

Chapter 15

Bacterial Biowarfare Agents

MARK SOBOLESKI, AUDREY GLYNN, AND LUCY CÁRDENAS-FREYTAG

1.	Biowarfare Agents and Historical Perspective	576
2.	Anthrax.	577
2.1.	Introduction	577
2.2.	Pathogenesis	578
2.3.	Virulence	581
2.4.	Treatment and Prevention.	586
2.5.	Vaccines and Immunity	587
3.	Plague.	589
3.1.	Introduction	589
3.2.	Pathogenesis	590
3.3.	Virulence	592
3.4.	Treatment	595
3.5.	Vaccines and Immunity	595
4.	Tularemia.	598
4.1.	Introduction	598
4.2.	Pathogenesis	599
4.3.	Virulence	599
4.4.	Vaccines and Immunity	601

Program in Molecular Pathogenesis and Immunity, Department of Microbiology and Immunology,
Tulane University, School of Medicine, New Orleans, LA 70112

Historical Landmarks

- 1346 Mongols catapult plague-infected corpses over the walls into Kaffa , with the intent of causing a plague epidemic upon the Genoan enemy (Derbes, 1966).
- 1767 During the French and Indian War, British forces in North America give blankets used by smallpox patients to the Native Americans (Christopher et al., 1997).
- 1917 Germans use anthrax and glanders (*Burkholderia mallei*) to infect livestock and animal feed for export to the Allied Forces (Christopher et al., 1997).
- 1937 Japan creates “Unit 731,” a BW (Biowarfare) research facility in Manchuria, where experimental infections were carried out on Chinese prisoners. More than 10,000 people die after exposure to plague, anthrax, tularemia, syphilis, and other agents. It is believed that the facility also had millions of rats infected with fleas carrying *Yersinia pestis* (Girdwood, 1985; Harris, 1992).
- 1939 Japan poisons Soviet water supply with intestinal pathogens at Mongolian border (Nomonhan incident) (Williams and Wallace, 1989).
- 1940 Japan drops rice and wheat mixed with plague-carrying fleas over China and Manchuria (Williams and Wallace, 1989).
- 1942 The U.S. begins biological weapons program and chooses Camp Detrick, Frederick, Maryland, as its research and development site. Research efforts initially concentrated on the use of anthrax and botulinum toxin as bioweapons (Christopher et al., 1997).
- 1943 England tests anthrax bombs to kill sheep on Gruinard Island (“Anthrax Island”) off the coast of Scotland. Viable anthrax spores were still found on the island 40 years later (Manchee et al., 1982).
- 1979 Outbreak of pulmonary anthrax in Sverdlovsk, U.S.S.R. caused by an accidental release of anthrax spores from a Soviet military microbiological facility. Hundreds are exposed and at least 67 die (Meselson et al., 1994).
- 1984 Outbreak of salmonellosis in Oregon, U.S., after members of the Rajneesh cult intentionally contaminate salad bars with *Salmonella typhimurium* (Torok et al., 1997).
- 1985 Iraq develops biological weapons including anthrax, botulinum toxin, and aflatoxin (Christopher et al., 1997).
- 1993 Members of the Japanese cult of Aum Shinrikyo attempt an aerosolized release of anthrax from the tops of buildings in Tokyo (Smithson, 2000).
- 1996 Outbreak of *Shigella dysenteriae* in Texas, U.S. after workers ingest food that was intentionally contaminated with this bacteria (Kolavic et al., 1997).

- 2001 Release of anthrax spores through the U.S. mail. A total of 22 confirmed cases of bioterrorism-related anthrax; 5 people die (Fennelly et al., 2004; Jernigan et al., 2001).

1. Biowarfare Agents and Historical Perspective

Bioterrorism can be defined as the dissemination of biological agents with the intention to induce disease and spread fear and panic. The release of microbial pathogens (such as *Bacillus anthracis* or *Yersinia pestis*) or biological toxins (such as botulinum toxin or ricin) can result in serious morbidity and mortality and perhaps, more importantly, it can cause great disruption to society, massive public health crises, and tremendous impact on worldwide economy.

According to the U.S. Centers for Disease Control and Prevention (CDC), potential biological biowarfare agents are classified into three categories: A, B, and C (Table 1).

Category A agents are the highest-priority pathogens. They pose the greatest risk to national security because they (a) can be easily disseminated or transmitted from person to person, (b) result in high mortality rates and have the potential for major public health impact, (c) might cause public panic and social disruption, and (d) require special action for public health preparedness. Category B agents include pathogens that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates, and require specifically enhanced diagnostic capacity. Category C agents include emerging pathogens, to which the general population lacks immunity, and that could be engineered for mass dissemination because of ready availability, ease of production, ease of dissemination, potential for high morbidity and mortality, and major public health impact. (CDC, 2005)

In this chapter, we present an overview of the most important aspects of virulence, pathogenicity, interactions with the host, and prevention of bacterial pathogens. These organisms are potential bioweapons because their intentional release in the environment can induce severe, debilitating, and potentially lethal infections. Our discussion will focus on *B. anthracis*, *Y. pestis*, and *Francisella tularensis*, which represent the most currently investigated bacterial pathogens in category A. The use of botulinum toxin as a bioweapon would cause “inhalational botulism,” which in essence is an “intoxication” rather than an infection, and therefore will not be discussed here.

The use of microbial pathogens as potential weapons of terrorism dates to ancient times. A recount of the use of bacterial bioweapons beginning with the known earlier instances to the most current events is given in the Historical Landmarks. Although the veracity and exact epidemiological and bacteriological data from some of the earlier reported events are impossible to confirm, this historical review reveals that humans have had and continue

TABLE 1. CDC classification of bioterrorism agents/diseases.

	Bacteria	Toxin	Virus
Category A	<i>Bacillus anthracis</i>	Botulinum toxin	Variola major (smallpox)
	<i>Yersinia pestis</i>	(from <i>Clostridium</i>	Filovirus (e.g., Ebola, Marburg)
	<i>Francisella tularensis</i>	<i>botulinum</i>)	Arenavirus (e.g., Lassa, Machupo)
Category B	<i>Brucella</i> species	Epsilon toxin (from	Viral encephalitis—alphavirus
	<i>Burkholderia mallei</i>	<i>Clostridium</i>	such as: Venezuelan
	<i>Burkholderia pseudomallei</i>	<i>perfringens</i>)	equine encephalitis
	<i>Chlamydia psittaci</i>	Staphylococcal	Western equine
	<i>Coxiella burnetii</i>	enterotoxin B (SEB)	encephalitis
	<i>Rickettsia prowazekii</i>	Ricin	Eastern equine
	<i>Salmonella</i> , <i>Shigella</i>		encephalitis
	<i>Escherichia coli</i> O157:H7		
	<i>Vibrio cholerae</i>		
	Category C		
			Hantavirus
			Severe acute respiratory syndrome (SARS)

to have a keen interest in the development and malicious use of biological weapons.

The terrorist events of September 11, 2001 in the U.S., followed by the dissemination of anthrax spores via the postal service, materialized the vulnerability of our society to these kinds of attacks and underscored the urgency for developing new strategies of countermeasurement, detection, diagnosis, treatment, and prevention of infections caused by microbial biowarfare agents. In this regard, the scientific community is in the presence of great challenges and unprecedented opportunities. The outcome of the renewed scientific initiatives on bioterrorism will undoubtedly be a more comprehensive understanding of the pathogenesis and host interactions of these pathogens, as well as the identification of new targets for the development of efficacious prophylaxis and therapeutics. This information will not be limited to bacterial bioweapons, but will be broadly applicable to other important human microbial pathogens.

2. Anthrax

2.1. Introduction

Anthrax is caused by the Gram-positive bacterium *B. anthracis*. In nature, anthrax is a disease that herbivores or other mammals acquire after contact with *B. anthracis* spores present in soil. Human disease results from contact

with infected animals or contaminated animal products, or after exposure to accidentally or intentionally released spores. Anthrax was originally called Woolsorter's disease because it occurred most frequently in people who worked in animal textile mills. *B. anthracis* derives its name from the morphology of the microscopic bacterium (baculum, Latin for rod), and the dry, black appearance of cutaneous anthrax lesions (anthrakis, Greek for coal).

Beginning in 1972, nations around the globe signed a treaty at the Biological and Toxin Weapons Convention banning the research of biological weapons for offensive purposes. Despite this written agreement, many nations secretly continued to develop bioweapons (Cole, 1996). In April and May of 1979 a military facility in the northwest region of Sverdlovsk, U.S.S.R. (now Ekaterinburg), accidentally released *B. anthracis* spores into the air. Prevailing winds carried the spores into the nearby town resulting in 96 reported cases of pulmonary anthrax and at least 64 deaths (Meselson et al., 1994). Russian officials initially claimed that most of the cases were gastrointestinal anthrax acquired as a result of consumption of contaminated meat; however, subsequent laboratory analyses of patient samples and epidemiological studies concluded that the infections resulted from inhalation of aerosolized spores originating from the nearby military base (Bezdenzhnykh and Nikiforov, 1980; Meselson et al., 1994). This was the first known incident of human infection resulting from weaponized *B. anthracis* spores. Most recently, in October 2001, 22 individuals became infected with anthrax after the intentional release of spores through the U.S. mail. As a result, 11 confirmed cases of inhalation anthrax and 11 confirmed or suspected cases of cutaneous anthrax were diagnosed. There were 5 fatalities, and prophylactic antibiotics were recommended to approximately 10,000 people (Shepard et al., 2002). The fifth and final pulmonary anthrax fatality occurred months after the initial cases in a 94-year-old Connecticut woman who could not be directly linked to a known infectious source (Jernigan et al., 2001). Since this chapter deals specifically with agents of biowarfare capability, the majority of this section will focus on pulmonary anthrax.

