factor (EF, 89 kDa), and a protective antigen (PA, 83 kDa). These virulence factors acting together play a key role in pathogenesis but are not toxic when present individually. In combination, PA, LF, and EF induce a dangerous cascade of events upon cell entry. As early as 1953, Smith and colleagues implicated toxic factors as the cause of death from anthrax infection (Smith *et al.*, 1953, 1955; Keppie *et al.*, 1953, 1955). It has since been shown that these three factors combine to form the two protein exotoxins of anthrax LT and ET.

PA binds to an as yet ill-defined cell receptor/lipid raft, mediating the entry of the other two components inside the cytoplasm via the endosomal pathway. Edema factor is a Ca^{2+}/calmodulin-dependent adenylate cyclase involved in producing a generalized accumulation of fluid into the interstitium, characteristic of anthrax. Lethal factor is a proteolytic enzyme involved in the inactivation of MAPK kinases (MAPKK), key players in secondary messenger signal transduction cascades. Both toxins are translocated into the cytosol of target cells by way of endosomes through a pore formed from a heptamer of PA molecules. They interfere with vital cellular responses to bacterial infection, disabling host immunity and promoting bacterial dissemination. As the disease progresses, the toxins accumulate to higher levels, causing respiratory distress, shock, widespread hemorrhage, and death.

V. TOXICOKINETICS

A. Inhalational Anthrax

To date, mice, rats, guinea pigs, rabbits, nonhuman primates, dogs, swine, and sheep have been used in inhalational animal model studies for *B. anthracis*. Routes of administration have included aerosol, intranasal, and intratracheal methods. In addition, various animal species differ in their natural resistance to infection (Welkos and Friedlander, 1988). Some of the most common findings in human inhalational anthrax concern the respiratory tract. A majority of patients experience mediastinal widening (70%), pulmonary infiltrates (70%), and pleural effusion (80%). These signs demarcate inhalational anthrax cases from influenza-like illnesses (Oncu *et al.*, 2003). Pathological findings from inhalational anthrax patients in a bioterrorism-related outbreak cited hemorrhage and necrosis in mediastinal lymph nodes, hemorrhage or inflammation of the pleurae and interhilar septae, and prominent intra-alveolar macrophages or inflammation in the lung parenchyma. There were no skin lesions. Bacilli were located in the lung, mediastinal tissues, and thoracic tissues, as identified by staining (Guarner *et al.*, 2003).

Early studies with mice were unable to identify the vegetative form of *B. anthracis* in the alveolar walls of the lungs. Aerosol exposure was achieved by exposing mice (mouse strain unreported) to clouds of spores (Albrink, 1961). Other aerosol studies involving mouse models indicated evidence of the spore form in lung sections but not the vegetative form (Young *et al.*, 1946; Barnes, 1947). The Barnes study highlighted the fact that not all of an aerosolized dose ends up in the lungs; spores can be swallowed to pass through or infect the alimentary tract.

Intratracheal administration of anthrax has demonstrated a respiratory lesion in mice (Lyons *et al.*, 2004). Inoculation of the Ames strain (5,000 vs 10,000 vs 50,000 spores) in BALB/c mice does not produce signs of early pulmonary lesions. Significant numbers of anthrax colony forming units were found in lung-associated lymph nodes harvested 5 h post-inoculation in a dose-dependent manner (Lyons *et al*, 2004). Airway damage did occur after 24 h post-inoculation with all anthrax doses; signs of toxicity include widespread edema vacuole formation, degeneration, airway epithelial cell sloughing, and necrosis. After 48 h, bacterial rods (vegetative form) were located within alveolar capillaries, suggesting active bacterial uptake from the respiratory tract.

Drysdale *et al.* (2005) investigated the role of *B. anthracis* capsule in uptake from the respiratory tract. These investigators deleted the capsule operon *capBCAD* in mutant strains of *B. anthracis* and inoculated female BALB/c mice intratracheally with ~4×10^4 mutant spores. All capsule-deficient strains germinated in the lungs (Drysdale *et al.*, 2005), indicating no role for the capsule at this particular stage in pathogenesis. This contradicts the accepted hypothesis that germination takes place in the lymph nodes for inhalational anthrax exposure.

In the only intranasal mouse model, Guidi-Rontani *et al.* (1999b) demonstrated uptake of *B. anthracis* by alveolar macrophages. A dose of 2.5×10^7 Sterne strain spores was given to Balb/c mice. Bronchial alveolar lavage (BAL) fluids were subsequently collected at 1, 3, and 24 h post-inoculation. Germination of spores in the fluid was assessed by exposing the material to heat (65°C) for 30 min. Ungerminated spores are resistant to this treatment. Germination was significantly evident in the alveolar macrophage fraction of BAL fluid by 24 h post-inoculation. Therefore, the uptake of bacteria by alveolar macrophages occurs rapidly in this system (Guidi-Rontani *et al.*, 1999b).

To date, evidence of respiratory lesions in humans does not exist. Spore cloud exposure studies by Ross (1957) determined the pathogenicity of anthrax in guinea pigs. Guinea pigs were exposed to high numbers of M.36 strain spores for 20 min and sacrificed at various time points to assess spore location. After 1 h, spores were indeed found in alveolar macrophages. Free spores were not, however, isolated from surrounding lymph nodes at this early time point. However, after 18–24 h, bacilli were observed throughout the lymphatic system. The results of these studies led to the idea that spore germination takes place in regional lymphatic nodes (i.e. tracheobronchial nodes) before spreading to the rest of the body via the blood circulatory system (Ross, 1957).

In a guinea pig/aerosol model, 6×10^5 spores of the vaccine anthrax strain STI were detected in the lungs 1 h after infection from a dose of 2.43×10^6 spores. *Bacillus anthracis* was not detectable in the tracheobronchial lymph nodes until 2 days post-infection. On day 36 post-infection, lung levels were down to 10^3 spores and tracheobronchial lymph nodes were again negative.

B. Cutaneous Anthrax

Dermal *B. anthracis* pathogenesis has been studied in mice, rats, hamsters, rabbits, guinea pigs, nonhuman primates, and

dogs. Several methods of inoculation have been tested, including subcutaneous, intradermal, epicutaneous, footpad, and scarification. Data on uptake/absorption from the skin, persistence in the circulation, transit to target tissues, and lethality will be discussed. The only animal models that have been used in bacterial uptake studies following dermal inoculation to date are the mouse and rabbit.

An extensive study on the interaction of *B. anthracis* with mouse skin following epicutaneous inoculation was conducted by Hahn *et al.* (2005). C57BL/6 and DBA/2 mice were inoculated with the Sterne strain. Epicutaneous inoculations were performed by applying an inoculum of 10^7 spores onto the shaved, tape-stripped, or abraded area of the mouse. A difference in foci development was observed, according to skin treatment prior to inoculation. Shaved-only inoculation sites did not have foci of vegetative bacilli. In contrast, abraded inoculation sites had readily apparent foci. Germination and proliferation occurred at the skin surface and in the epidermis and hair follicles. Hair follicles had deeper foci of infection, >200 μm below the skin surface. In animals inoculated with 2×10^8 spores onto unshaved skin, foci appeared only in the hair follicles and not in the epidermis or dermis (Hahn *et al.*, 2005). According to reviews of human infection, germination and proliferation also occur, to some degree, at the site of inoculation (Cranmer and Martinez, 2001). Proliferation also occurs in the draining lymph nodes near the site of inoculation (Anon, 2000). Zaucha *et al.* (1998) subcutaneously inoculated New Zealand white rabbits with 43 to 1.56×10^5 CFU of the Ames strain of *B. anthracis*. At the inoculation site, the main sign of bacterial uptake involved dermal and subcutaneous edema, signs observed in human cutaneous anthrax.

C. Gastrointestinal Anthrax

Despite the rarity in documented human cases of gastrointestinal anthrax (Beatty *et al.*, 2003), this form is common in underdeveloped areas of the world where infected carcasses are consumed (Sirisanthana and Brown, 2002). Gastrointestinal anthrax infection carries a 25–60% mortality rate (Mansour-Ghansei *et al.*, 2002). Gastrointestinal anthrax can be divided into intestinal and oropharyngeal forms. In the intestinal form, following an incubation period of 1–7 days, there is severe abdominal pain, hematemesis, melena and/or hematochezia, ascites and watery diarrhea. Intestinal anthrax carries a greater risk of mortality. In contrast, the milder, oropharyngeal form can be contracted following consumption of infected cattle and water buffalo. In this form, there is marked neck edema and ulcerative lesions in the oropharynx. Clinical signs include nausea, loss of appetite, emesis, and fever. In one natural outbreak of oropharyngeal anthrax, only three out of 24 patients died (Anon, 2000; Pile *et al.*, 1998).

