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Current and Future Medical Approaches To Combat the Anthrax Threat

Dimitrios G. Bouzianas*

Laboratory of Molecular Endocrinology, Division of Endocrinology and Metabolism, AHEPA University Hospital, 1 S. Kyriakidi Street, P.C. 54636, Thessaloniki, Macedonia, Greece

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The terrorism events of September 11, 2001, and the subsequent *B. anthracis* spore attacks through the U.S. mail system have demonstrated their feasibility as a bioterrorism weapon. Moreover, they have shown that the West needs to be prepared for an increasing number of terrorist attacks, which may include the use of biological warfare. In the absence of specific intelligence and integrated real-time detection systems, the unpredictable nature of bioterrorism events calls for the development of medical countermeasures, which will enable the authorities to treat the exposed individuals.[1,2] Following a biological anthrax attack, vaccination in conjunction with antibiotic administration appears to be the most effective and economical form of mass protection. However, such a countermeasure is not as effective as it may seem because the current vaccines have drawbacks and the interval between initial exposure and the onset of treatment can be lengthy[3] as well. For these reasons, therapeutic strategies that emphasize toxin inactivation are seen to be worth pursuing.

At present, physicians have antibiotic options at their disposal to eliminate an anthrax infection, but they have no therapeutic options to combat the lethal factor (LF[a]) mediated toxemia and tissue destruction or the residual toxemia that persists even after the bacteria have been eliminated by antibiotics.[4] New details regarding anthrax pathogenesis and host responses have emerged since the terrorist attack in 2001, which demonstrate that not only a better understanding of the biology of virulence factors but also their interaction with the host during infection could lead to the development of improved options against anthrax.[5,6]

Research efforts for the development of improved treatment modalities have increased dramatically, leading to a huge expansion in the existing literature, and it is precisely this that makes reviewing such a topic so difficult. The present study focuses on the most important medical countermeasures against inhalational anthrax that have been developed mainly since 2001 and also highlights current problems and the possible development of improved human anti-infective strategies.

1. *Bacillus anthracis* as a Biological Weapon

In the early days of microbiology, about 130 years ago, anthrax was a serious and damaging disease in domesticated animals and the farming economy, but this problem was realized and effective countermeasures with vaccines were deployed. Once again, it is necessary to focus on anthrax but this time as a bioterrorist agent.[1,2,7]

Primarily, anthrax is a zoonotic disease but it has long been considered a potential biological warfare agent because it can cause massive mortality through the intentional release of aerosolized spores. Naturally present in soil, the *B. anthracis* endospores can survive in a dormant state for decades and their small size, about 1−2 μm in diameter, renders them optimal for inhalation and deposition in the alveolar spaces.[8] Resilient properties such as resistance to a wide range of extreme environmental conditions (heat, desiccation, UV, γ-irradiation, and oxidation), the efficiency with which they infect via an aerosol route, and the potentially life-threatening nature of the disease make spores an ideal biological weapon.[9]

Infective spores can be obtained from fermentation cultures, and after undergoing specific treatments, large quantities of a purified powder are collected that are suitable for aerosol disseminaton over a wide area from a flying aircraft.[9,10] Data from animal experiments show that 2500−55000 spores of anthrax powder constitute the median lethal inhalation dose (LD_{50}) for humans.[9] Although no official analysis of the concentration of spores has been published, anthrax powder has been reported to contain between 100 billion and 1 trillion spores per gram; hence, an envelope similar to that sent to Senator Daschle in 2001, containing 2 g of *B. anthracis* powder spores properly distributed, could kill large numbers of people.[2] Depending on the weather conditions and means of delivery, a kilogram of aerosolized spores has the potential to eventually cripple a metropolitan area, killing hundreds of thousands of people.[11] For these reasons the organism and the disease it causes have recently come under increased scrutiny[12] and the intelligence community and civilian experts consistently rank anthrax spores as the leading bioweapon threat.[2]

*Phone: +30 2310 934 094. Fax: + 30 2310 934 094. E-mail: bouzianas@the.forthnet.gr.