2.2. Pathogenesis

Three distinct clinical forms of anthrax disease can develop in humans depending on the mode of infection (Figure 1). Cutaneous anthrax is the most common type of infection, usually acquired by people who are in frequent contact with infected animals or animal products. Disease results after entry of infectious spores into abrasions or cuts in the skin. Initially the infection appears as a small painless lesion that resembles an insect bite. The lesion is often pruritic, and develops into a larger inflamed papule. The raised area results from local fluid accumulation, caused at least in part by the action of anthrax toxins. As the tissue becomes necrotic, a dry black eschar forms and

eventually dries and sloughs off a few weeks after formation (Kalamas, 2004). Antibiotic treatment confers a 99% survival rate for patients diagnosed with cutaneous anthrax (Karwa et al., 2003). Dissemination to the bloodstream can occur in the absence of treatment, but is very rare if treatment is sought promptly. Gastrointestinal anthrax results from the ingestion of meat contaminated with *B. anthracis* spores. The infection is characterized by the presence of ulcers, massive edema, and necrosis of the intestinal mucosa. This is the rarest form of anthrax and, although treatable when diagnosed promptly, death can occur due to intestinal perforation and systemic dissemination of the toxins.

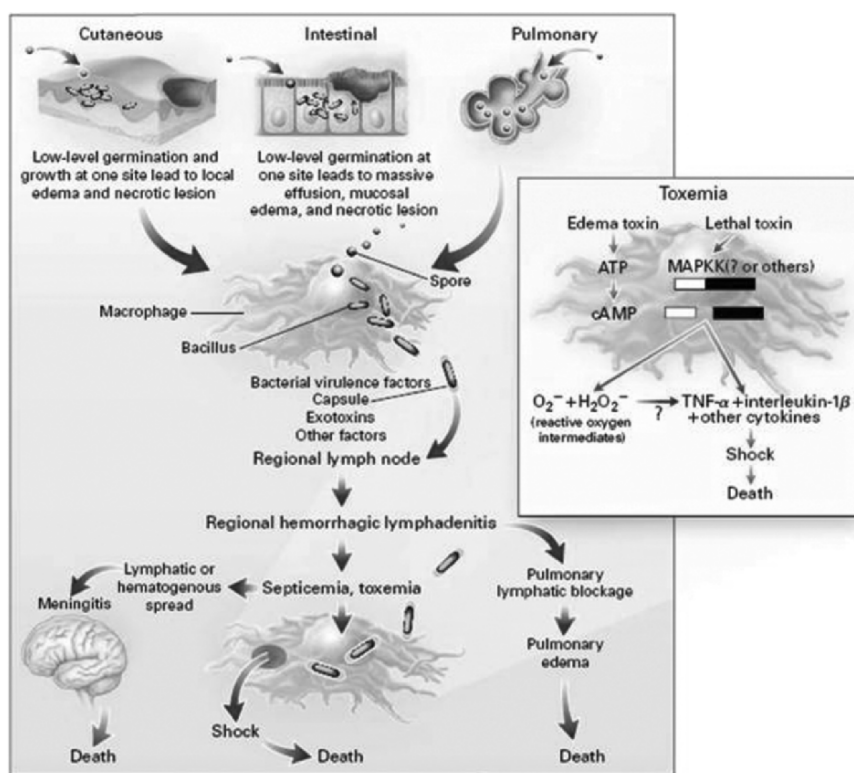


FIGURE 1. *Bacillus anthracis* endospores reach a primary site in the subcutaneous layer, gastrointestinal mucosa, or alveolar spaces. For cutaneous and gastrointestinal anthrax, low-level germination occurs at the primary site, leading to local edema, necrosis and, occasionally, to systemic spread. In cases of pulmonary anthrax, peribronchial hemorrhagic lymphadenitis blocks pulmonary lymphatic drainage, leading to pulmonary edema. Death results from septicemia, toxemia, or pulmonary complications. The action of Edema toxin and Lethal toxin are shown on the right. (Reproduced with permission from Dixon et al., 1999.)

Pulmonary anthrax is the most serious manifestation of the disease and results from inhalation of aerosolized *B. anthracis* spores. To induce a productive infection, the spores must reach the alveolar spaces of the lung where resident macrophages phagocytize them and germination occurs. To reach the alveolar spaces and avoid the mechanical innate barriers of the host, spores must be between 1µm and 5µm in diameter (Hatch, 1961). This requirement represents one of the major technological hurdles for the “weaponization” of *B. anthracis* and may be one of the reasons why weaponized anthrax can only be produced with considerable financial and technological resources. Pulmonary anthrax is the most likely outcome in instances of biological warfare, since once the microscopic spores are released in the environment they can be rapidly disseminated and inhaled by humans (Jernigan et al., 2001; Meselson et al., 1994). Although pulmonary anthrax is highly fatal and easily contracted from aerosolized spores, it has not been shown to be transmissible from person to person and is thus limited in its ability to cause mass casualties. This is in contrast to pneumonic plague, which can be acquired after inhalation of aerosolized infectious bacteria and subsequently disseminated from infected individuals to close contacts.

Following inhalation, alveolar macrophages ingest the dormant spores and germination is triggered by a series of host-and/or bacteria-dependent events, most of which are not yet completely understood (Bergman et al., 2005; Guidi-Rontani et al., 1999; Weiner and Hanna, 2003). From studies in a murine model it was determined that germination begins within 1 h of intranasal or intratracheal inoculation, and after 5 h vegetative bacilli can be found in regional lymph nodes (Lyons et al., 2004). Some of the spore-containing macrophages migrate to the peribronchial and mediastinal lymph nodes where vegetative bacilli continue to replicate and induce initial hemorrhagic mediastinitis (Abramova et al., 1993). Once outside macrophages, the bacilli multiply rapidly, and synthesize copious amounts of toxins that are hematogenously disseminated, leading to the clinical manifestations and the mortality associated with the disease. Pulmonary anthrax has been described as a biphasic illness in which the initial symptoms occur within days of infection (Abramova et al., 1993; Brookmeyer et al., 2005). Symptoms in the first phase are nondistinct, resemble a mild flu infection, and include fever, chills, headache, cough, and malaise. The second phase is a fulminant disease characterized by chest pain, and significant respiratory distress evident by severe cough, shortness of breath, and harsh, labored respiration (Kalamas, 2004). In the absence of treatment, death often follows 2–3 days after onset of the severe symptoms. Characteristic clinical presentations of pulmonary anthrax include pleural effusions and mediastinal widening observed on chest X-radiograms (Dixon et al., 1999; Jernigan et al., 2001). Death apparently results from multiorgan failure similar to systemic shock.

Pathology evaluation of tissues obtained from the pulmonary anthrax victims of the 2001 bioterrorist attack identified common abnormalities such as mediastinal edema, necrotizing mediastinitis, liver hypoxia, lymphadenopathy,

and hemorrhagic bilateral pleural effusions. Cultures of nasal swabs, serosanguinous fluid, blood, and cerebral spinal fluid were not always positive for *B. anthracis*, particularly in early stages of the disease. In fact, in several cases, samples did not test positive for *B. anthracis* until postmortem analysis. Confirmation of *B. anthracis* DNA by PCR was often successful and represented a more sensitive test than bacterial culture or Gram stain analyses (Jernigan et al., 2001).

2.3. Virulence Determinants

The ability of *B. anthracis* to induce a fulminant disease has been primarily attributed to the presence of an antiphagocytic capsule and the production of two toxins: anthrax lethal toxin (LeTx) and anthrax edema toxin (EdTx). A number of genes important in the pathogenesis of *B. anthracis* have been identified, but the most extensively studied are found on two virulence plasmids called pXO1 and pXO2 (Green et al., 1985). Plasmid pXO1, originally named pBA1, is 184 kb and contains the genes necessary for synthesis of the three components of the anthrax toxins. Also present on pXO1 is the *atxA* gene, which encodes a transactivator protein involved in the regulation of many *B. anthracis* genes (Bourgogne et al., 2003; Dai et al., 1995; Uchida et al., 1993). Experimental evidence indicates that the primary determinant of anthrax virulence is the presence of lethal factor (LF) and protective antigen (PA) which together form LeTx (Beall et al., 1962; Cataldi et al., 1990; Ezzell et al., 1984; Klimpel et al., 1994; Park et al., 2002; Pezard et al., 1991; Pitt et al., 2001; Reuveny et al., 2001). The other virulence plasmid, pXO2, contains the genes responsible for synthesis of the bacterial capsule (Green et al., 1985).

2.3.1. Capsule

Wild-type *B. anthracis* synthesizes a unique proteinaceous capsule composed exclusively of gamma-linked D-glutamic acid residues. Although the exact role of the capsule in infection has not been elucidated, it is known to be effective at preventing phagocytosis by macrophages and is also necessary for bacterial systemic dissemination (Drysdale et al., 2005; Makino et al., 1989). The enzymes responsible for capsule synthesis are encoded by the genes *capA*, *capB*, *capC*, and *capD* located on the 96 kb pXO2 virulence plasmid (Makino et al., 1989, 2002). Collectively these four genes make up the capBCAD operon. A strain with the capBCAD operon deleted is highly attenuated in a murine model of inhalation anthrax (Drysdale et al., 2005). Since the capsular material has been shown to be poorly immunogenic, it is believed that the capsule is necessary to avoid recognition by antibodies *in vivo*, and therefore the lack of capsule can result in accelerated clearance by the host (Leonard and Thorne, 1961). It has been postulated that pXO2 may carry other genes important for the virulence of the organism. Transposon mutagenesis of

pXO2 produced mutants that synthesized wild-type levels of capsule *in vitro* but exhibited reduced virulence when injected subcutaneously in mice (Welkos, 1991).