Limited data regarding the pathogenesis of gastrointestinal *B. antracis* infection in any animal model system are available. Barnes (1947) observed that 1 h following inhalational exposure to anthrax spores, the majority of spores were found in the stomach. Therefore, regardless of the route of exposure, there is a high risk of spores transiting to the gastrointestinal tract. Unfortunately, further pathophysiology was absent from the study.

The guinea pig is the only animal model to date for which there are data following oral exposure. There is a need for more oral animal models of *B. anthracis* infection. Gastrointestinal anthrax is perhaps far underdiagnosed in humans; however, it remains an important disease due to the significant risk of ingesting spores after exposure to inhalation anthrax. Since there are limited data regarding pathogenesis of gastrointestinal anthrax in humans, evaluation of future animal models will be difficult. Stability of the organism as a function of pH in the various compartments of the gastrointestinal tract, method of inoculation, presence of stabilizers, and gastrointestinal physiology will become important factors to analyze for oral anthrax.

Aloni-Grinstein *et al.* (2005) assessed the stability of spores and vegetative cells in the gastric fluid and gastrointestinal tract. Female Hartley guinea pigs were given 5×10^9 spores or 5×10^8 vegetative cells of the anthrax MASC-13 variety *per os*. This vaccine strain is nontoxigenic, devoid of capsule, and lacking a nonfunctional form of PA. Bacteria in feces and gastric fluid were charted according to days post-ingestion. Gastric fluid incubations were conducted at 37°C with fluid taken from the guinea pig stomach. Anthrax spores exhibited much greater stability to the harsh environment created by the gastric mucosa than their alternate form. Vegetative cells were barely detectable on day 1 post-ingestion in feces and gastric fluid. There are no animal models or human studies that have examined bacterial persistence in circulation and transit to target tissues following oral exposure to *B. anthracis*. Similarly, there are no animal models that examine oral lethality, clinical signs, or epidemiology following oral exposure.

VI. MECHANISM OF TOXICITY

The significance of the capsule in virulence was demonstrated early last century when anthrax strains lacking a capsule were shown to be avirulent (Bail and Weil, 1911; Bail, cited by Sterne, 1959). The genes encoding synthesis of the capsule were found to be encoded on a 110-kilobase (kb) plasmid. Anthrax strains lacking the plasmid no longer produced the capsule and were attenuated (Ivins *et al.*, 1986), confirming the role of the capsule in pathogenesis.

The capsule contains a polymer of poly-D-glutamic acid, conferring resistance to phagocytosis by macrophages (Keppie *et al.*, 1963). This linear polymer of the capsule is weakly immunogenic (Goodman and Nitecki, 1967). The negatively charged capsule enables the bacterium to inhibit phagocytosis of bacilli by macrophages (Tomcsik and Szongott, 1933; Sterne, 1937; Keppie *et al.*, 1963; Ezzell

and Welkos, 1999). In conjunction with LF and EF, whose target cells include macrophages, dendritic cells, and other immune cells, the capsule allows *B. anthracis* to grow virtually unimpeded in the infected host. The capsule functions as a "one-way" filter, allowing bacilli to diffuse the other three virulence factors through the capsule, resulting in host cell intoxication without causing self-harm.

A. Protective Antigen

Bacillus anthracis secretes three plasmid-encoded soluble toxin proteins collectively referred to as anthrax toxin; these are PA, LF, and EF. LF and EF function individually and in combination as catalytic enzymes in susceptible host cells. In an unusual twist of nature, they both have evolved to share PA as a common receptor binding moiety for translocation into the cytosol of the host. PA is a dominant component of the three-part protein toxin secreted by *B. anthracis* (Liddington *et al.*, 1999; Petosa *et al.*, 1997). The mature form of PA, a secreted 735 amino acid protein, has a molecular weight of 83 kDa. The ribbon structure is illustrated in Figure 31.5 along with a detailed description of its functional domains. PA forms a membrane-insertion heptamer that translocates other toxic enzymes (EF and LF) into the cytosol of host cells.

The mechanisms by which *B. anthracis* toxins work at the cellular level are illustrated in Figure 31.6 and are described elsewhere in great detail (Mock and Fouet, 2001; Duesbery and Vande Woude, 1999). In this model, PA released from the vegetative form of the bacilli binds to cell surface receptors, namely tumor endothelium marker (TEM) 8 and capillary morphogenesis protein (CMG) 2, which are expressed as different isoforms by many cell types, including immune cells (Collier and Young, 2003; Bradley *et al.*, 2001; Liu and Leppla, 2003; Scobie *et al.*, 2003; Baldari *et al.*, 2006). Upon binding to the receptor, PA is cleaved into a 20 kDa C-terminal domain (PA_{20}) and a 63 kDa fragment (PA_{63}) by furin or similar host cell surface-associated protease (Klimpel *et al.*, 1992; Molloy *et al.*, 1992). The PA_{20} fragment is released, resulting in spontaneous oligomerization of truncated PA (PA_{63}) into heptamers that bind to EF and LF. It was once thought that a PA heptamer complex may bind up to seven molecules of LF and/or EF (Beauregard *et al.*, 2000; Duesbery *et al.*,

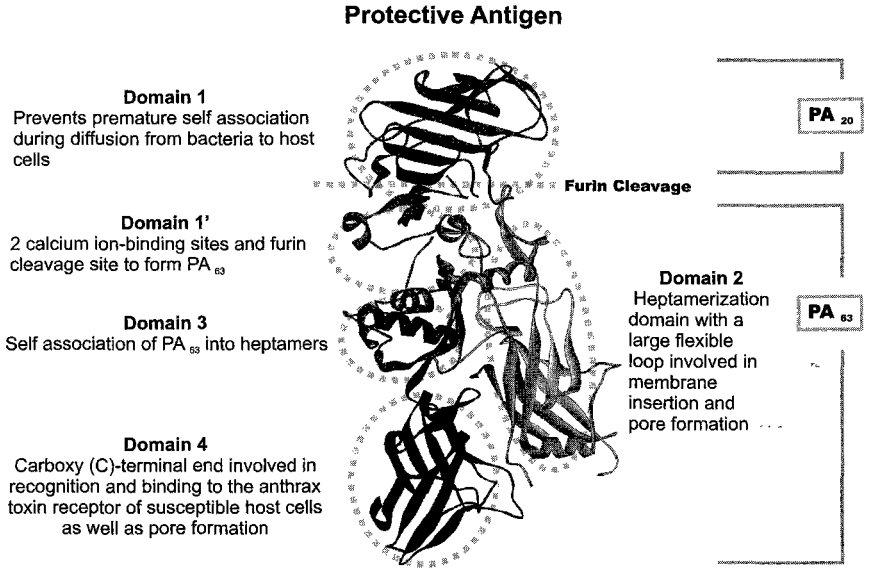


FIGURE 31.5. Three-dimensional ribbon structure of protective antigen (PA). PA, an 83 kDa protein with 735 residues, is organized into antiparallel β-sheets comprising four major domains (Petosa *et al.*, 1997). Domain 1 contains the first 258 residues and the furin enzyme cleavage site between residues 164 and 167. Prior to translocation of soluble factors into the host cell, PA is cleaved by a cell-surface protease (furin enzyme) into PA20 (20 kDa portion containing residues 1–167) and PA63. PA20 is responsible for maintaining PA as a soluble monomer and preventing premature self-association (Collier and Young, 2003). Domain 1′ (residues 168–258) designates the N-terminal end of PA63. This domain contains two charged calcium (Ca^{2+}) atoms which function to maintain PA63 in a conformation capable of self-association into heptamers and binding to the host ligand anthrax toxin receptor (Gao-Sheridan *et al.*, 2003, Petosa *et al.*, 1997; Collier and Young, 2003. Domain 2, comprising residues 259–487, is illustrated with a β-barrel core structure and large flexible loops. This structure enables membrane insertion and pore formation (Petosa *et al.*, 1997; Benson *et al.*, 1998). Domain 3, comprising residues 488–595, is involved in self-association of PA63 into heptamers. Domain 4 contains the remaining C-terminal end of PA63 (residues 596–735) and binds to the anthrax toxin receptor on the host cell membrane (Singh *et al.*, 1991; Collier and Young, 2003). The various domains of PA63 enable the protein anchor to the plasma membrane of the host, form heptamers, bind EF or LF, and translocate these soluble factors into the cytosol through a pore. The structure of PA was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Berman *et al.*, 2000; PDB ID: 1acc; Petosa *et al.*, 1997) and rendered using Accelrys DS Visualizer 2.0 software.