[a] Abbreviations: AIG, (polyclonal) anthrax immunoglobulin; AMs, alveolar macrophages; ATXR-1/TEM-8, anthrax toxin receptor-1/tumor endothelium marker-8; ATXR-2/hCMG-2, anthrax toxin receptor-2/human capillary morphogenesis protein-2; AVA, anthrax vaccine adsorbed; CDC, Centers for Disease Control and Prevention; CFUs, colony forming units; DHHS, Department of Health and Hunan Services; DNI, dominant negative inhibitor; EF/ET, edema factor/edema toxin; FDA, Food and Drug Administration; γ-D-PGA, γ-D-poly-glutamic acid; IL-1, interleukin-1; Ig, immunoglobuline; LDCs, lung dendritic cells; LF/LT, lethal fctor/lethal toxin; MAPKKs, mitogen-activated protein kinase kinases; mAbs, monoclonal antibodies; NIAID, National Institute of Allergy and Infectious Diseases; PA, protective antigen; PMNs, polymorphonuclear neutrophils; rPA, recombinant protective antigen; sCMG2, soluble capillary morphogenesis protein-2; sTEM-8, soluble tumor endothelium marker-8; TNF-α, tumor necrocis factor-α; VWA, von Willebrand factor type A.

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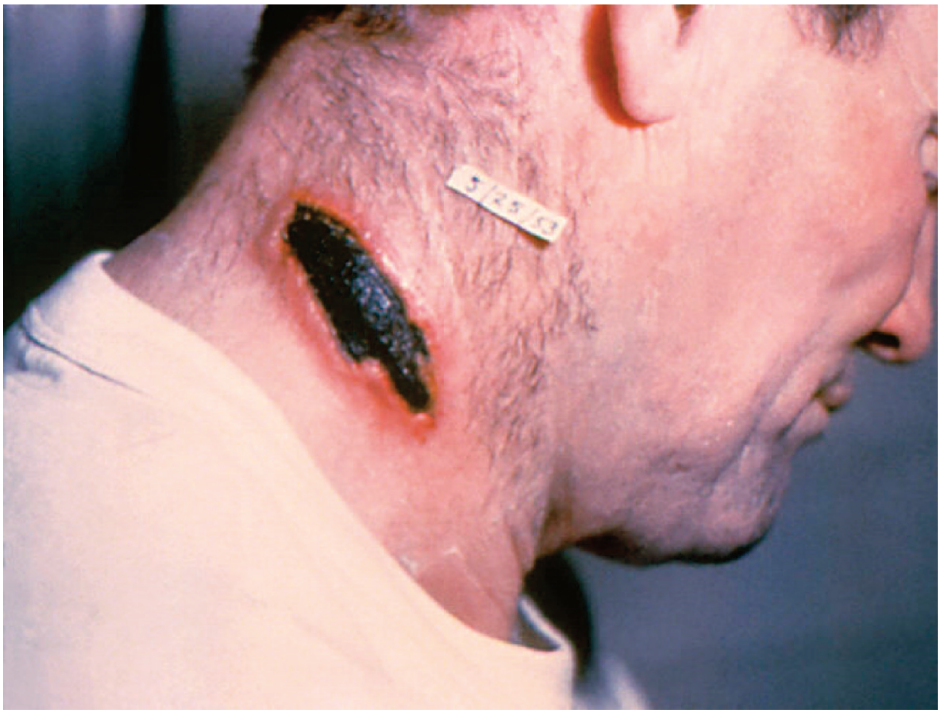

Figure 1. Typical black eschar of a cutaneous anthrax lesion on the neck of a male patient as a result of LT action. The term *anthrax* is derived from the Greek word *anthracites* and refers to coal-like, cutaneous lesion. The eschar dries, loosens, and falls off during the following 2−4 weeks, often resulting in complete healing with minimal or no scar formation. The photograph is Public Domain. Courtesy of CDC/Public Health Image Library (http://phil.cdc.gov/phil/home.asp).

Research on anthrax as a biological weapon began more than 80 years ago, and various countries have developed anthrax as a biological warfare agent, such as Japan, the U.K., the U.S., the former Soviet Union, and more recently, Iraq, which admitted to the United Nations in 1995 that it fielded weapons containing *Bacillus anthracis* spores.[8,11] Today at least 17 nations are believed to have offensive biological weapons programs that include anthrax. Moreover, some independent terrorist groups have indicated their intent to use anthrax spores as a biological weapon.[2,7]