2.3.2. Toxins

Virulence of *B. anthracis* is complemented by the action of two toxins: LeTx and EdTx. EdTx and LeTx conform to the classical model of bacterial A-B toxins, since both are composed of a receptor-binding B subunit and a catalytically active A subunit. Unlike most binary toxins, the two components are not covalently linked, but instead exist as three separate proteins, before binding to the host cell. Initial binding to the host cell receptor is accomplished by the 83 kDa protective antigen (PA) protein encoded by the *pagA* gene located on virulence plasmid pXO1 (Vodkin and Leppla, 1983). The recently identified receptors for anthrax toxin are the tumor endothelial marker (TEM8) (Bradley et al., 2001) and the capillary morphogenesis protein2 (CMG2) (Scobie et al., 2003). These receptors are present in a broad range of tissues and contain a common extracellular von Willebrand factor type A domain, also called integrin-like domain (I-domain), which constitutes the PA binding site. The crystal structure of the interaction between PA and its receptors has been described in detail (Santelli et al., 2004).

After binding to the host cell receptor, a furin-like endoprotease cleaves the PA monomer at residue R167 releasing a small, N-terminal, 20 kDa fragment (Klimpel et al., 1992; Singh et al., 1989). The larger peptide (63 kDa) left on the cell surface, assembles into heptamers that can bind to either of the two A subunits: edema factor (EF) or lethal factor (LF); or some combination of the two (Pimental et al., 2004), producing a functional toxin molecule. PA-deficient strains of *B. anthracis* are avirulent in animal models attesting to the importance of the binding subunit in toxin-mediated pathogenesis (Cataldi et al., 1990; Pezard et al., 1991). When a molecule of LF attaches to a bound, activated molecule of PA, the complex is referred to as LeTx (lethal toxin). If a molecule of EF binds, the resulting complex is called EdTx (edema toxin). *B. anthracis* is unique in this respect since both catalytic proteins share the same binding subunit. Current evidence suggests that a maximum of three ligand molecules can bind per each heptamer, although it is not known if both EF and LF can bind simultaneously to one heptamer (Cunningham et al., 2002; Mogridge et al., 2002; Mourez, 2004). Following binding of the EF or LF subunits, the cell engulfs the PA/LF/EF complex via receptor-mediated endocytosis (Gordon et al., 1988). Each member of the PA heptamer then inserts a β -barrel hairpin loop into the vesicle membrane forming a pore that allows the translocation of the catalytic subunit(s) to the cytosol where they initiate their toxic effects (Nassi et al., 2002). Acidification of the endosome has been shown to be essential for the toxic effects *in vitro* suggesting that formation of the pore and/or translocation of the A subunit is pH-dependent (Friedlander, 1986; Milne et al., 1994). The insertion of PA

into the membrane seems to occur soon after the toxin complex is internalized, while the delivery of LF or EF to the cytosol is a later event in the endocytic pathway (Abrami et al., 2004) (Figure 2).

Whether LeTx is injected directly in purified form or synthesized by vegetative bacilli, it is believed to be the primary cause of mortality in animal models (Beall et al., 1962; Ezzell et al., 1984; Pezard et al., 1991). The catalytically active component of LeTx is the LF protein encoded by the *lef* gene. Work by Klimpel et. al. identified this 776-amino acid protein as a metalloprotease with essential zinc-binding histidine residues at positions H686 and H690 and a glutamate residue at position E687 required for LF's catalytic activity (Duesbery et al., 1998; Klimpel et al., 1994). These residues make up the HExxH consensus sequence, a motif that is common to many metalloproteases (Klimpel et al., 1994). It is likely that the PA subunit of LeTx exists

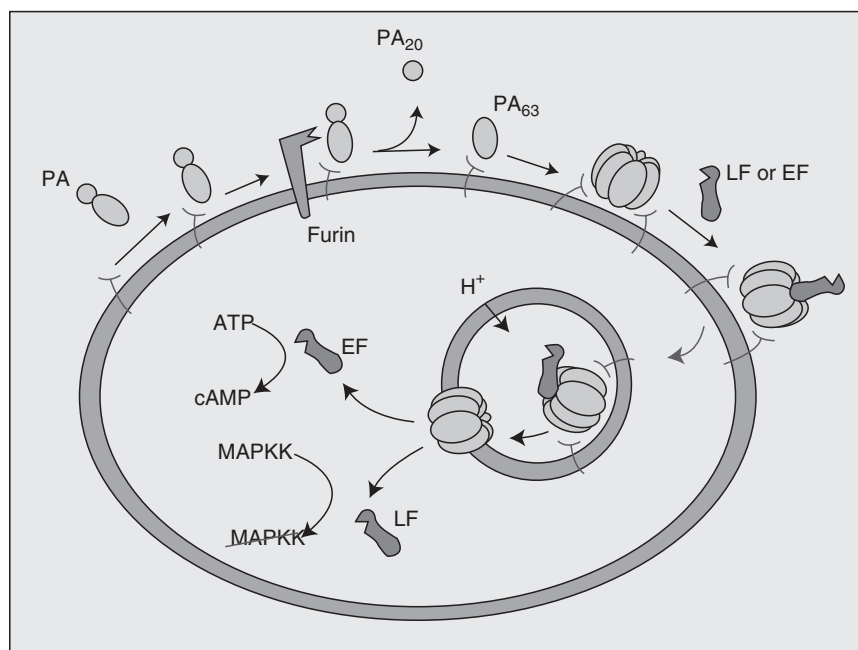


FIGURE 2. Anthrax toxin action. Protective antigen binds to a cellular receptor and is activated by a furin-like protease. The PA₆₃ fragment oligomerizes on the cell surface to form a ring-shaped heptamer, which binds lethal factor (LF), edema factor (EF), or both. The complexes are endocytosed and trafficked to an acidic compartment. Subsequently, the heptamer inserts into the membrane and forms a transmembrane pathway for transfer of EF and LF to the cytosol, where they catalyze their respective reactions. PA, protective antigen; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate. (Reproduced with permission from Starnbach and Collier, 2003.)

solely for the purposes of binding and entry since purified LF protein is fully capable of performing its catalytic activity when delivered directly into the cytosol of susceptible cells (Duesbery et al., 1998; Friedlander et al., 1993a).

LF targets members of the mitogen-activated protein kinase kinase (MAPKK) family of signaling proteins (Fig. 1). Within this family, MAPKK1, MAPKK2, and MAPKK3 (aka Mek 1–3) have been shown to be specific targets of LF proteolytic activity (Duesbery et al., 1998; Pellizzari et al., 1999; Vitale et al., 1998). Cleavage of these molecules near their terminus prevents interaction with their respective targets, thus altering vital intracellular signaling functions such as those involved in cell proliferation, cell differentiation, and cell survival (Duesbery et al., 1998). Although cleavage of Mek 1–3 is likely to be an advantage in suppression of the host immune response, it is unknown whether this particular function of LeTx is directly involved in the mortality associated with the disease. It is also possible that LF acts on other intracellular targets; however, no other substrates outside of the MAPKK family of signal transduction proteins have been identified.

Among the downstream effects of MAPKK are the impairment of dendritic cells in their ability to prime CD4+ T cells, inhibition of pro-inflammatory cytokine production, and macrophage lysis via inhibition of the p38 MAPK signaling pathway (Agrawal et al., 2003; Park et al., 2002; Pellizzari et al., 1999). In 1986 Friedlander observed that LeTx could induce rapid lysis of certain murine macrophages, leading to the release of pro-inflammatory cytokines (Friedlander, 1986). This observation gave rise to the hypothesis that macrophages play an important role in the progression of the disease and that a cascade of macrophage-derived pro-inflammatory cytokines was responsible for the shock-like effects observed in toxin-challenged animals. However, this theory is currently the subject of intense debate, because it is now known that humans or certain animals that are very sensitive to both anthrax infection and anthrax toxin have macrophages that are resistant to the action of the toxin *in vivo* (Cui et al., 2004; Friedlander et al., 1993a; Moayeri et al., 2003; Roberts et al., 1998). Therefore, LeTx-mediated death cannot be explained solely as a result of macrophage cytotoxicity. The observations that (1) LF negative strains of *B. anthracis* are avirulent in animals and (2) catalytically inactive LF mutants fail to cleave MAPKK members *in vitro* suggest that a strong correlation exists between the disruption of intracellular signaling events by LF and the mortality caused by LeTx (Duesbery et al., 1998; Pezard et al., 1991).

Despite the growing amount of information on the molecular mechanisms of LF and the multiple effects of the toxin observed *in vitro* and *in vivo*, the specific series of events that lead from LeTx exposure to sudden death remain unknown. In animals and humans, death has been associated with liver hypoxia and respiratory failure (Borio et al., 2001; Moayeri et al., 2003). Strong evidence arguing against the inflammatory cytokine-induced shock theory was presented in experiments in which TNF-receptor knockout and

wild-type mice were equally susceptible to infection after intraperitoneal injection of anthrax spores (Kalns et al., 2002). There are numerous conflicting reports regarding whether the inflammatory cytokine production is induced, suppressed, or unaffected after LeTx exposure (Cui et al., 2004; Erwin et al., 2001; Hanna, 1999; Hanna et al., 1993; Popov et al., 2004).

B. anthracis is one of several species of bacteria that produces adenylate cyclase toxin (Ahuja et al., 2004). Edema factor encoded by the *cya* gene is a 767-amino acid protein that functions as a calmodulin-dependent adenylate cyclase (Fig. 1) (Leppa, 1982, 1984; Robertson et al., 1988). Direct injection of purified EdTx into animals results in local fluid accumulation and swelling (Stanley and Smith, 1961). It has been suggested that an important function of EdTx may be the functional suppression of neutrophils. This was demonstrated when EdTx-treated neutrophils showed a decreased ability to phagocytose killed and opsonized *B. anthracis* (O'Brien et al., 1985). Theoretically, this would aid in survival and spread of the bacteria, and in conjunction with LeTx would disable the first line of protection against infection. The contribution of EdTx to systemic anthrax is not known, but it is assumed that the most significant role for EdTx is in the cutaneous form of the disease. A possible synergistic effect of the two toxins on the host immune system has also been proposed (Pezard et al., 1991; Smith and Stoner, 1967).