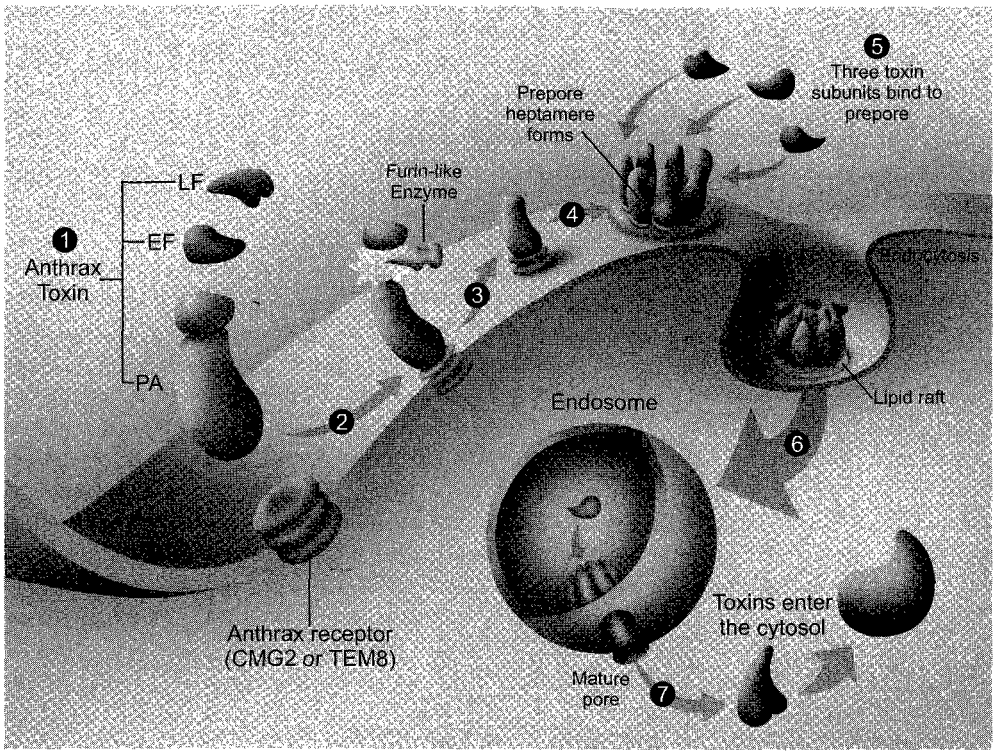


FIGURE 31.6. Anthrax toxin entry. Cellular entry of LF and EF into susceptible host cells involves an unusual mechanism for sharing PA. Binding of PA to the host cell, cleavage to form PA63, heptamerization of PA in the plasma membrane, organization into lipid rafts, binding of EF and LF to PA, internalization, PA pore formation, and translocation of anthrax soluble factors across the vesicle membrane are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

1998; Mock and Fuet, 2001); however, evidence suggests that LF or EF binding sites on the PA_{63} heptamer span the interface between adjacent PA_{67} subunits. Therefore, it is believed that a single heptamer can interact with a maximum of three EF or LF molecules (Baldari *et al.*, 2006). The heptamer PA-LF and PA-EF complexes enter lipid rafts, illustrated as a shaded disc in Figure 31.6, in the membrane. Binding and oligomerization trigger a receptor-mediated endocytotic event followed by internalization of the hetero-oligomeric toxin complex in a membrane-bound vesicle (Beauregard *et al.*, 2000) via clathrin-dependent, receptor-mediated endocytosis. The internalized vesicle becomes an acidic endosome. The acidic pH of the endosome triggers a conformational change in the complex, leading to insertion of a flexible loop of each PA molecule into the lipid bilayer to form a pore. Formation of the pore allows for translocation of LF and EF out of the late endosome and into the cytoplasm of the host cell. Once inside the cytoplasm, LF and EF reach their respective targets.

B. Structure and Activity: Edema Factor

EF (89 kDa) is a calmodulin (CaM)-dependent adenylate cyclase, increasing intracellular cyclic adenosine monophosphate (cAMP) levels in the infected host cell cytosol. The first 261 N-terminal residues of EF are responsible for CaM binding (Duesbery and Vande Woude, 1999; Labruyere, 1990). The catalytic domain resides in amino acids 265–570 of the EF peptide sequence (Betsou *et al.*, 1995; Escuyer *et al.*, 1988). The ribbon structure of EF is illustrated in Figure 31.7. The N-terminal sequence of EF is highly conserved to a similar region in LF; this domain is essential for binding to PA. Fusion of this highly conserved N-terminal sequence to other toxins, such as Shiga and diphtheria, can cause toxic effects in mammalian cells (Arora and Leppla, 1994). Edema toxin (ET), the combination of PA and EF, causes edema when injected into the skin of experimental animals (Stanley and Smith, 1961; Beall *et al.*, 1962). Injection of EF alone, in contrast, has no toxic activity.

C. Structure and Activity: Lethal Factor

LF has a molecular weight of 90 kDA and is one of the key agents of anthrax disease. The structure of LF is illustrated in Figure 31.8. LF consists of several domains, including a PA-binding domain, a Zn^{2+}-binding domain, an imperfect repeat region, and a catalytic domain. LF functions as a highly specific Zn^{2+}-binding metalloprotease that can cleave MAPKKs near their amino (N)-termini. This cleavage reaction can potentially inactivate one or more host cellular signaling pathways (Pannifer *et al.*, 2001). Similar to EF, the LF N-terminal residues (1–254) serve as the binding domain for PA (Arora and Leppla, 1994). Adjacent to the PA-binding domain is a region of imperfect repeats containing 19 amino acids each. This region was demonstrated to be essential for toxic activity (Arora and Leppla, 1993). Deletion of the first of the four imperfect repeats of residues 308–383 eliminated LF toxicity. The LF catalytic domain resides in the C-terminus where a zinc-metalloprotease consensus sequence has been identified

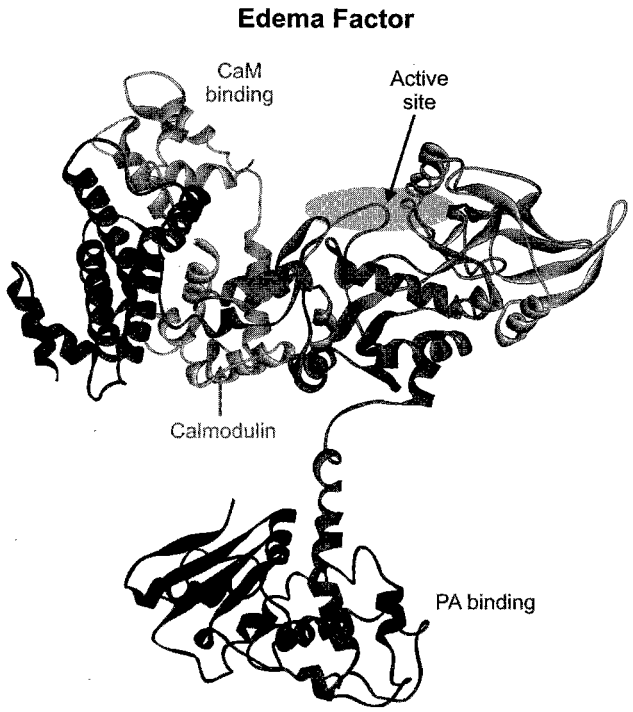


FIGURE 31.7. Three-dimensional ribbon structure of edema factor (EF). The purified and crystallized structure of EF has been previously reported (Shen *et al.*, 2005). EF is a calmodulin (CaM)-activated adenylyl cyclase and is another key factor in anthrax pathogenesis. EF contains a finger-like projection comprising a CaM-binding region, an active site to bind Mg^{2+} and 3′ deoxy-ATP, and a separate PA63 binding region. The structure of EF with bound CaM is illustrated. CaM binding causes a conformational change and activation of EF. The PA63 binding region of EF is separate from the remainder of the protein. It is shown in the same approximate position as the homologous PA-binding domain of LF in Figure 31.8 for comparison. The structure of EF was provided free of copyright restrictions from the RCSB PDB (Berman *et al.*, 2000; PDB ID: 1xfv; Shen *et al.*, 2005) and rendered using Accelrys DS Visualizer 2.0 software.