The Sverdlovsk accident in 1979 provides data on the only known aerosol release of *B. anthracis* spores resulting in an epidemic.[13] In 1970, the World Health Organization (WHO) provided a grim estimate of the numbers of casualties that there would be if dried anthrax were released in aerosolized form on large cities; it estimated that 50 kg of *B. anthracis* released over an urban population of 5 million would sicken 250 000 and kill 100 000.[14] Moreover, in 1993 the U.S. Congressional Office of Technology (COT) analyzed the potential scope of larger attacks; it calculated that between 130 000 and 3 million deaths would follow the release of 100 kg of *B. anthracis*, a lethality matching that of a hydrogen bomb. It is anticipated that anthrax would be unlikely to cause severe disruptions in a military operation, although residual contamination of the ground would occur. In the U.S., New York City, with one of the most advanced detection and response systems in the country, is at the forefront of confronting an anthrax threat. It is using the next generation of sensors that the federal BioWatch program hopes to distribute nationwide by 2010. Most of the devices require up to 34 h to detect a lethal bug. However, experts in the field say that the nation's ability to detect biological weapons is still inadequate. Hospitals warn that the volume of casualties from an effective attack could simply overwhelm facilities.[15]

The use of aerosol-delivery technologies inside buildings or over large outdoor areas is another method that has been studied. The relatively small-scale dissemination of anthrax spores in a few paper envelopes that may have exposed as many as 30 000 people could only indicate that a more organized attack focused on delivering spores by aerosolization could result in a proportionately larger number of deaths and social and political chaos.

Concerning the legal aspect of this bioterrorism event, on August 7, 2008, the FBI released a wealth of new information about the troubled life of Bruce E. Ivins: Scientist Bruce E. Ivins, 62 years old, apparently killed himself on July 29, 2008, after learning that federal prosecutors were preparing to indict him on murder charges in the 2001 anthrax attacks that left five people dead. He had worked on the investigation of the anthrax attacks, although this meant that he, like other scientists at the Army's defensive biological laboratory at Fort Detrick, was scrutinized as a possible suspect.

Most experts agree that the ability to manufacture anthrax aerosol is beyond the capacity of individuals who lack access to advanced biotechnology laboratories. However, since the attacks on the World Trade Center on September 11, 2001, and the subsequent 11 cases of inhalational anthrax, concerns have been raised that these events mask the beginning, rather than the end, of terrorism.

2. Consideration of the Organism and the Disease

2.1. Historic Data. *B. Anthracis* holds a special place in the history of medicine and biology because its study has resulted in the foundation of medical microbiology.

Anthrax is a virulent and highly contagious disease[2] caused by *Bacillus anthracis*, an aerobic, Gram-positive, spore-forming, and large, nonmotile, rod-shaped bacterium.

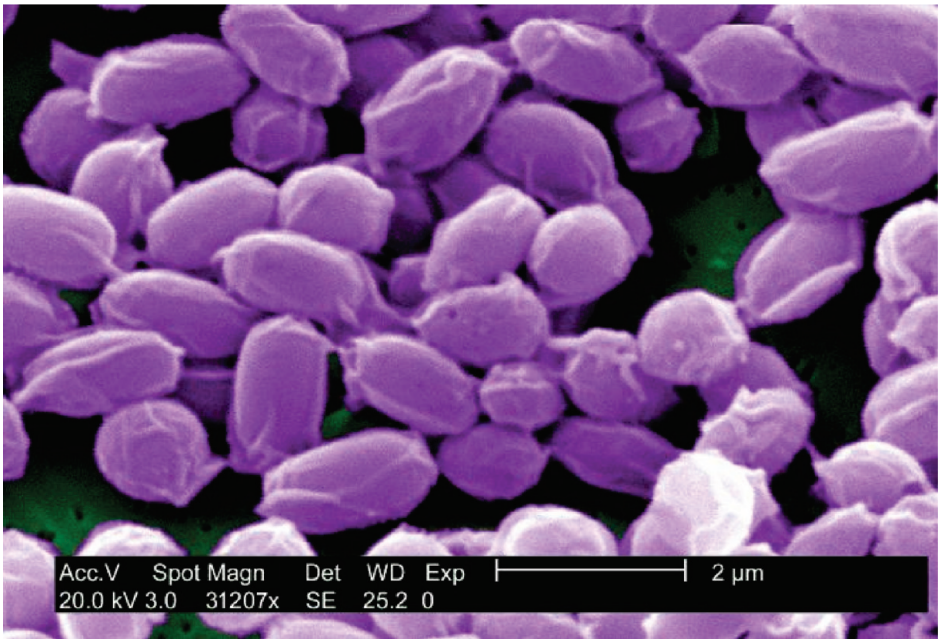

Figure 2. Scanning electron micrograph (SEM) of spores from the Sterne strain of *Bacillus anthracis*. The exosporium, namely, the outermost spore layer, is composed of 20 different protein species, and its wrinkled appearance represents a key characteristic of the spores of this *Bacillus anthracis* strain (magnification 31207×). The micrograph is Public Domain. Courtesy of CDC/Laura Ros/Public Health Image Library (http://phil.cdc.gov/phil/home.asp).