2.3.3. Gene Regulation

B. anthracis relies on a combination of environmental signals and responder molecules to control expression of genes critical to survival and virulence. Studies of capsule expression in a pXO1⁻ pXO2⁺ strain revealed that *acpA*, a gene present on pXO2 was essential for the transcription of the *cap* operon and expression of the encapsulated phenotype (Vietri et al., 1995).

The transcriptional transactivator gene, *atxA*, has been shown to regulate expression of the three toxin components of *B. anthracis* and to play a role in expression of the bacterial capsule (Uchida et al., 1993, 1997). Consistent with the importance of *atxA* in toxin gene expression is the observation that an *atxA*-null mutant is avirulent in mice (Dai et al., 1995). Shortly after *atxA* was identified, work by a separate group noted that temperature and CO₂ levels also influence toxin gene expression (Sirard et al., 1994). This is not surprising since germination of spores in the lungs of susceptible individuals is closely followed by toxin production. It is very likely that bacteria use the change in temperature and CO₂ levels as stimulus to turn on molecular switches that upregulate genes necessary for vegetative growth and protection against immune effector cells. One possible explanation is that temperature and/or CO₂ levels affect *atxA* expression, which in turn leads to toxin production. A later publication reported that temperature affected production of the transactivator protein but CO₂ levels did not (Dai and Koehler, 1997; Dai et al., 1995; Uchida et al., 1993).

The precise mechanisms by which CO₂ and *atxA* regulate expression of toxin genes remain unclear.

2.4. Treatment and Prevention

In the absence of previous immunity, resolution of anthrax requires antibiotic treatment prior to the onset of fulminant disease (Jernigan et al., 2001). Treatment is typically initiated with oral antibiotics, although for pulmonary anthrax or systemic dissemination, intravenous administration of antibiotics is recommended. Limited human data obtained from the U.S. anthrax patients in 2001 suggests that early detection and swift application of antibiotics can reliably prevent mortality and progression of the disease (Jernigan et al., 2001).

In August 2000 the Food and Drug Administration (FDA) approved the use of ciprofloxacin for treatment of postexposure anthrax (Meyerhoff et al., 2004). The CDC recommends ciprofloxacin or doxycycline in combination with at least one other antibiotic such as rifampin, vancomycin, or chloramphenicol for proper treatment (CDC, 2001a). Penicillin-resistant strains are emerging, and susceptibility to antimicrobials should be verified before deciding the course of treatment (Lalitha and Thomas, 1997). A 30 to 60-day course of antibiotics is the standard treatment. Data obtained from monkey models of inhalation anthrax indicate that spores can take as long as 100 days to germinate; therefore, close clinical observation should follow completion of antibiotic treatment (CDC, 2000, 2001a, b; Henderson et al., 1956). Alternative options include an extended 100-day regimen or postexposure vaccination in combination with antibiotic treatment (CDC, 2000; Friedlander et al., 1993b).

Although in the 2001 outbreak patients treated with antibiotics in early stages of the infection survived, Brookmeyer et al. (2004) speculate that swift antibiotic treatment alone will be at best 70–80% effective in larger outbreak scenarios. Therefore, preexposure immunization may be necessary to achieve higher survival rates in the event of a large-scale bioterrorist attack. The Advisory Committee on Immunization Practices (ACIP) has recommended making anthrax vaccine available to individuals who are at risk of exposure to aerosolized anthrax spores (CDC, 2000).

Studies by Marcus et al. (2004) indicate that postexposure vaccination as a sole treatment is unlikely to be beneficial. The mean time to death in guinea pigs is 2–4 days after infection while the time required to achieve protection following booster immunizations is 8 days. Also, monkeys vaccinated immediately following aerosol exposure were no better protected than naive controls, while animals that received the vaccine along with antibiotics were protected (Friedlander et al., 1993b). These data strongly suggests that vaccination alone in a postexposure scenario will not allow sufficient time for anti-PA antibody titers to develop, and treatment should be supplemented with antibiotics and/or passive immunotherapy to increase chances of survival.

Failure of antibiotic treatment in late-stage disease suggests that the infection has persisted long enough to raise toxin concentrations to lethal levels. Furthermore, it is unlikely that antibiotics combined with vaccination in patients with advanced disease will prevent death. Therefore, immediate neutralization of anthrax toxin may be the only method of preventing mortality associated with the late phase of pulmonary anthrax. Although the CDC has recognized the potential benefit of having preimmune sera on hand in the event of future outbreaks, it remains to be determined how effective this treatment could be in late-stage human disease (Enserink, 2002).

2.5. *Vaccines and Immunity*

Immunologic protection against anthrax infection is primarily determined by the level of anti-PA antibody present at the time of exposure. In the absence of significant anti-PA antibodies, pulmonary anthrax or systemic LeTx intoxication invariably leads to death. The crucial nature of anti-PA antibodies to host survival can be attributed at least in part to their ability to (1) prevent germination of extracellular spores; (2) neutralize toxin; and (3) enhance macrophage-mediated killing of engulfed spores (Welkos et al., 2001, 2002). Although the individual contributions of each of these events to survival of the host are not completely understood, collectively they afford significant protection against the most serious form of anthrax disease. Lasting host immunity is therefore dependent on the establishment of B-memory and B-effector cell populations capable of providing a continuous supply of anti-PA-specific plasma cells.

The only anthrax vaccine licensed for human use in the U.S. is Anthrax Vaccine Adsorbed (AVA). It was licensed by the FDA in 1970 and is currently provided only to military personnel, individuals at high risk of occupational exposure, and to those recently exposed or suspected to have been exposed to aerosolized spores. The vaccine is derived from the cell-free filtrate of a nonencapsulated strain of *B. anthracis*. Although AVA contains multiple bacteria and media-derived components, the primary immunogen is PA-adsorbed onto aluminum hydroxide adjuvant (Ivins et al., 1996). This vaccine is far from ideal because it requires multiple vaccine doses and it has been reported to induce adverse effects in some vaccinees (Belyakov et al., 2000; Kerrison et al., 2002)

Patients receiving AVA are administered three subcutaneous injections at 0, 2, and 4 weeks followed by three more injections at 6, 12, and 18 months. In addition to the 6 injections, annual boosters are recommended for as long as immunity is required. Since immunological memory to PA in the absence of anti-PA antibodies is not necessarily protective in animal models, annual boosters are recommended to prevent titers from falling below minimal protective levels, which could leave vaccinated individuals at risk of infection.

Intramuscular vaccination with AVA induces primarily a systemic immune response characterized by high serum IgG levels capable of efficiently neutralizing toxin in passive transfer experiments (Sawada-Hirai et al., 2004). Studies in rhesus macaques have demonstrated that animals vaccinated with AVA were protected against aerosol challenge with 93 lethal doses (LD50) of the highly virulent Ames strain of *B. anthracis* (Ivins et al., 1998). Challenge studies in nonhuman primates represent the most reliable data available regarding the efficacy of AVA in humans since there are no confirmed cases of AVA vaccinees being exposed to infectious spores.

AVA and an equivalent vaccine available in the U.K. are both prepared from the culture filtrate of nonencapsulated strains of *B. anthracis* according to procedures that have been in place for over 40 years (Puziss et al., 1963). Both vaccines are administered parenterally (intramuscular injection) and provide robust systemic immunity but limited mucosal or cell-mediated immunity. Since most microorganisms typically enter via mucosal surfaces, it is believed that local immunity may aid in preventing the initial establishment of infection and, in combination with systemic immunity, increase the minimal infectious dose required to cause disease. Advances in protein purification, adjuvants, immunology, and vaccinology have provided opportunities for researchers to design more effective vaccines that can be administered through nonparenteral (needle-free) routes and that potentially provide both mucosal and systemic immunity to pathogens.

Given the evidence that protection against anthrax correlates strongly with anti-PA antibody titers, researchers are currently exploring the effectiveness of purified PA protein in next-generation vaccines with increased safety and efficacy (Pitt et al., 2001; Reuveny et al., 2001). Besides the obvious benefit of vaccinating people with well-defined formulations, expression of PA from vectors other than *B. anthracis* is safer and easier to standardize. In anticipation of phase I clinical trials, researchers at the National Cancer Institute have demonstrated successful adsorption and desorption of recombinant PA (rPA) to FDA-approved adjuvants with little loss of biologic viability (Jendrek et al., 2003). Other researchers have characterized the immune response to intranasal immunization using rPA in combination with various experimental adjuvants (Boyaka et al., 2003). Intranasal immunization of mice using cholera toxin (CT) as adjuvant elicited mucosal and systemic IgG responses as well as high levels of type 2 cytokines. Other novel approaches to vaccination include the use of transcutaneous immunization using a patch-based vaccine with *Escherichia coli* heat-labile toxin (LT) adjuvant (Kenney et al., 2004).

Continuing research into adjuvants, novel antigens, alternative routes of administration, and mechanisms of immune development will likely lead to improved anthrax vaccines for humans. Besides providing a benefit to overall public health, research on improved vaccines and therapeutics will provide the necessary tools to protect soldiers and civilians from the threat of biological warfare.

3. Plague

3.1. Introduction

Plague is a zoonotic infection caused by the Gram-negative coccobacillus *Y. pestis*, a Gram-negative rod that has a bipolar “safety pin” appearance on Giemsa or Wayson stains (Figure 3). Several rodent and lagomorph species are natural reservoirs for plague, and the disease is transmitted between animals by hematophagous fleas or by direct contact. Humans are accidental hosts and are commonly infected after the bite of an infected flea or via respiratory droplets from animals to humans or between humans. *Y. pestis* is believed to be responsible for the great epidemic of “Black Death” which killed millions of people in Europe in the Middle Ages. Infection with *Y. pestis* is no longer a preamble to death, since most infections are resolved after antibiotic treatment; however, cases of pneumonic plague that are not promptly diagnosed and treated can be fatal.