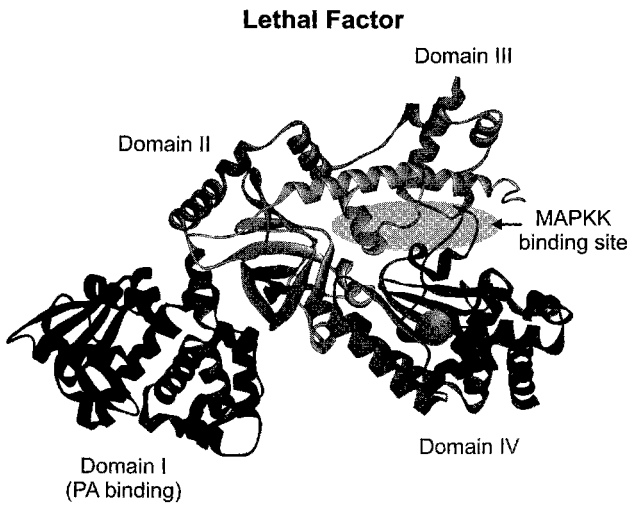


FIGURE 31.8. Three-dimensional ribbon structure of lethal factor (LF). The purified and crystallized LF has been reported (Bernardi *et al.*, 2000; Pannifer *et al.*, 2001). LF contains four recognized regions, termed domains I–IV. Residues 28–263 comprising domain I are illustrated as being entirely separate from the rest of the protein. Domain I binds the membrane-translocation component of PA63 (Lacy *et al.*, 2002). It is homologous to the same PA binding domain of edema factor (EF). Domains II–IV function together to create a long, deep pocket that holds the 16-residue N-terminal tail of MAPKK before the cleavage reaction takes place. Members of the MAPKK proteins are the only known cellular substrates of LF. The cleavage reaction removes the docking sequence for the downstream mitogen-activated protein kinase (MAPK) and therefore blocks cellular signaling via the MAPK pathway. Domain II has an ADP-ribosyltransferase active site. Domain III contains an α-helical bundle, and domain IV contains both a Zn^{2+}-binding motif and a catalytic center of the protease. The structure of LF was provided free of copyright restrictions from the RCSB PDB (Berman *et al.*, 2000; PDB ID: 1jky; Pannifer *et al.*, 2001) and rendered using Accelrys DS Visualizer 2.0 software.

within residues 686–692 (Klimpel *et al.*, 1994). The zinc metalloprotease activity is responsible for the cytotoxicity of LF. LF binds Zn^{2+} (Klimpel *et al.*, 1994; Kochi *et al.*, 1994), and mutations in LF which decrease zinc binding are poorly cytotoxic to cultured cells (Brossier *et al.*, 2000; Klimpel *et al.*, 1994).

LF interferes in the MAPK pathway which relays environmental signals to the machinery required for transcription in the nucleus and therefore modulates gene expression and protein synthesis. Specifically, LF inhibits MAPKKs; the identified substrates for LF enzymatic activity are MAPKK1, MAPKK2, MAPKK3 (Duesbery *et al.*, 1998; Pellizzari *et al.*, 1999; Vitale *et al.*, 1998), MAPKK4, MAPKK6, and MAPKK7 (Vitale *et al.*, 2000); MAPKK5 has never been demonstrated to be cleaved by LF. MAPKK cleavage occurs within the N-terminal proline-rich region preceding the kinase domain, subsequently inhibiting protein–protein interactions essential for assembly of host cell signaling complexes (Hammond and Hanna, 1998).

D. Mechanism of Toxicity: Edema Toxin

Edema toxin does not produce major tissue damage. In fact, its major role is to impair phagocyte function (Leppla, 2000). This is consistent with other toxins which function to elevate cAMP concentrations. Edema toxin inhibits phagocytosis of spores by human PMNs (O'Brien *et al.*, 1985) similar to LF; this is in contrast to spores which promote immune cell uptake (see Table 31.1). Increased intracellular cAMP induced by EF inhibits neutrophil chemotaxis, phagocytosis, superoxide production, and microbicidal activity (Crawford *et al.*, 2006; Turk, 2007; O'Brien *et al.*, 1985; Friedman *et al.*, 1987; O'Dowd *et al.*, 2004; Ahmed *et al.*, 1995). EF has been shown to inhibit TNFα and increase IL-6 production (Hoover *et al.*, 1994). Increased cAMP levels also block LPS-induced activation

of extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) MAPK pathways in monocytes through protein kinase A (PKA) activation. This might explain suppression of cytokine production by EF (Willis and Nisen, 1996; Dziarski *et al.*, 1996; Delgado and Ganea, 2000). EF also activates guanine nucleotide-exchange proteins directly activated by cAMP 1, known as Epac1, through elevated cAMP levels. Epac1 inhibits phagocytosis by both alveolar macrophages and monocyte-derived macrophages (Aronoff *et al.*, 2005; Bryn *et al.*, 2006), EF also inhibits T-cell proliferation and subsequent cytokine production through inhibition of ERK and JNK MAPK pathways (Comer *et al.*, 2005). Therefore, EF impairs the adaptive immune response as well. EF affects other cell types; it causes endothelial cell barrier dysregulation, coagulopathy, and RBC death (Banks *et al.*, 2006).

E. Mechanism of Toxicity: Lethal Toxin

The mechanism of death accredited to lethal toxin is unclear. A summary of the cellular targets and effects of lethal and edema toxins is provided in Table 31.2. LF has similar actions on phagocytes; it disables phagocytosis by macrophages and dendritic cells. Once internalized, LF cleaves the N-termini of MAPKKs, kinases for MAPKs and ERKs, and MEKs (MAPKs and ERKs) (Turk, 2007; Collier and Young, 2003). As a result, LF blocks three critical cell signaling pathways downstream of MAPKKs and MEKs. These include the ERK1/2, JNK, and p38 MAPK pathways. In addition, LF has additional targets within host cells.

There is evidence that LT suppresses pro-inflammatory cytokine production in macrophages (Erwin *et al.*, 2001) and decreases TNFα (Pellizzari *et al.*, 1999). These reports, which suggest impairment of innate immunity in the host cell, are in contrast to those which suggest LT functions to increase cytokines by macrophages (Hanna *et al.*, 1993). LT has also been shown to cause an increase in ion permeability and rapid depletion of ATP in J774 macrophage-like cells, leading to cell lysis by osmotic mechanisms (Hanna *et al.*, 1992). Inhibition of MAPKKs may block induction of NF-κB target genes, causing apoptosis of activated macrophages (Park *et al.*, 2002).

LF effects on monocytes and macrophages are multiple. LF seems to decrease innate immune responses by blocking maturation of monocytes, which differentiate into both macrophages and dendritic cells, and promote death of activated macrophages (Banks *et al.*, 2006). LF causes reductions in pro-inflammatory cytokines and inhibits the ability of dendritic cells to activate T cells *in vivo* (Agrawal *et al.*, 2003). This negates an important mechanism of inducing adaptive immunity. Alileche *et al.* (2005) demonstrated that LF can be cytotoxic to human and murine dendritic cells. Cytotoxicity was demonstrated to be through either a caspase-dependent apoptotic mechanism in the case of human dendritic cells or a necrotic pathway. LF alters PMNs, important mediators of the adaptive and innate immune response. LT also slows neutrophil mobility (During *et al.*, 2005).

LF promotes lysis of red blood cells (RBCs) when PMNs are present (Banks *et al.*, 2006). It is believed that LT induces these immune cells to release toxic factors that are hemolytic, causing RBC lysis (Wu *et al.*, 2003). Vascular damage is pathognomonic of anthrax disease. Signs of endothelial damage include hemorrhages and leaky blood vessels, leading to vascular collapse, shock, and death. LF appears to induce a caspase-dependent apoptotic pathway in endothelial cells derived from large vessels or so-called human umbilical vein endothelial cells; LF has been shown to cause similar apoptosis in small vessels, namely neonatal dermal vascular endothelial cells (Kirby, 2004).

F. Interactions Between Lethal and Edema Toxins

Since their expression is coordinately regulated, both anthrax toxins circulate together during infection (Turk, 2007). These toxins have distinct mechanisms of toxicity, yet they target similar cell types. Both LF and EF cause endothelial cell barrier dysregulation, coagulopathy, RBC death, inhibition of neutrophil mobility and phagocytosis, and alterations in cytokine modulation. Acting together, EF and LF inhibit superoxide production by neutrophils and cytokine production by dendritic cells (Crawford *et al.*, 2006; Tournier *et al.*, 2005). EF up-regulates PA receptors, TEM 8 and CMG 2, in macrophages and increases their sensitivity to LF cytotoxicity (Comer *et al.*, 2005; Maldonado-Arocho *et al.*, 2006). Finally, the appearance of black pigmentation characteristic of anthrax disease might be explained by the combined effects of LF and EF on melanocytes. LF has been shown to produce melanin in melanoma cell lines, an effect enhanced by the addition of EF (Koo *et al.*, 2002).