It is primarily a disease of herbivores, which are infected by ingestion of spores from the soil. Humans can become infected either by handling products from contaminated animals or by inhaling spores as a purified powder. The term *anthrax* is derived from the Greek word *anthracites*, meaning coal-like, referring to the typical black eschar formation seen in the cutaneous form of the disease as a result of the toxin action in the primary lesions[7,16] (Figure 1). Although anthrax can be found globally in temperate zones, it is more often a risk in countries with less standardized and less effective public health programs (South and Central America, Southern and Eastern Europe, Asia, Africa, the Caribbean, and the Middle East).

The first recorded description of anthrax is in the Book of Genesis, where the disease was referred to as the fifth plague (1491 B.C.) and was responsible for killing Egyptian cattle. Additional descriptions of anthrax affecting both animals and humans have been recorded in the early literature of the Hindus, Greeks, and Romans. The first reports of outbreaks associated with occupational cutaneous and respiratory anthrax occurred in the mid-1800s in the industrialized parts of Europe, namely, England and Germany. A pandemic, referred to as the "black bane", swept through Europe at that time and was responsible for many animal and human deaths; the disease resulted from handling hides, wool, and hair.

During the 19th century, several distinguished microbiologists characterized the pathologic basis of the disease and attempted to develop a vaccine to combat anthrax in the livestock industry. With the advent of modern microbiology, historically, the first live bacterial vaccine was developed by L. Pasteur in 1881 as an attenuated spore vaccine.[7] It was followed in 1939 by Sterne's development of a vaccine consisting of a spore suspension of an avirulent, nonencapsulated live strain of *B. anthracis*. This vaccine remains in use today for the vaccination of livestock, and widespread vaccination of domesticated animals with it has drastically reduced the animal mortality rate and has controlled anthrax in industrialized countries.[7]

Before the anthrax attacks in 2001, modern experience of inhalational anthrax was limited to an epidemic in Sverdlovsk, Russia, in 1979 following an unintentional release of *B. anthracis* spores from a Soviet bioweapons factory and to 18 occupational exposure cases in the U.S. during the 20th century.[13] Zimbabwe has seen the largest human epidemic to date, in which more than 10 000 human cases, nearly all of them cutaneous, were reported between 1979 and 1985.[14] Information about the potential impact of a large, covert attack using *B. anthracis* or the possible efficacy of post-attack vaccination or therapeutic measures remains limited.

2.2. Anthrax Virulence Factors. Virulence factors have always been at the heart of anthrax research. They are the specific tools used by the pathogen to fight and defeat host defenses. Our understanding of the interactions, at the cellular level, between the toxins and target cells has increased tremendously in recent years[17−20] and has provided targets for antitoxin therapies.[21]

The anthrax spore consists of several morphologically distinct layers, as follows (from outermost to innermost): exosporium, spore coat, cortex, spore membrane, and core. These structures jointly provide a highly protective "lock-box", protecting the core that houses the spore's genetic material[22] (Figure 2). The genome of the best studied *B. anthracis* strain, the Ames strain, consists of a chromosome and two plasmids, pXO1 and pXO2.[13] Both plasmids are required for full virulence, the loss of either resulting in an attenuated strain.[14]

Plasmid pXO1 harbors the structural genes for the anthrax toxin proteins LF, EF, and PA that functions as a ligand. These proteins are individually nontoxic, but when acting in binary combinations, they produce two distinct toxic responses in the host: edema and cell death (Figure 3). The PA is a long molecule with four distinct domains (domain-1, -2, -3, and -4). It is a 735-amino-acid protein