The causative agent of plague, *Y. pestis*, was discovered by French bacteriologist Alexandre Yersin in 1894. In 1898 Paul-Louis Simon observed that fleas were the natural vector of transmission to animals (Mollaret, 1999). Plague is one of the oldest recorded infectious diseases, and a recent publication points

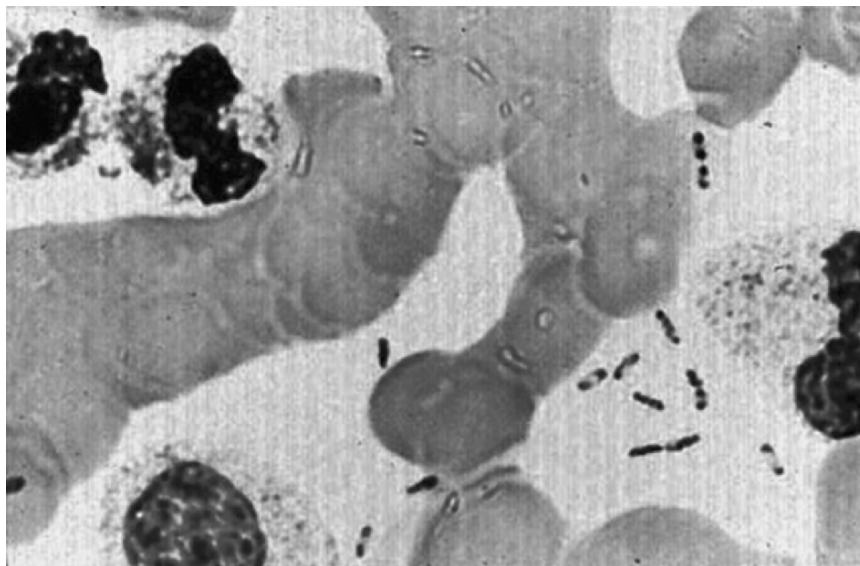


FIGURE 3. Wayson stain of *Yersinia pestis* showing the characteristic “safety pin” appearance of the bacteria. (From CDC web page <http://www.cdc.gov/ncidod/dvbid/plague/wayson.htm>).

to its origin in ancient Egypt (Panagiotakopulu, 2004). Although many unconfirmed records of ancient plague epidemics abound in the literature, it is known that the first recorded pandemic or “Justinian” plague (AD 541–767) started in Egypt and spread to Europe and Africa. The second pandemic, known as the ‘Black Death’ (1346 to the early 1600s) spread from the Caspian Sea to Europe, and the third pandemic began in 1855 in the Yunnan region of China and spread globally via ships leaving from Hong Kong (Achtman et al., 1999; Perry and Fetherston, 1997).

Y. pestis can be found in more than 200 species of wild rodents present in most continents, and over 80 species of fleas are proven vectors of plague (Anisimov, 1999, 2002a, b; Brubaker, 1972, 1991; Perry and Fetherston, 1997).

Plague first entered the U.S. in 1899 when infected rats from a ship sailing from Hong Kong to San Francisco left the vessel and entered the city’s sewer system. The San Francisco epidemic lasted 4 years and killed 118 people (Lipson, 1972). Plague in North America occurs in 15 western states of the U.S., in southwestern Canada on the border with the U.S., and in northern Mexico.

Although the improvement of living and sanitary standards worldwide has decreased the incidence of plague, the World Health Organization (WHO) reports that from 1954 to 1997 plague occurred in 38 countries, with 80,613 cases and 6,587 deaths (Dennis et al., 1999). The WHO recently reported that an outbreak of plague in Congo, Africa, started in October 2004 and caused over 20 deaths. In the U.S. bubonic plague occurs at a rate of 10–15 cases per year, mostly in veterinarians and other animal handlers (Chase, 2003; MMWR, 1996). It is clear from these epidemiological studies that this ancient scourge is far from being eradicated from the modern world.

Plague has been used as a biological weapon throughout history. Two of the most notable examples are Tartars in 1346, catapulting plague-infected corpses into Kaffa at the end of a 3-year siege, and the Japanese in the 1940s, breeding *Y. pestis*-infected fleas with the intention to release them from aircrafts over Chinese cities (Beeching et al., 2002; Christopher et al., 1997; Inglesby et al., 2000). The palpable threat of bioterrorism combined with the continuing occurrence of natural plague outbreaks emphasizes the need to understand the pathogenesis of this organism and to develop new prophylactic and therapeutic tools to control plague.

3.2. Pathogenesis

The genus *Yersinia* consists of 11 species, three of which, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, are human pathogens. Evolutionary studies indicate that 20,000 years ago *Y. pestis* evolved from *Y. pseudotuberculosis*, and that both *Y. enterocolitica* and *Y. pseudotuberculosis* evolved from a common predecessor 1 million years earlier (Achtman et al., 1999; Wren, 2003). *Y. pestis* causes plague and is transmitted by insects or by droplets, whereas

Y. enterocolitica and *Y. pseudotuberculosis* are enteropathogens that are transmitted by food and water and enter the host through M cells in intestinal Peyer's patches (Autenrieth and Firsching, 1996; Clark and Jepson, 2003), followed by invasion of lymphoid tissues (Autenrieth and Schmidt, 2000; Wren, 2003) (Figure 4).

Depending on the mode of transmission and the immune status of the infected individual, *Y. pestis* can cause bubonic, septicemic, pneumonic, pharyngeal, cutaneous, or enteric plague. Bubonic, septicemic, and pneumonic plague are the most recognized forms and will be the only ones discussed here.

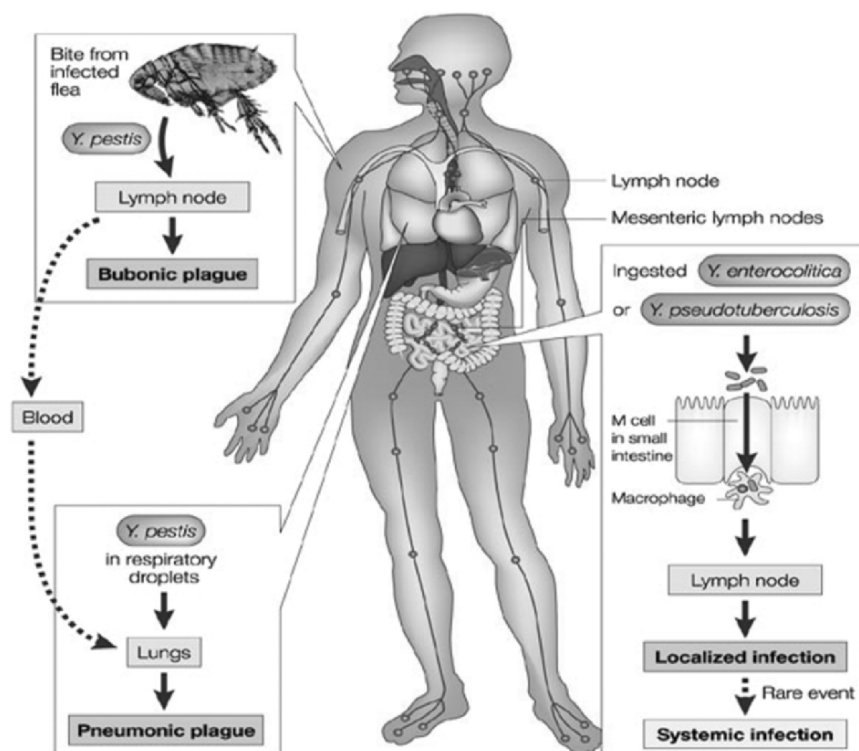


FIGURE 4. Steps in the transmission of pathogenic *Yersinia* in humans. *Yersinia pestis* has a rodent reservoir. Fleas infesting the rodents acquire *Y. pestis* from infected blood and transmit the bacterium to other rodents or occasionally to humans, causing bubonic plague. Pneumonic plague is transmitted from human to human through respiratory droplets or by artificially generated aerosols. In contrast, *Y. enterocolitica*, and *Y. pseudotuberculosis* are transmitted orally and enter the lymphatic system through the M cells of the small intestine. (Reproduced with permission from Wren, 2003.)

The bubonic plague is the most common form of the disease and occurs following a bite from a flea that has previously taken a blood meal from an infected animal (Brubaker, 2003). *Y. pestis* disseminates from the initial site of infection and drains to the local lymph nodes, especially axillary or inguinal, which rapidly become swollen, necrotic and painful. The swollen lymph nodes, known as “buboes”, can get larger than a golf ball and constitute the classical pathognomonic feature of bubonic plague. Bacteremia often develops as bacteria from the bubo spill into the bloodstream, and large numbers of bacteria can be usually cultured from the blood (Butler, 1989; Crook and Tempest, 1992).

Septicemic plague arises when bacteremia occurs without notable bubo formation. It is characterized by fever, headache, malaise, and gastrointestinal disturbances. These symptoms are nonspecific, making the initial diagnosis difficult and delaying the start of antibiotic therapy. Even with medical treatment, about 30–50% of the cases are fatal (Hull et al., 1987).

Pneumonic plague, the most lethal form, arises after inhalation of bacteria-containing droplets, followed by colonization of the alveoli in the lungs with *Y. pestis*. The incubation period is 1–3 days, and patients with this type of infection produce bloody sputum that is highly contagious. Plague acquired this way has a 100% mortality rate if antibiotic treatment is not administered at the first signs of illness. *Y. pestis* pneumonia can also develop after initial bubonic or septicemic plague. Regardless of how it is acquired, pneumonic plague can spread rapidly within a population (Perry and Fetherston, 1997). This contagious and deadly form of plague would be the most likely outcome of an intentional bioterrorism attack using aerosolized bacteria.