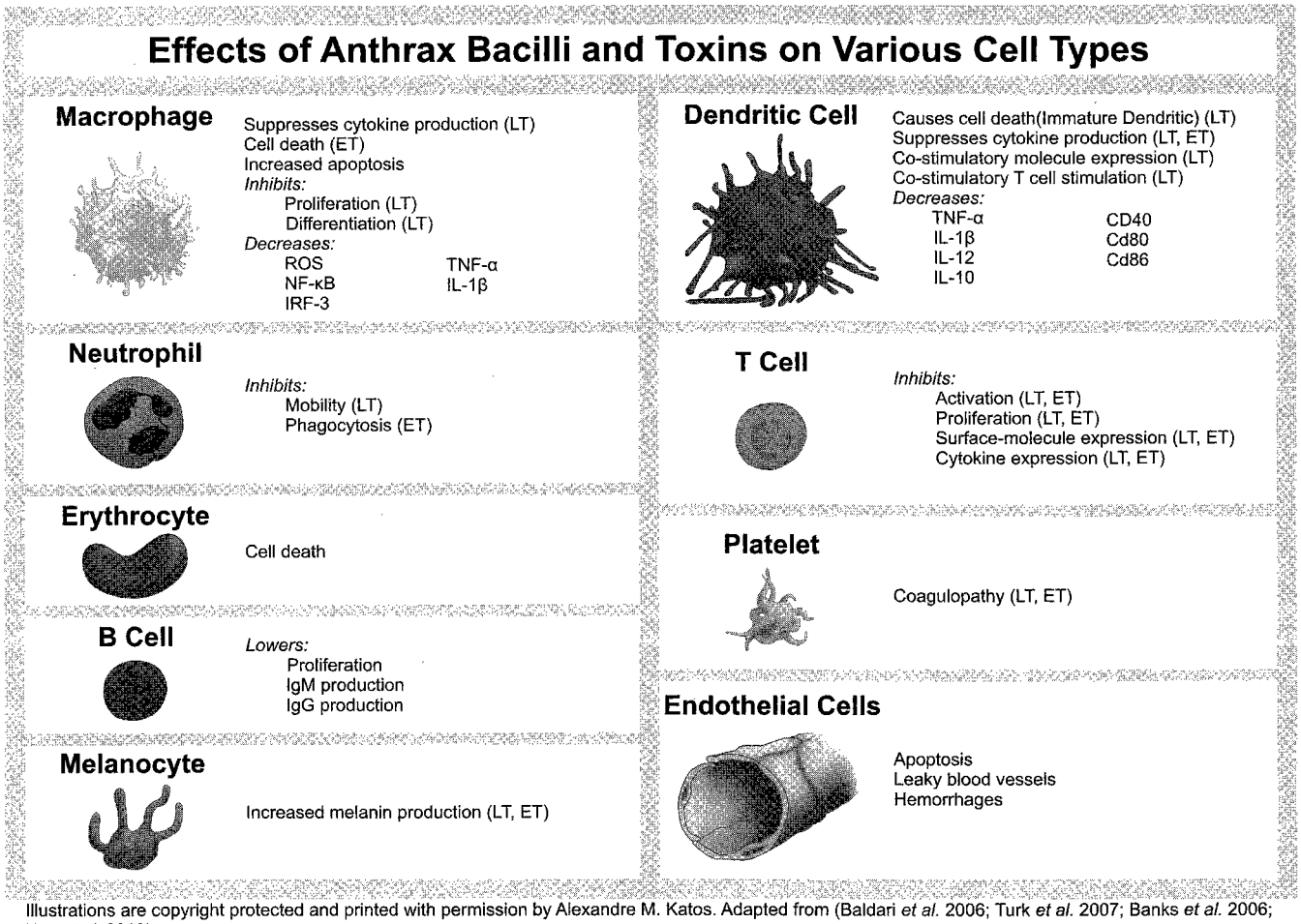
VII. TOXICITY

A. Cutaneous Anthrax

Cutaneous anthrax is the most common form of anthrax encountered worldwide, consisting of >95% of anthrax cases. The most obvious sign of bacterial uptake/absorption from the skin in human anthrax cases is the developing papule. This papule progresses to become vesicular in nature and then ulcerative. Finally, a black eschar forms, which is the hallmark of human cutaneous anthrax infection (Bell *et al.*, 2002). A more detailed look at the lesion discloses subepidermal edema, vessel thrombosis, tissue destruction, and hemorrhagic interstitium (Oncu *et al.*, 2003).

Symptoms of cutaneous anthrax infection begin with a painless papule at the site of infection 3–5 days after exposure. After 24–36 h, the papule progresses to a vesicle 1–2 cm in diameter. Once the lesion ruptures, it slowly

TABLE 31.2. Effects of anthrax bacilli and toxins on various cell types



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erodes, leaving a necrotic ulcer with a black central scab, termed an eschar. Eschar lesions typically form at exposed areas of the body (arms, hands, neck, and face). The formation of edema around the eschar lesion is also characteristic of cutaneous anthrax. Two to three weeks later, the eschar sloughs off the skin. Other signs and symptoms of cutaneous disease in humans include fever, headache, malaise, toxemia, and regional lymphadenopathy. Painful swelling of regional adrenal glands can occur.

B. Inhalational Anthrax

Signs and symptoms of inhalational anthrax follow a biphasic course. The initial phase is characterized by fever, malaise, and unproductive cough characteristic of an upper respiratory infection. Following this flu-like phase, the patient will typically recover after 2–4 days. The second phase proceeds rapidly with the following constellation of signs and symptoms: acute dyspnea, pleural effusion, fever, progressively worsening respiratory failure, cyanosis, circulatory collapse, shock, and death, if left untreated. Death occurs 24 h after the onset of this second phase secondary to toxemia and suffocation (Atlas, 2002; Bales, 2002; Dixon *et al.*, 1999; Friedlander, 1999). Although the lung is the primary site of infection here, inhalational anthrax is not considered a true pneumonia. In most, but not all cases, there is no infection in the lungs (Abramova *et al.*, 1993; Albrink, 1961). An infectious dose is estimated at 8,000 to 50,000 spores via aerosol (Franz *et al.*, 1997).

C. Gastrointestinal and Oropharyngeal Anthrax

Although extremely rare, gastrointestinal anthrax has a high estimated mortality rate (25–60%). Symptoms occur 1–7 days post-ingestion, presenting as either oropharyngeal or intestinal anthrax. Oropharyngeal anthrax presents as tongue lesions, lymphadenopathy, fever, and dysphagia. Intestinal anthrax presents 2–5 days after infection with abdominal pain, fever, nausea, emesis, and diarrhea. Abdominal pain and diarrhea are a direct result of ulceration, edema, and hemorrhaging of the gastrointestinal tract

due to edema and lethal factors (Friedlander, 1997). Therefore, diarrhea may be bloody with extensive fluid loss and circulatory collapse. If treatment is not started early, toxemia and shock may develop, resulting in death. Oropharyngeal anthrax produces a milder infection than the intestinal form and leads to a better outcome. Treatment for either form reduces symptoms and typically leads to full recovery 10–14 days after infection.

D. Meningitis

Meningitis is a complication of all anthrax infections and is associated with high mortality. Anthrax meningitis occurs through either a hematogenous or lymphatic spread from the infection site to the central nervous system (CNS) (Sejvar, 2005). High bacterial counts from anthrax bacteremia allow for *B. anthracis* to cross the blood–brain barrier into the CNS to infect the meninges and cerebrospinal fluid. Anthrax meningitis is most common with inhalational anthrax and seen in ~50% of those cases. Development of anthrax-associated meningitis is a very grave sign and invariably fatal. Even with antibiotic treatment, death occurs approximately 1–6 days after signs and symptoms of meningitis appear (Dixon *et al.*, 1999), and the mortality rate is estimated to be 94% (Sejvar, 2005).

Symptoms at the onset of anthrax meningitis include fever, headaches, nausea, vomiting, chills, malaise, agitation, and nuchal rigidity (Sejvar, 2005). Delirium, coma, refractory seizures, and neurological degeneration occur within 2–4 days (Kim *et al.*, 2001; Sejvar, 2005). Neurological degeneration can be seen, with signs that include cranial nerve palsies, myoclonus, fasciculations, decerebrate posturing, and papilloedema (Sejvar, 2005). A notable feature of anthrax meningitis infection includes subarachnoid and intraparenchymal hemorrhages. Hemorrhage can be observed in the cerebrospinal fluid, as well as polymorphonuclear pleocytosis, an increase in protein concentration, and a decrease in glucose concentration. *Bacillus anthracis* can be observed in the cerebrospinal fluid (CSF), meninges, subarachnoid space, and brain parenchyma.

VIII. DETECTION AND DIAGNOSIS

A. Detection

Detection and diagnosis of anthrax infection can be difficult. Diagnosis is made predominantly on a positive history of exposure to contaminated animal products and a physical exam of the presenting signs and symptoms. Tests to detect *B. anthracis* are typically never ordered unless there is evidence of exposure. The disease typically progresses to an advanced stage prior to initiating appropriate treatment. The formation of a black eschar with hyperemic and edematous borders is a hallmark of cutaneous anthrax infection. Unfortunately, the black eschar occurs in the later stages of infection. The appearance of painless, pruritic papules with edematous borders is suggestive of possible cutaneous anthrax infection. Further tests to identify *B. anthracis* as the infectious agent should be attempted but should not delay initiation of antibiotic treatment. Evidence of pulmonary involvement on chest x-ray can provide suspicion of inhalational anthrax. The common triad of findings is mediastinal widening, pulmonary infiltrates, and/or pleural effusions (Friedlander, 1997; Dixon *et al.*, 1999). Without a proper diagnosis, progression of some forms (i.e. inhalational anthrax) to the second stage of infection is nearly 100% fatal.