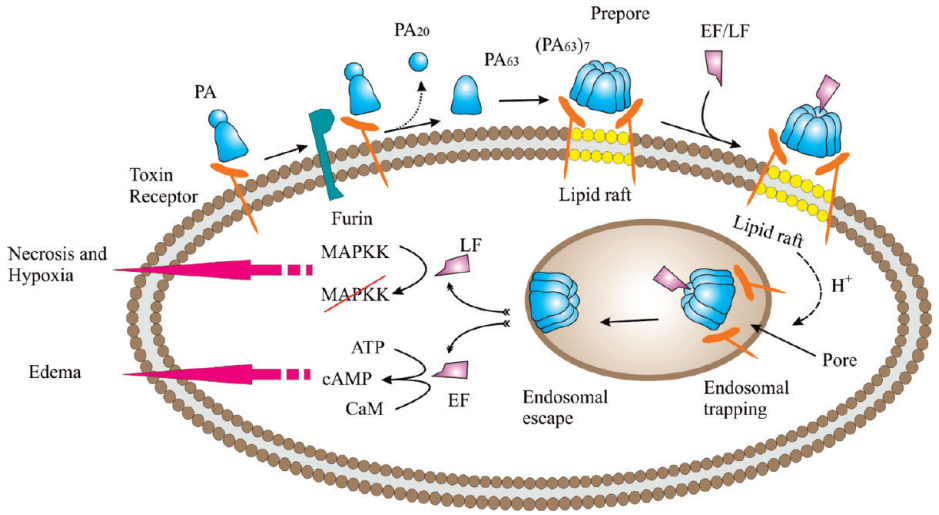

Figure 3. Current model of cellular toxin effects. Combined data from structural and cellular studies have resulted in a general model for anthrax toxin entry into host cells. This figure summarizes the fundamental steps in the uptake of anthrax toxins and their intracellular effects. Vegetative anthrax bacteria use a three-component nontoxic protein system that self-assembles at the surface of receptor-bearing mammalian cells or in solution, yielding a series of toxic complexes that act on cytosolic substrates. The main component, the PA, helps transport two secreted exotoxins into the host cell. LF is a zinc-dependent metalloprotease that cleaves most mitogen-activated protein kinase kinases (MAPKK), and EF is a calcium- and calmodulin-dependent adenylate cyclase that increases intracellular cAMP concentration and alters water homeostasis, resulting in massive edema. Upon binding cellular toxin receptor, PA83 is activated through cleavage by a surface furin-family protease into two fragments. The small fragment (PA_{20}) diffuses away, and the receptor-bound monomer fraction (PA_{63}) oligomerizes and self-assembles into a ring-shaped heptameric core termed prepore $(PA_{63})_7$. Recently, it has been shown that PA20 has an effect on human peripheral blood leukocytes and can induce apoptosis in the absence of other PA components.[244] Heptamerization of PA_{63} triggers endocytosis. Initially, it induces clustering of the toxin receptors, association of the $(PA_{63})_7$ complex with lipid rafts, and exposure of binding domains to the EF or LF followed by subsequent internalization.[19] Upon acidification of the endosomal compartment, a conformational change in the $(PA_{63})_7$ complex is triggered and the prepore is converted into a "ring" pore that inserts into the membrane and translocates EF and LF into the cytosol[23] (a striking 1.0 pH unit difference was found in the pH thresholds for pore formation when PA is bound to ATR2 (pH 5.2) versus ATR1 (pH 6.2). Nonelectrolyte polymers of poly(ethylene glycol) (PEG) were used to estimate the diameter of the ion channel formed by $(PA_{63})_7$. The results suggest that the limiting diameter of the PA(63) pore is < 2 nm, which is consistent with an all-atom model of the $(PA_{63})_7$ channel.[245] Although the molecular details of toxin entry are still being elucidated, the exact cascade of events that result in cellular death and death of the infected host remains unclear. Currently, how the MAPKK cleavage contributes to tissue necrosis is not well understood, but it is clear that LF plays a critical role in all stages of anthrax infection. In the early stages, LF helps the bacteria evade the host immune system, promoting its survival and release from macrophages where the spores germinate.[18] EF increases intracellular levels of cAMP, and this is believed to alter water homeostasis, resulting in massive edema. Illustration adapted from *Expert Rev. Infect. Ther.* **2007,** *5* **(4), 665−684 with permission of Expert Reviews Ltd.**