One of the most interesting aspects of the pathogenesis of *Y. pestis* is the strategy used by the bacterium to ensure propagation from the insect vector. When a flea ingests blood containing *Y. pestis*, the bacteria start multiplying rapidly in the midgut of the insect. Two days after ingestion of infected blood, the stomach of the fleas have large masses containing fibrin, hemin, and bacteria, which continue to grow over a period of about 1 week. This produces a “blockage” that prevents the flea from ingesting food. To avoid starvation the insect needs to get rid of the blocking material by regurgitating the *Y. pestis*-laden mass. As the flea takes its next meal, it delivers the bacteria onto the wounded skin of the host (Gage and Kosoy, 2004).

3.3. Virulence

The most significant differences between *Y. pestis* and the other *Yersinia* species evolved after *Y. pestis* gained several genes by lateral transfer, and in the process acquired the ability to be transmitted by insect vectors and to disseminate through lymph and interstitial spaces. It is known that *Y. pestis* carries two plasmids not present in the enteropathogenic *Yersiniae*: pPla and pMT1 (Ferber and Brubaker, 1981). Additionally, some of the genes that facilitate invasion of enteropathogenic *Yersiniae* to the small intestine are

inactive in *Y. pestis* (*YadA*, *Inv*) (Brubaker, 2003). It has been postulated that the loss of these genes may contribute to the severity of plague (Perry and Fetherston, 1997).

Despite the genetic similarities with *Y. pseudotuberculosis*, *Y. pestis* causes a very different and much more severe disease (Parkhill et al., 2001). All pathogenic *Yersinia* species express virulence factors that enable them to enter, colonize, and multiply in lymphatic tissues and organs. Among these virulence factors are iron-binding siderophores, toxins, adhesins, and invasion molecules. Most importantly, all three pathogenic *Yersinia* species use a type III secretion system to inject effector proteins (Yops) into the host cell.

The pathogenicity of *Y. pestis* results from its ability to overcome the defenses of the host and to multiply rapidly, reaching high numbers in infected tissues and blood. *Y. pestis* is a facultative intracellular pathogen that is thought to be intracellular only during the early stages of infection, followed by extracellular growth at later stages (Brubaker, 1991; Straley et al., 1993). Therefore, to be a successful pathogen, *Y. pestis* needs effective mechanisms to survive both in the intracellular and extracellular compartments. Here, we present a summary of the most important virulence factors described in the plague bacillus.

3.3.1. *Yersinia* Outer-membrane Proteins

All pathogenic *Yersinia* species contain the 70 kb virulence plasmid pYV, also called low-calcium response plasmid (pLcr), which enables the bacteria to survive and multiply in the lymphoid tissue of their host (Brubaker, 1983; Cornelis et al., 1998). Curing of this plasmid results in total avirulence of the *Yersinia* strains studied thus far (Brubaker, 1983; Kutyrev et al., 1989). This plasmid encodes the Yop virulon, a system consisting of secreted effector proteins called *Yersinia* outer-membrane proteins (Yops) and a type III secretion apparatus that serves to inject Yops into the host cell. The injected Yops disturb the dynamics of the cytoskeleton and block the production of pro-inflammatory cytokines, thereby favoring the survival of the invading *Yersinia* (Bliska and Black, 1995; Fallman et al., 1995; Grosdent et al., 2002; Rosqvist et al., 1990). Of particular interest, YopH has a direct effect on inflammation by its ability to downregulate expression of costimulatory molecule B7.2, thereby modulating T cell- and B cell-mediated responses and possibly repressing monocyte chemotactic protein 1 (MCP-1).

3.3.2. V-antigen

The V-antigen, or LcrV protein, which is also encoded by the pYV plasmid, was assumed to be a protective antigen shortly after its discovery in 1956 by T. W. Burrows (Burrows, 1956; Burrows and Bacon, 1958; Une and Brubaker, 1984). In 1994, Motin demonstrated passive immunity mediated by antibodies against a staphylococcal A-LcrV fusion (PAV) (Motin et al., 1994). He also showed that injection of PAV prevented upregulation of IFN- γ and TNF- α by

LcrV-deficient mutants, providing additional evidence that this protein blocks the inflammatory response (Nakajima et al., 1995). It has since been shown that V-antigen serves two broad functions. First, V antigen (along with YopB and YopD) is responsible for selective targeting of effector proteins into the cytosol of infected cells by the type III secretion system (Cornelis, 2000; Cornelis et al., 1998). Second, V antigen is secreted into the environment (Fields et al., 1999), and can act on macrophages to induce the immunoregulatory cytokine IL-10 (Sing et al., 2002a). While the exact relevance of V antigen on virulence and immune evasion is still unclear, recent evidence points to V-antigen signaling through CD14 and TLR2 (Sing et al., 2002b).

3.3.3. F1 Capsule

The 96 kb pMT1 (also known as pFra) plasmid encodes the *Yersinia* murine toxin Ymt and the F1, or Caf1, capsule. The murine toxin has been shown to facilitate colonization of the flea and has phospholipase D activity that is toxic to mice (Hinnebusch et al., 2000; Rudolph et al., 1999). F1 is the major protein component of the capsule (Baker et al., 1952; Burrows, 1963) and lends resistance to *Y. pestis* against phagocytosis by macrophages (Cavanaugh and Randall, 1959; Du et al., 2002). Prolonged growth at 37°C is necessary for capsular development (Du et al., 2002). Not surprisingly, *Y. pestis* does not synthesize F1 in the flea vector (Cavanaugh and Randall, 1959). Mutants unable to synthesize F1 occur in nature, but in general are not highly attenuated; probably because their ability to secrete type III Yops is enhanced (Davis et al., 1996; Drozdov et al., 1995; Du et al., 1995; Welkos et al., 1995). It has been reported that *Y. pestis* F1-negative strains are virulent in mice (Friedlander et al., 1995), African green monkeys (Davis et al., 1996), and humans (Winter et al., 1960).

3.3.4. Plasminogen Activator Pla

The 9.6 kb pPla, or pPst, encodes the plasminogen activator Pla, a coagulase/fibrinolysin that interferes with blood coagulation and complement pathways (Beesley et al., 1967; Sodeinde and Goguen, 1988). Pla is very important for the dissemination of *Y. pestis* from peripheral infections; Pla mutants injected subcutaneously produced only a localized infection with high numbers of inflammatory cells in lesions when compared with nonmutated strains (Sodeinde et al., 1992; Welkos et al., 1997). It is believed that during the emergence of *Y. pestis* from *Y. pseudotuberculosis*, fibrillar adhesin YadA and cell invasin Inv were replaced by tissue invasin Pla (Finlay and Falkow, 1997; Perry and Fetherston, 1997).

In summary, the Pla protease allows *Y. pestis* to be highly invasive, the capsule offers immediate resistance to phagocytosis by macrophages, Yop injection cripples the immune response of surrounding cells, and secreted LcrV causes a systemic anti-inflammatory response by reducing IFN- γ and TNF- α and increasing IL-10 production.

3.4. Treatment

The key to treating plague, particularly the pneumonic or septicemic form, is rapid diagnosis and administration of prompt antibiotic therapy. Most therapeutic guidelines suggest using streptomycin or gentamicin as first-line therapy against plague, with ciprofloxacin as optional treatment. Additionally, patients should be monitored for septic shock in an intensive care unit. Persons who come into contact with plague patients should receive antibiotic prophylaxis for 7 days. In order to prevent human-to-human transmission, quarantine should be observed until the patient has received at least 4 days of antibiotic treatment. For the other clinical manifestations of the disease, e.g., bubonic plague, patients should be isolated for the first 48 h after the initiation of treatment (Bossi et al., 2004).

3.5. Vaccines and Immunity

The development of an effective vaccine against plague is desirable since plague is endemic in many parts of the world and there is an ever-present likelihood of the illegitimate use of *Y. pestis* by bioterrorists. An ideal vaccine should be safe, require few doses, cause little or no side effects, be easy to administer, and be inexpensive to manufacture in mass quantities. More specifically, an ideal vaccine would induce sufficient levels of effector memory cells capable of rapid expansion upon exposure to plague. A number of candidate vaccines have been described and tested, among which the most recently described subunit vaccines have great potential for successful development into a practical vaccine for human use.

The Cutter USP, or killed whole cell (KWC), vaccine is a formaldehyde KWC preparation developed from a virulent *Y. pestis* strain. This preparation is expensive, has a high degree of heterogeneity, has variable endotoxin content, requires frequent boosters, and can cause a variety of local and systemic side effects (Russell et al., 1995). More importantly, there have been cases of pneumonic plague reported in vaccinated individuals indicating inadequate immunity at mucosal surfaces (Meyer, 1970). The Cutter vaccine is no longer approved for use in the U.S.

Live attenuated EV76 vaccine, which has been used since 1908, is a non-pigmented mutant of *Y. pestis* that is unable to assimilate chromogenic substances such as Congo Red (Meyer, 1970). The live EV76 vaccine induces better protection against *Y. pestis* challenge than the KWC vaccine, but the live vaccine strain is not fully avirulent and can cause disease in the mouse model (Russell et al., 1995). In humans, the EV76 vaccine has been tested extensively but its effectiveness is questionable (Meyer, 1970; Meyer et al., 1974). Furthermore, significant variability in efficacy and side effects is seen between various EV76 preparations (Russell et al., 1995).

While the EV76 vaccine is not safely attenuated for use in humans, it does yield protection against both bubonic and pneumonic plague, suggesting that

attenuated bacteria may be a possible venue for a safe and effective plague vaccine. Other attenuated bacterial species carrying plasmids coding for plague antigens can elicit significant systemic and mucosal anti-*Yersinia* immune responses. Studies have shown increased protection against plague infection following subcutaneous immunization of mice with attenuated *Salmonella typhimurium* expressing F1 protein (Oyston et al., 1995; Titball et al., 1997). However, mice and African green monkeys immunized this way are not protected against all *Y. pestis* strains (Friedlander et al., 1995).