Depending on the form of disease suspected, certain specimens should be collected. If cutaneous anthrax is suspected, swabs of the lesion are warranted. A sterile, dry swab should be used to collect vesicular fluid if the lesion is in the vesicular stage. If the lesion is in the eschar stage, the edge of the eschar should be lifted and the swab should rotate underneath for 2 to 3 seconds. However, if the lesion is not in either the vesicular or eschar stage, the base of the ulcer can be swabbed with a sterile swab moistened with saline. Blood cultures should be collected regardless of the route of exposure or signs of disease (Beatty *et al.*, 2003). If gastrointestinal anthrax is suspected, ascitic fluid should be taken, and if oropharyngeal anthrax is suspected, swabs from oropharyngeal lesions should be taken (Beatty *et al.*, 2003). If pleural effusions are seen, pleural fluid should be collected, and if meningeal signs/symptoms are present, CSF should be collected.

B. Diagnostics

Laboratory tests for anthrax can include Gram staining, differential plating, γ-bacteriophage plaque assay, blood cultures, motility tests, enzyme-linked immunosorbent assays (ELISA), and fluorescent covalent microsphere immunoassay (FCMIA). Although microbial tests take 24 h to perform, often delaying the diagnosis, these tests may be necessary to confirm a diagnosis of anthrax. If there is a high index of suspicion for anthrax, initiation of therapy should not be delayed for results of these confirmatory tests.

1. Microbiological Tests

Simple tests to rule out anthrax infection are Gram staining of cultured bacteria, differential plating, and a γ-bacteriophage plaque assay. Gram staining is nearly always used in the identification of bacteria (CDC, ASM, APHL, 2002). *Bacillus anthracis* will appear as large, Gram-positive rods in short chains. The size of the bacterium is approximately 1–1.5 by 3–5 μm. *Bacillus anthracis* capsules will not take up India ink stain and will cause the bacilli to appear as clear zones on a black, stained background. *Bacillus anthracis* can be distinguished from other *Bacillus* species by culturing the organism on blood agar plates. It is the only *Bacillus* species that does not cause hemolysis. In addition,

B. anthracis cells are lysed by the bacterial virus γ-bacteriophage to form small plaques on nutrient agar plates.

Blood cultures should follow normal laboratory protocol. Specimens collected for cutaneous anthrax can be plated on 5% sheep blood agar (SBA), MacConkey agar (MAC), or any media normally used for surface wounds (CDC, ASM, APHL, 2002). Stool specimens from gastrointestinal anthrax cases should be plated on phenylethyl alcohol agar (PEA), MAC, and SBA. Sputum specimens from inhalational anthrax patients should be plated on chocolate agar, SBA, and MAC. Cultures will show isolated *B. anthracis* colonies 2–5 mm in diameter, flat or slightly curved, with a wavy border after 15–24 h. *Bacillus anthracis* shows growth on SBA and not on MAC. Colonies of *B. anthracis* plated on SBA will appear as a "beaten egg white" when teased with a loop (basic diagnostic testing). These blood cultures are useful, especially in the differential diagnosis of anthrax, and should be completed before antibiotic treatment is given.

A motility test is useful because *B. anthracis* is a nonmotile bacterium. Two motility tests available are the wet mount and motility medium variety. In a wet-mount preparation, organisms with Brownian movement or no movement will support the presence of *B. anthracis*. The presence of *B. anthracis* in a motility medium preparation would be a single line of growth along the original inoculum stab (CDC, ASM, APHL, 2002).

2. Molecular Tests

Acute and convalescent serum samples for serological ELISA testing should be collected for diagnosis. The CDC developed an ELISA for the detection of *B. anthracis* protective antigen (PA) using immunoglobulin G (IgG) antibodies in response to the bioterrorist anthrax plot in 2001. ELISA proved extremely useful in the detection of cutaneous and inhalational anthrax (Quinn *et al.*, 2002). The diagnostic sensitivity and diagnostic specificity for this test are 97.6% and 94.2%, respectively.

A more recent study reports another tool that detects anthrax better than ELISA detection of PA (Biagini *et al.*, 2004). The FCMIA can be multiplexed, meaning that numerous analytes can be measured simultaneously. In anthrax cases, anti-PA and anti-LF can be examined in serum samples at the same time. FCMIA is more sensitive, rapid, and reliable than ELISA.

3. Histopathology

Punch biopsies can confirm cutaneous anthrax if Gram stain and culture results are negative, due to antibiotic treatment (Godyn *et al.*, 2005), and a suspicion of cutaneous anthrax remains (Celia, 2002). The center of the eschar, the erythematous region, and the skin margin should all be included in the biopsy when an eschar is present (Godyn *et al.*, 2005).

4. Radiology

Radiological tests are available to narrow the differential diagnosis for anthrax. A chest radiograph will almost always exhibit a widened mediastinal contour that includes unilateral or bilateral hilar enlargement, as well as prominent periobronchovascular markings and pleural effusions (Frazier *et al.*, 2006). The size of the widened mediastinum contours and pleural effusion can increase rapidly over hours/days. However, findings from the radiograph might be subtle and even appear normal in the early stages of infection (Frazier *et al.*, 2006). Noncontrast CT will exhibit high-density mediastinal and hilar lymphadenopathy, which can rapidly increase in size over days (Friedlander, 1997; Frazier *et al.*, 2006). This indicates both hemorrhage and edema in the mediastinal lymph nodes. In addition, contrast-enhanced CT on mediastinal nodes can display rim enhancement and central hypodensity (Frazier *et al.*, 2006). The role of radiological tests has not been identified in the diagnosis of gastrointestinal/oropharyngeal anthrax (Beatty *et al.*, 2003). Radiographs of patients with gastrointestinal anthrax displayed findings indicative of obstruction; however, further studies need to be completed to determine if radiology can be used to help diagnose gastrointestinal anthrax (Beatty *et al.*, 2003). Patients with anthrax disease can also acquire complications of anthrax meningitis. CT or magnetic resonance imaging (MRI) shows hemorrhages in the deep gray matter, subarachnoid space, and ventricles (Sejvar *et al.*, 2005).

IX. RISK ASSESSMENT

The risks posed by an intentional outbreak of anthrax cannot be minimized, as the potential effects go beyond merely the medical. The primary costs of a biological terrorism-related anthrax event in terms of lives lost, medical resources required to treat affected individuals, and economic burden to decontaminate spores are significant. Certainly, the subsequent psychological and societal costs could be as high or higher, based on the US Federal government experience with the 2001 anthrax letters. "On a collective level, a major epidemic of anthrax can destroy the social order" (Guillemin, 1999).

The distribution of *B. anthracis* endospores in mailings through the US Postal Service in the fall of 2001 served to ignite public awareness concerning anthrax as a weapon of mass destruction. Deliberate contamination of the mail resulted in 22 cases of anthrax (11 inhalational and 11 cutaneous). These mailings led to five deaths among the inhalational anthrax cases and an enormous economic burden associated with decontamination. The media coverage and public fallout unveiled the deficiencies in our current risk assessment of anthrax.

The effects on public order, as well as economic and social effects, should be considered in an assessment of weaponized anthrax risk. Decontamination of the Hart

Senate Office Building in Washington, DC required 7 months at a cost of $23 million. Decontaminaton of the postal facilities that processed the "anthrax letters" in Brentwood, DC and Hamilton Township, NJ required more than a year at a cost in excess of $100 million (Fernandez, 2002). The psychological effects of an anthrax attack could exceed the medical or economic effects, especially in the short term. Panic caused by seemingly random outbreaks of symptoms in apparently unrelated persons or events "could degenerate into panic, flight, communications breakdown, general societal dysfunction" (Wein *et al.*, 2003).

A comprehensive risk assessment is dependent in part on determining the health risk posed to the individual by this biological agent. Animal models play a critical role by providing key sources of information for predicting consequences of human exposure since comparable naturally occurring human indices are rare. These animal models have been reviewed in previous sections throughout this chapter. Another method to assess risk is to use an anthrax stimulant. The Defense Research Establishment Suffield (DRES) in Canada undertook a series of experiments to assess the risk of envelopes filled with anthrax spores (Kournikakis *et al.*, 2001). In their experiments, envelopes containing spores of the nonpathogenic *Bacillus globigii* were opened in a mock mail room inside an aerosol test chamber to estimate aersol release from the envelopes. Investigators showed that dispersal by passive letter opening was far more effective than initially thought. A lethal dose could be inhaled within seconds of opening the content (Kournikakis *et al.*, 2001). Not only would the mail handler opening the envelope receive a lethal dose (LD) of between 500 and 3,000 LD_{50}s, but other workers in the room would inhale lethal numbers of spores.