Other studies showed that an oral vaccine with attenuated *S. typhimurium* expressing V antigen protected mice from subcutaneous plague challenge (Garmory et al., 2003). Immunization studies with *S. typhimurium* expressing both F1 and V antigen induced 1,000 times greater protection than that induced by *S. typhimurium* expressing F1 alone (Leary et al., 1997).

The use of plasmid DNA encoding protective antigens has been demonstrated to be an effective method of generating immune responses against several viral and bacterial pathogens. A DNA construct encoding the V antigen was shown to induce immune responses in the mouse after delivery of the plasmid either intramuscularly or intradermally with a gene gun (Bennett et al., 1999; Garmory et al., 2004). It has also been determined that the inclusion of other antigens (*B. anthracis* PA) in the first DNA immunization and in the subsequent protein boost enhanced the protective immune response to *Y. pestis* (Williamson et al., 2002). Significant antibody responses were also observed following a 3 or 4 immunization regimen with optimized DNA vaccines encoding the F1 protein (Grosfeld et al., 2003).

Despite the potential reported from the vaccine strategies listed above, there are many known disadvantages to killed, live attenuated, and DNA vaccines, including negative side effects, lack of protection against aerosolized plague, and the need for frequent boosters. To overcome these disadvantages and to improve efficacy, numerous studies are now being conducted with acellular vaccines made up of recombinant plague proteins (Heath et al., 1998; Williamson et al., 1997). Many surface-expressed plague proteins have been studied and have been shown to induce high levels of specific antibody (Andrews et al., 1999; Benner et al., 1999). However, only immune responses against F1 and V antigen proteins provided protection against *Y. pestis* challenge (Titball and Williamson, 2001). F1 and V antigen have been expressed as recombinant proteins (Leary et al., 1995; Miller et al., 1998) and have been shown to induce protective responses when used separately (Anderson et al., 1996; Leary et al., 1995; Roggenkamp et al., 1997).

A more recent approach to vaccination is the use of a genetic F1-V fusion (Heath et al., 1998), coadministered with alum. This vaccine induces significant protection in several animal models (Anderson et al., 1998; Heath et al., 1998; Jones et al., 2000, 2003; Williamson et al., 1995, 1996, 1997, 2000). A study in mice, evaluating the effect of using different routes of vaccination for the initial priming and the subsequent boosting, indicated that long-lasting high titer

specific antibody levels were developed whether mice were boosted by the same or different route of administration (Glynn et al., 2005).

Another novel approach to antiplague vaccination is the microencapsulation of antigenic peptides. This provides an adjuvant effect by improving uptake into antigen presenting cells (APCs) (Morris et al., 1994) and sustained release of the antigen over long periods of time (Eldridge et al., 1991; Nakaoka et al., 1996). Several studies have demonstrated successful inclusion of F1 and V antigen into microspheres (Eyles et al., 1998a, b; Spiers et al., 1999; Williamson et al., 1996). Immunization with these microcapsules leads to increased protection, which is further enhanced by inclusion of a mucosal adjuvant (Eyles et al., 1998a; Williamson et al., 1996).

Many studies have indicated that the strongest correlate of protection is the presence of antibodies against *Y. pestis* antigens (Elvin and Williamson, 2000; Williamson et al., 1999). Circulating anti-F1 and anti-V antibody presumably functions to bind surface-exposed or secreted *Y. pestis* proteins. It is known that innate immune responses are necessary to resolve the necrotic lesion(s) caused by a natural *Y. pestis* infection (Brubaker, 2003). However, upon *Y. pestis* infection, the V antigen inhibits innate immunity by inducing upregulation of IL-10 and downregulation of IFN- γ and TNF- α , thereby preventing granuloma formation and instead allowing the development of necrotic lesions (Moore et al., 2001; Wang et al., 1995). This dramatic decrease of Th1 cytokines is shown to be very characteristic of plague. Studies have shown that passive administration of anti-V serum prevents the downregulation of IFN- γ and TNF- α (Nakajima and Brubaker, 1993; Nakajima et al., 1995). A vaccine that generates a type 2 antibody response against V antigen could conceivably protect against disease by neutralizing the physical and chemical effects of the V antigen on innate immunity. A positive correlation between decreasing levels of antibody titers, especially IgG1, and decreasing protection against challenge has been established (Williamson et al., 1999).

Recent studies, however, suggest greater involvement of a type 1 response in protection against plague. Delivery of F1 and V with Ribi adjuvant to IL-4 receptor knockout mice induced a predominantly type 1 response. Yet, transfer of serum from these animals into B cell knockout mice was sufficient to protect the passively transferred animals from challenge (Elvin and Williamson, 2000). Elvin and Williamson (2004) observed that F1- and V-immunized Stat 4 knockout mice (with a defect in the type 1 cytokines IFN- γ and IL-12) do not survive *Y. pestis* challenge, whereas immunized Stat 6 knockout mice (with a defect in type 2 cytokines IL-4 and IL-13) do not show compromised protection compared to immunized normal syngeneic mice.

The involvement of both type 1 and 2 immune responses in protection against plague is a practical finding in the sense that Th1/Th2 protective responses rarely, if ever, exist separately in humans. These new studies illuminate the importance of examining both types of immune responses in experimental immune response and challenge models.

4. Tularemia

4.1. Introduction

Tularemia is a severe and potentially lethal zoonotic disease in humans caused by the Gram-negative bacterium *Francisella tularensis*. It is considered a dangerous potential biological weapon because it is highly infectious and can cause serious illness and death, especially after inhalation of the aerosolized bacteria.

Tularemia was first recognized in Japan in 1837. In 1911, American investigators described a plague-like illness killing rodents in Tulare County, California. The isolated causative organism was named *Bacterium tularense*. In 1921, Edward Francis linked the etiological agent as being the cause of multiple tularemia syndromes in animals and humans. In honor of Francis, who devoted his life to researching the pathogenesis of this organism, the bacterium was later renamed *F. tularensis* (Dennis et al., 2001).

F. tularensis has been recognized as a biothreat for decades. Between 1932 and 1945, the Japanese studied tularemia in their biowarfare research facilities in Manchuria (see Historical Landmarks). It has been suggested that the outbreaks of tularemia in Russian and German soldiers during World War II might have been intentionally caused. In the 1950s and 1960s, there were programs in the U.S. and in the former U.S.S.R. to develop tularemia as a biological weapon, and the organism was grown and stockpiled by both countries. After the U.S. signed the Biological and Toxic Weapons Convention in 1972, all the stockpiled biological weapons were destroyed (Dennis et al., 2001).

Tularemia is a disease that occurs naturally in many parts of the world and is particularly prevalent in the northern hemisphere. In the U.S., the number of cases declined from several thousand in the 1930s to less than 200 in 2000. Natural reservoirs of *F. tularensis* include multiple species of animals such as rodents, rabbits, hares, voles, and water rats; but the bacteria can also be found in insects and protozoans, and water transmission of the disease from wild animals to humans can occur via an arthropod vector. Several species of ticks (*Dermacentor reticulatus*, *Ixodes ricinus*), mosquitoes (*Anopheles*, *Aedes*), and biting flies have been implicated in transmission (Ellis et al., 2002; Oyston et al., 2004). Direct transmission from person to person has not been documented.

F. tularensis is a nonmotile, encapsulated, nonspore-forming, fastidious, Gram-negative coccobacillus. Four distinct subspecies of *F. tularensis* have been described based on virulence, O-antigen structure, biochemical tests, and geographical location where the organism is found. The subspecies are *tularensis* (type A), *holartica* (type B), *novicida*, and *mediasiatica*. Of these, the subspecies *tularensis* is the most virulent, with an infectious dose of less than 10 CFU and a mortality rate of 5–30% in the absence of treatment. Subspecies *holartica* and *mediasiatica* have low virulence, and subspecies *novicida* is only reported to cause disease in immunocompromised humans (Ellis et al., 2002; Titball et al., 2003).

4.2. Pathogenesis

F. tularensis is a zoonotic disease found mostly in rodents and lagomorphs. Humans are incidental hosts that can become infected by different routes of exposure. Infection might occur after: the bite from an infected insect, handling infected animal carcasses, drinking contaminated water, eating contaminated meat, or inhaling infectious aerosolized droplets. The route and site of transmission, the virulence of the infecting organism, and the immune status of the host determine the clinical presentation of the disease. The incidence of tularemia is higher in people with an occupational risk, such as hunters, butchers, farmers, and laboratory workers (Dennis et al., 2001; Feldman et al., 2001; Lawler, 2005). The six clinical types of tularemia described have significant symptomatology overlap between them (Lamps et al., 2004). The clinical presentations are: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal, and septic tularemia. The pneumonic form is the most severe and has the highest rate of mortality, but most clinical types can include pulmonary involvement.

The most common type is ulceroglandular tularemia, which appears after an arthropod bite, or after handling contaminated material. An inflamed local ulcer develops at the site of infection, which later becomes a pustule. Although the ulcer heals, bacteria can disseminate to the local lymph nodes, which become grossly swollen and tender. These enlarged lymph nodes closely resemble the characteristic “buboes” seen in bubonic plague.

Oculoglandular tularemia occurs after accidental inoculation of the conjunctiva with infected fingers. The disease is manifested by inflammation and the appearance of ulcers in the eye. Glandular tularemia is characterized by lymphadenopathy in the absence of an ulcer.

Oropharyngeal or gastrointestinal tularemia usually develops after the ingestion of contaminated water or food. Infected patients develop pharyngitis, tonsillitis, and cervical lymphadenopathy. Typhoidal and septic tularemia are similar clinical syndromes. They are severe, potentially lethal infections and are manifested after systemic dissemination of the organism, without evident cutaneous or mucosal involvement.

Respiratory tularemia, the most serious form of the disease, occurs after inhalation of infected aerosols or after hematogenous dissemination from a localized infection. This produces a severe, febrile illness, with initial flu-like symptoms progressing to bronchiolitis, pleuropneumonitis, and hilar lymphadenitis, with many patients developing acute symptoms 3–5 days after exposure. Pulmonary tularemia can progress rapidly and lead to acute respiratory failure and death (Oyston et al., 2004; Titball et al., 2003).

4.3. Virulence

The virulence factors that enable *F. tularensis* to be such a successful pathogen have not yet been identified. *In vivo*, *F. tularensis* is a facultative intracellular parasite with a predilection for macrophages (Figure 5). The

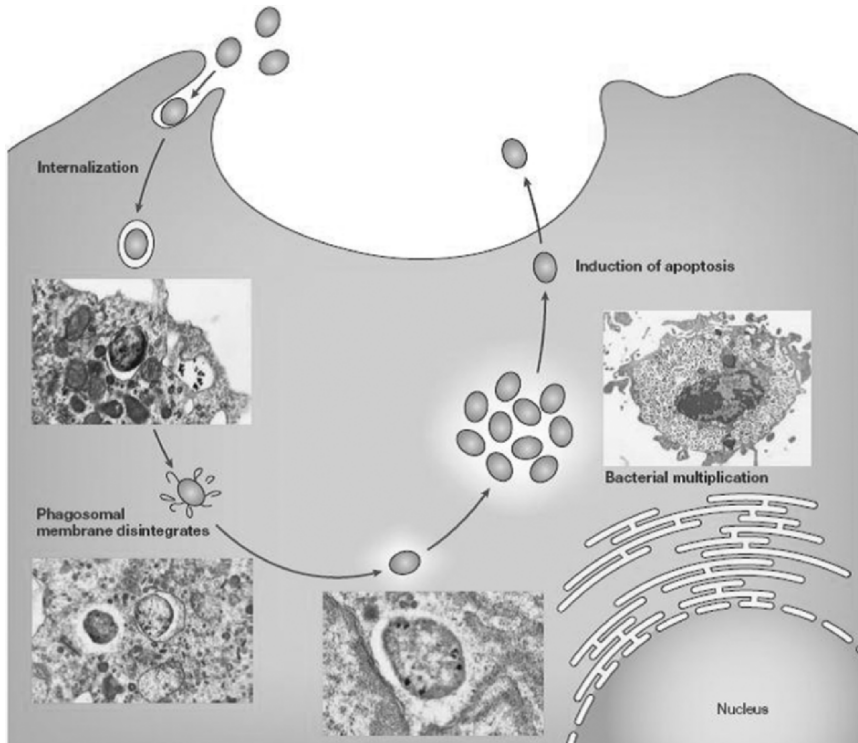


FIGURE 5. The intracellular lifestyle of *Francisella tularensis*. Steps in the intracellular replication cycle of *F. tularensis* resemble those observed for replication of *Listeria monocytogenes* inside macrophages. (Reproduced with permission from Oyston et al., 2004.)

bacteria enter macrophages without activating the respiratory burst, disrupts the phagosome membrane, and gains access to the macrophagic cytoplasm where rapid multiplication takes place. A bacterial acid phosphatase encoded by AcpA has been shown to inhibit the respiratory burst (Reilly et al., 1996). However, the role of this protein in virulence is tentative at best, since mutant Acp strains of *F. tularensis* subspecies *novicida* are still virulent in mice and can replicate in macrophages (Baron et al., 1999).

Proteomic studies have revealed that four *F. tularensis* proteins ranging in size from 20 to 70 kDa are upregulated when the bacteria are grown inside macrophages. The identity of these proteins and the role they play in virulence have not yet been elucidated (Golovliov et al., 1997). It is known that a 23 kDa protein encoded by IglC plays a role in intracellular multiplication of the subspecies *holartica* and *novicida*. Other genes such as MinD (Anthony et al., 1994) and those controlled by the operon *mgIAB* (Lauriano et al., 2004) have been implicated in pathogenicity.

The LPS of *F. tularensis* is different from other Gram-negative bacteria LPS, as it does not seem to have a role as a typical proinflammatory endotoxin. *Francisella* LPS displays phase variation *in vitro*, producing phenotypically distinct colonies. However, the significance of this LPS change to virulence and to human disease is not known (Cowley et al., 1996). Also, it is known that the capsule is essential for serum resistance, but not necessary for survival after phagocytosis by polymorphonuclear leukocytes (Sandstrom et al., 1988).

The recently published complete genomic sequence of *F. tularensis* strain SchuS4 (the subspecies *tularensis* type strain) constitutes a new powerful tool for the identification and characterization of virulence factors in *Francisella*. The sequence revealed previously uncharacterized genes encoding type IV pili, a surface polysaccharide and iron-acquisition systems, as well as several virulence-associated genes located in a putative pathogenicity island (Larsson et al., 2005).

4.4. Vaccines and Immunity

A live vaccine strain (LVS) derived from attenuated *F. tularensis* has been used since 1960 to protect at-risk individuals. This vaccine has been used in the U.S. and Europe as an investigational new drug (IND), but it has never been fully licensed. LVS vaccine, administered by scarification, was shown to reduce the incidence of aerosolized tularemia from 5.7 to 0.27 cases per 1,000 at-risk workers, although it did not reduce the frequency of ulceroglandular disease (Burke, 1977). Further studies in human volunteers indicated that vaccination with LVS did not afford complete protection against a high-dose *F. tularensis* aerosol challenge (Eigelsbach et al., 1967). Other routes of administration of LVS (such as oral and nasal) have been shown to induce equal or better levels of protection against aerosolized *F. tularensis* challenge.

Although strong evidence indicates that LVS induces significant levels of protection, the FDA withdrew the IND status of LVS and is currently reviewing its use. Because the nature of the attenuating mutations in LVS is not known, there are legitimate concerns about the possibility of reverting to full virulence, or about the effects of this live vaccine on immunocompromised individuals.

In search for a better tularemia vaccine, other approaches have been investigated. Killed whole cell *F. tularensis* vaccines are protective only against low systemic challenges in animals, while in humans this vaccine reduced the number of infections and altered the course of infection (Ellis et al., 2002). The development of purified subunit vaccines has been hampered by our limited knowledge of the virulence factors and/or the protective immunogens of this organism. Vaccination of mice with LPS obtained from LVS induces varying degrees of protection against aerogenic or systemic challenge (Conlan et al., 2002b, 2003). Several outer-membrane proteins have been tested as potential vaccines against tularemia, but so far, no definite protective antigens have been identified.

An important piece of the puzzle for rational vaccine design is the understanding of the necessary immune responses to evoke protection. The immune correlates of protection against tularemia have not yet been identified. It has been reported that CD4+ and CD8+ cells (Conlan et al., 1994) as well as IFN- γ and IL-12 are necessary for protection (Anthony et al., 1989; Conlan et al., 2002a; Duckett et al., 2005; Elkins et al., 2002), but the exact contribution of these responses to clinical protection is not known.

The recently published genome sequence of *F. tularensis* strain SchuS4 (Larsson et al., 2005) is likely to provide new insights into the nature of previously uncharacterized protective antigens or might reveal new targets for immuno intervention or treatment against tularemia.

Questions to Consider

1. What would be the ideal vaccination approach against bacterial biowarfare agents?

An ideal vaccine should be safe, require few doses, induce long-lasting and rapidly developed protection, cause little or no side effects, be easy to administer, and be inexpensive to manufacture. In addition, this ideal vaccine should prevent the infection instead of resolving an established infection. For example, an ideal vaccine against pulmonary anthrax would prevent the germination of spores within the alveolar macrophages, thereby eliminating the chances of vegetative bacilli ever reaching internal organs.

2. What is the advantage for the anthrax bacilli to make a toxin that can bind to a receptor present on a wide variety of eukaryotic cells?

A recurring theme on the pathogenicity of many bacterial toxins is the subversion of ubiquitous host membrane molecules as specific receptors for their own toxin-binding subunits. In the case of the two identified anthrax toxin receptors, TEM8 is a membrane protein implicated in the neovascularization of human colon tumors and developing mouse embryos, while CMG2 is expressed in endothelial cells during capillary morphogenesis. However, the exact role of these proteins in normal cellular function and the reason why anthrax PA binds to them with such high affinity are not yet exactly known.

3. Theoretically speaking, which bacterial pathogen would be the most efficient “weapon of mass infection”?

The dissemination of anthrax via the U.S. postal system is an example of how even very small quantities of a biological pathogen spread intentionally can create a state of panic, produce great disruption to society and result in huge economic losses. Although many of the potential biotreats have this capacity, the most feared biowarfare agents are those that are highly infectious and can disseminate from person to person. Among the three category A bacterial pathogens discussed here, *Y. pestis* is the only one that can be spread by aerosol droplets between humans.

4. Considering that several bacterial biowarfare agents are easily obtainable from nature, why do you think there have not been many instances of successful mass bioterrorism using these agents?

The manufacture of lethal biological aerosols is, for the most part, beyond the capacity of individuals or groups without access to advanced biotechnology. The “weaponization” of microorganisms requires the concerted effort of professionally trained microbiologists, and industrial and manufacturing engineers. The process would need to be carried out in highly sophisticated laboratories to ensure that the aerosolizable microbial particles have the physical characteristics (e.g., density, weight, size), are viable, highly infectious, and virulent, and are properly contained, packed, and distributed.

5. Could anthrax toxin have beneficial uses for human health?

The use of various bacterial toxins as weapons to kill specific populations of cells (e.g., tumor cells) has been amply investigated using other potent bacterial toxins (i.e., diphtheria toxin and botulinum toxin). Although the potential therapeutic uses of anthrax toxin have not been investigated, it is conceivable to imagine that the toxin could be genetically modified to target specific cells for therapeutic purposes.

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