In silico models of anthrax release can be used to bridge the gap to help predict consequences of human exposure in the event of an outbreak or biological attack scenario. Several modeling studies have been published (Wein *et al.*, 2003) in attempts to assess anthrax risk. The model of Wein *et al.* (2003) assumes a point-release of 1 kg of spores at a height of 100 m over a city of 10 million inhabitants. The effects on mortality of different antibiotic treatment strategies in an urbanized anthrax release are examined. This *in silico* biological weapon attack results in >100,000 deaths, given both symptomatic and asymptomatic persons receive antibiotics and the model incorporates availability and distribution parameters. In the base case, an approximation of current treatment and distribution strategies, deaths are estimated at >1% (Wein *et al.*, 2003). The model predicts significantly higher death rates (≥7 times) in cases where less aggressive distribution or administration is modeled (Webb, 2003).

One of the greatest fears of a scenario involving anthrax is the application of genetic engineering to enhance its pathogenicity. Several studies have demonstrated the ability to generate antibiotic-resistant anthrax (Pomerantsev *et al.*, 1992, 1993). In addition to developing multiple antibiotic-resistant strains of *B. anthracis*, biotechnology offers a highly likely scenario that the very nature of *B. anthracis* could be altered. It is possible that *B. anthracis* can be engineered to produce novel toxin activity (Mesnage *et al.*, 1999; Sirard *et al.*, 1997a, b). Other approaches may focus on the modification of PA such that it remains functional but immunologically distinct from the PA used in current vaccines.

X. TREATMENT

A. Overview

Treatment of any form of anthrax infection is generally the same: aggressive antibiotics and supportive care. Rapid definitive diagnosis of anthrax is critical for effective treatment, though in cases where anthrax is suspected prior to confirmation antibiotic therapy should not be withheld pending test results. Post-exposure treatment should be given in cases of putative exposure to anthrax spores to prevent systemic disease. Antibiotics and supportive management are the mainstays of treatment to prevent septic shock, fluid and electrolyte imbalance, and dyspnea associated with systemic anthrax disease. Initial administration of ciprofloxacin or doxycyclin is recommended (USFDA, 2001; CDC, 2001a, b, c). A duration of 60 days has been recommended for prophylaxis, though the most efficacious duration has not been determined (Brook, 2002).

Intravenous ciprofloxacin or doxycycline are recommended for treatment of anthrax, usually as part of a cocktail of antibiotics (CDC, 2001a, b). Multiple antibiotics are usually indicated in anthrax cases with signs of septicemia, extensive edema, or for cases with cutaneous lesions in the head and neck (Brook, 2002). Penicillin may be included in the antibiotic cocktail, but is not recommended as a standalone therapy due to *B. anthracis* β-lactamase production. *Bacillus anthracis* can express β-lactamase variants, penicillinases and cephalosporinases, which would undermine a lone-penicillin therapy (Lightfoot *et al.*, 1990). *Bacillus anthracis* has shown *in vitro* resistance to cephaloporins and trimethoprim-sulfamethoxazole (Inglesby *et al.*, 2002). Corticosteroid therapy may help treat edema from head and neck lesions or prevent airway obstruction. Table 31.3 contains therapeutic guidelines, based on CDC recommendations (CDC, 2001a, b, c), for pharmacologic management of *B. anthracis* infection.

B. Inhalational, Oral, and Gastrointestinal Anthrax

At the time of writing, the recommended initial therapy for inhalational, oral, and gastrointestinal anthrax in adults is 400 mg of ciprofloxacin every 12 h or 100 mg of doxycycline every 12 h, administered intravenously (Inglesby *et al.*, 2002). In addition to these treatments, one or two

TABLE 31.3. Anthrax therapeutic guidelines[a]

Therapy		Adults		Children		Pregnant women	
		Initial therapy[e] (intravenous dosing)	**Oral dosing**	**Initial therapy (intravenous)**	**Oral dosing**	**Initial therapy**	**Duration**
Recommended first line treatment – inhalational, GI, and oropharyngeal anthrax[b]	Ciprofloxacin (use in combination with one or two additional antimicrobials listed below)	IV treatment initially (400 mg q 12 h) in combination with one or two additional antimicrobials[f]	Switch to oral dosing when appropriate (500 mg PO BID)	IV treatment initially (10–15 mg/kg q 12 h)[h] in combination with one or two additional antimicrobials	Switch to oral when appropriate (500 mg PO BID)	Same for adults	
		Duration: Continue for 60 days total (IV and PO combined)[i]					
	Doxycycline[d] (use in combination with one or two additional antimicrobials listed below)	IV treatment initially (100 mg q 12 h) in combination with one or two additional antimicrobials[f]	Switch to oral when appropriate (100 mg PO BID)	IV treatment initially >8 yrs & >45 kg (100 mg q 12 h) >8 yrs & <45 kg (2.2 mg/kg q 12 h) <8 yrs (2.2 mg/kg q 12 h)	Switch to oral therapy when appropriate >8 yrs & >45 kg (100 mg PO BID) >8 yrs & <45 kg (2.2 mg/kg PO BID) <8 yrs (2.2 mg/kg PO BID)		
		Duration: Continue for 60 days total (IV and PO combined)[i]					

Therapy		Adults		Children		Pregnant women	
		Initial therapy (oral)[g]	**Duration**	**Initial therapy (oral)**	**Duration**	**Initial therapy**	**Duration**
Recommended first line treatment – cutaneous anthrax[b]	Ciprofloxacin	500 mg BID	60 days[i]	10–15 mg/kg q 12 h (not to exceed 1 g/day)	60 days[i]	500 mg BID	60 days[i]
	Doxycycline[d]	100 mg BID		>8 yrs & >45 kg (100 mg q 12 h) >8 yrs & <45 kg (2.2 mg/kg q 12 h) <8 yrs (2.2 mg/kg q 12 h)		100 mg BID	
Alternative option in cutaneous anthrax[c]	Amoxicillin		500 mg PO TID		80 mg/kg/day divided every 8 h		

[a]Therapeutic treatment protocol for immunocompromised persons is the same for immunocompromised adults and children
[b]Ciprofloxacin or doxycycline are first line therapeutics for all forms of anthrax
[c]Amoxicillin is an alternative option in adults and children for completion of therapy only after clinical improvement
[d]If meningitis is suspected, doxycycline may be less optimal due to poor central nervous system penetration
[e]Steroids may be considered in as adjunct therapy for patients with severe edema and for meningitis. [Dexamethosone: Adults (0.75–0.90 mg/kg/day orally, i.v., or i.m. in divided doses every 6 h); Children (0.25–0.50 mg/kg every 6 h)] [Prednisone: Adults (1–2 mg/kg or 5–60 mg orally/day); Children (0.5–2 mg/kg/day)]
[f]Other agents with *in vitro* activity include rifampin, vamcomycin, penicillin, ampicillin, chloramphenicol, imipenem, clindamycin, and clarithromycin. Because of concerns for beta-lactamases in *Bacillus anthracis*, penicillin and ampicillin should not be used alone. Consultation with an infectious disease specialist is advised
[g]Cutaneous anthrax with signs of systemic involvement, extensive edema, or lesions of the head or neck require intravenous therapy, and a multidrug therapeutic approach
[h]In children, ciprofloxacin dosage should not exceed 1 g/day
[i]Due to the potential for spores to persist after an aerosol exposure, antimicrobial therapy should be continued for 60 days

additional antimicrobials should be administered (list mentioned above) (Inglesby *et al.*, 2002). These guidelines should be followed until the condition of the patient improves. The treatment should then be switched to either 500 mg of ciprofloxacin twice a day or 100 mg of doxycycline twice a day (Inglesby *et al.*, 2002). This stage of treatment should begin intravenously and switch to oral dosing when appropriate (Inglesby *et al.*, 2002). Treatment should continue for 60 days (Inglesby *et al.*, 2002). Oral amoxicillin can be used as an alternative in adults and children for completion of therapy only after clinical improvement (Inglesby *et al.*, 2002; CDC, 2001a, b). Pediatric guidelines for medical management of anthrax are provided in Table 31.3, in addition to recommendations for pregnant women and immunocompromised patients.

C. Cutaneous Anthrax

The recommended initial therapy for adults with cutaneous anthrax is either 500 mg of ciprofloxacin orally administered twice a day or 100 mg of doxycycline orally administrated twice a day (Inglesby *et al.*, 2002). The duration of therapy is suggested to be 60 days (CDC, 2001a, b), although previous guidelines recommend 7 to 10 days (Inglesby *et al.*, 2002). Intravenous therapy and a multidrug regimen approach are recommended for patients with signs of extensive edema, or head/neck lesions (CDC, 2001c). The guidelines for pharmacologic management of cutaneous anthrax in pediatric patients and pregnant women are also provided (Table 31.3). With early treatment, systemic disease does not occur, and laboratory cultures will yield negative results for *B. anthracis*; however, treatment will not curtail formation of the black eschar and progression of the edematous skin ulcer (CDC, 2001c).

D. Bacteremia

Bacteremia often occurs with anthrax infection, especially with gastrointestinal/oropharyngeal and inhalational anthrax. A multidrug approach consisting of ciprofloxacin or doxycycline, along with one or two other antimicrobials, is suggested when bacteremia is suspected (CDC, 2001c). Other drugs that are recommended for use with ciprofloxacin or doxycycline include rifampin, vancomycin, imipenem, chloramphenicol, penicillin/ampicillin, clindamycin, and clarithromycin (CDC, 2001c).

E. Anthrax Meningitis

There is limited clinical experience for treating patients with anthrax meningitis (Sejvar *et al.*, 2005). However, the recommended therapy is also a multidrug treatment, including the use of a fluoroquinolone and two additional drugs with excellent CNS penetration. The fluoroquinolone that is recommended for use is ciprofloxacin. The ciprofloxacin level in CSF is 26–50% of serum levels. Other fluoroquinolones include levofloxacin, gatifloxacin, moxfloxacin, and ofloxacin. Doxycycline is not recommended as a first-line agent for anthrax meningitis. Although doxycycline has a low MIC (0.03 mg/l), it has low CNS penetration and a lower percentage in the CSF of serum levels than ciprofloxacin (10–26%). These drugs have been shown to work against *B. anthracis*; however, these drugs have not been observed in humans and have not been tested in animal models. Drugs with significant CNS penetration include ampicillin, meropenem, rifampicin, or vancomycin. Although penicillin is a beta-lactam, like ampicilin and meropenem, it is not suggested for use against anthrax meningitis for reasons stated above. Similar to the treatment suggestions above, the recommended duration of therapy is 60 days. Even with the effectiveness of antibiotic treatment, there is no guarantee that anthrax will be diagnosed in time to initiate therapy. Therefore, anthrax represents a significant risk to the public because it is lethal, potent, and induces public anxiety.

F. Vaccines

While vaccines are promising, further research needs to be conducted with the goal of updating vaccine technology. Conclusive demonstration of seroconversion against an anthrax infection was demonstrated in animals previously immunized with an active anti-PA vaccine and later given post-exposure antibiotic therapy (Friedlander *et al.*, 1993). Therefore, other therapies, such as injection of anthrax-specific monoclonal antibodies (Maynard *et al.*, 2002), may lead to a promising treatment following exposure to *B. anthracis*. Both of the current anthrax vaccines, *anthrax vaccine adsorbed* (AVA) and a newer one based on recombinant PA (rPA), offer long-lasting protection against an aerosol challenge of *B. anthracis* in rhesus macaques. Efficacy of both vaccines is due primarily to the PA. The AVA vaccine offers similar protection from challenge in rabbits; however, the duration of immunity is unknown (Phipps *et al.*, 2004). Various negative side effects have been reported after administration of the current anthrax vaccine to humans, and it would be beneficial to develop a vaccine that has fewer side effects before instituting a massive vaccination plan. Recent reports suggest that the addition of CpG oligonucleotides may offer an improved response. These CpG motifs interact with various receptors on B cells and dendritic cells, which improve antigen presentation and up-regulate pro-inflammatory cytokine expression. After co-administration of CpG oligonucleotides with the AVA vaccine, this combination triggered a fast and higher-titer immune response in comparison with the vaccine alone (Klinman *et al.*, 2004). These findings suggest a potential for using oligonucleotides as an adjuvant in a post-exposure vaccine.

Other studies have used an adenoviral delivery system to invoke a protective immune response in mice (Tan *et al.*,

2003). Immunization with this form of vaccine demonstrated a rapid anti-PA antibody response at a higher level than that of the current vaccine. Further, this method of delivery offered a longer protection time in comparison to that of the rPA vaccine. However, current public fears of using a virus as an immunization vector coupled with the lack of similar findings with other model systems make it imperative to further research this potential area of vaccine technology.

XI. CONCLUDING REMARKS AND FUTURE DIRECTION

The fear of *B. anthracis* has only been heightened in recent years as a result of the terrorist events in 2001 and the increase in acts of terrorism worldwide. There are few infectious agents that are more feared or notorious than *B. anthracis*, the causative agent of the zoonotic disease anthrax. *Bacillus anthracis* would be an ideal weapon for a terrorist attack not only because of the disease pathology but also because this organism can sustain desiccation and survive long term outside of its host as a dormant spore. Moreover, infection of humans by inhalation of spores has been reported. Thus, *B. anthracis* disseminated in an aerosol could be a feasible means by which to target and infect a large population.

The processes of *B. anthracis* infection and pathogenesis have been studied in various animal models. During the initial phases of infection, regardless of the route of infection (i.e. inhalational, dermal, or oral), the host animal may display general symptoms that are characteristic of numerous infections or disease states, making a positive diagnosis difficult. Pathogenesis involves initial uptake of dormant spores by phagocytes. Germination takes place soon afterwards either at the site of inoculation or later after transport to the lymph nodes. It is the vegetative cell that moves through the circulatory system and lymphatics to infect other target organs. Lungs and gastrointestinal tract are major targets for vegetative bacilli. Later the spleen, brain, liver, and almost every other organ in the body can be colonized. In systemic anthrax, there is overwhelming septicemia. While septicemia is important, lethality from *B. anthracis* is believed to be mediated by the actions of anthrax toxins (LT and ET). The toxins are thought to be primarily responsible for causing immune system suppression, necrosis of critical cells, vascular leakage, hemorrhage, shock, and death.

There are numerous gaps in our understanding of anthrax. While the general pathogenesis of *B. anthracis* has been studied in a variety of animals, a number of important questions still remain unanswered. Although spore uptake has been demonstrated in macrophages and dendritic cells, the molecular interactions involving uptake, spore germination, and escape require additional research. Further *in vitro* and *in vivo* studies will help to elucidate these mechanisms. Understanding of the molecular mechanisms of TLR signaling and its relationship to LT-mediated apoptosis may lead to new therapies. The inhibition of TLR-mediated signaling of apoptosis may inhibit macrophage cell death and assist in controlling the pathogen.

Several studies have shown bacilli in many organs of the body following various routes of exposure. However, the time course of transit to target organs and the targeting order of these organs may be helpful in further defining therapeutic windows of opportunity. Lastly, the molecular mechanisms of toxicity and death caused by LT and ET remain unclear. There is no direct evidence that proves the importance of macrophage apoptosis in the course of disease progression. Recent reports suggest that LT may induce apoptosis of endothelial cells, which suggest that macrophages are not the sole cell type affected by LT (Kirby, 2004). In addition, reports which demonstrate LT reduces cytokine expression (Erwin *et al.*, 2001; Moayeri and Leppla, 2004; Pellizzari *et al.*, 1999) suggest that death may not be due to an inflammatory reaction.

Several animal model studies have focused on the immune response to infection and also to vaccination. Most studies identify antibody titer, but some also show cytokine production or possible mechanisms of bacterial evasion. A focus on immune response studies will help to further define *B. anthracis* pathogenesis and provide insight into the design of future vaccines and therapeutics. Most countermeasure studies have been conducted on vaccines, as antibiotic studies have typically been performed *in vitro*. Primary concerns are the length of required treatment, efficient prophylaxis methods, and less complicated dosing regimens for vaccines. In a theoretical bioterrorist attack scenario, it will be essential to distribute effective treatment and prophylaxis to infected or potentially exposed persons in an expedient manner.

The choice of an appropriate animal model system that most closely mimics the human response to *B. anthracis* infection is crucial to the development of improved treatments and more effective vaccines. Such development relies on testing in appropriate animal models in which disease, infection, and progression mimic that seen in humans. The importance of selecting an appropriate animal model for human anthrax is magnified by the potential of a genetically engineered *B. anthracis* bacterium made more virulent than its predecessor. The addition of antibiotic resistance genes would render prophylactic treatment against infection by modified organisms difficult, if not futile. Further, the inclusion of genes that encode toxins not naturally found in *B. anthracis* may assist the bacterium in escaping host immunosurveillance. Therefore, defining the pathogenesis of *B. anthracis* and designing appropriate countermeasures is a critical necessity for treatment of those exposed in occupational settings, national security, and the global community. Whether medical systems would be able to provide treatment early enough to prevent widespread disease in the event of a bioterrorist attack involving anthrax remains questionable.

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