(83 kDa), also known as PA_{83}, and it is so named for its ability to provide experimental protective immunity against *B. anthracis* germinated spores. Each domain is required for a specific step in the intoxication process.[23] Lethal factor (LF) is a zinc-dependent metalloprotease that cleaves the N-terminus of mitogen-activated protein kinases (MAPKKs), and its crystal structure was solved in 2001 at 2.2 Å.[24] Lethal factor is organized into four domains. The description of the LF structure provided an innovative basis for the development of high-throughput screening of LF inhibitors. Moreover, the elucidation of the consequences of LF activity, downstream of proteolytic cleavage of MAPKKs leading to the host death, would be of great interest to the design of therapeutics targeting the clinical effects of the toxins. EF is an adenylate cyclase converting intracellular ATP into cAMP, an activity that is dependent on the eukaryotic protein calmodulin.[25] In 2002, a major advance was made with the determination of the crystal structure of the 58 kDa carboxy terminal catalytic part of EF (EF58) at 2.6 and at 2.75 Å in complex with calmodulin. EF58 is organized into three domains, two of which are connected to form a clamp around calmodulin.[26]

Plasmid pXO2 is smaller in size and carries three genes, *capB*, *capC*, and *capA*, required for capsule synthesis. The capsule is composed of a polypeptide, consisting of D-glutamic acid monomers joined by γ-peptidyl bonds (γ-D-PGA) and coats vegetative *B. anthracis* cells; it protects them from further phagocytosis and from complement-mediated killing by virtue of its negative charge and enables them to disseminate. This prevention can be done with a depolymerase, encoded by the *dep* gene, that catalyzes the hydrolysis of poly-γ-D-glutamic acid into lower molecular weight polyglutamates, which inhibit the host defense mechanisms.[27,28] In vivo observations show that dissemination by toxinogenic noncapsulated strains differs markedly from that by nontoxinogenic capsulated strains, suggesting that the capsule may be necessary for dissemination in human inhalation anthrax.[29] In plasma from infected African Green monkeys, rabbits, and guinea pigs the antiphagocytic poly-γ-D-glutamic acid capsule (γ-DPGA) was associated with LT in variable amounts. A portion of these LT/γ-DPGA complexes retained LF protease activity, suggesting that the co-inclusion of γ-DPGA in the effects of LT on specific immune cells in vitro may reveal novel and important roles for γ-DPGA in anthrax pathogenesis.[30]

Integrated regulation of two processes plays the key role in anthrax development in the early stages: (a) synthesis of the capsule whose presence promotes rapid escape of bacterial cells from macrophages and prevents phagocytosis and (b) synthesis of the toxin components participating in the transition of the germinating cells from phagosomes into cytoplasm.[31]

While the capsule passively protects the bacilli from host defense, toxins actively impair the host to further ensure a favorable environment for bacillar growth.[20]

2.3. Toxin Receptors. The first step of toxin entry into host cells is the recognition by PA of a receptor on the surface of the target cell. Since the anthrax attacks of 2001, much has been learned about the interactions between toxins and their cellular receptors.[23] They play a crucial role in anthrax pathogenesis, and their identification has made possible new avenues of research in antitoxin development and cancer biology.

The primary cellular receptors for PA have been identified by an elegant genetic approach,[32] and the molecular details of the toxin−receptor interactions have been revealed through crystallographic, biochemical, and genetic studies, which have revealed the existence of two distinct and closely related host cellular receptors for PA. They are designated anthrax toxin receptor-1/tumor endothelial marker 8 (ATR1/TEM8) and anthrax toxin receptor-2/capillary morphogenesis protein 2 (ATR2/CMG2). ATR is encoded by the tumor endothelial marker 8 (TEM8) gene, which is selectively up-regulated during blood vessel formation and in tumor vasculature, raising the possibility that this protein normally functions in angiogenesis. Both receptors bind to PA with high affinity and are capable of translocating EF and LF into the cytosol. ATR2 binds PA more tightly than ATR1.[23,33]

The anthrax toxin receptor (ATR) is a type I membrane protein with an extracellular von Willebrand factor A (VWA) domain, which mediates the receptor−PA interaction. The receptor type can dictate the pH threshold of anthrax toxin pore formation. The pore is formed at pH values of approximately 6.2 when PA is bound to the ATR1 but at pH 5.2 when it is bound to ATR2; LF can be translocated into cytosol under mildly acidic pH conditions.[23]

Northern blot analysis indicates that both host cellular receptors are expressed ubiquitously in a wide array of human cell types and normal tissues, including heart, lung, small intestine, spleen, liver, kidney, skeletal muscle, and skin.[34] Thus, many cell types could be damaged in a toxin-treated or infected animal, yet only macrophages and endothelial cells are killed by the toxin.[19,35]

2.4. Pathogenesis of Inhalational Anthrax. Given the defensive role of the immune system, a common strategy used by pathogenic bacteria to combat the unwelcoming environment in their mammalian hosts and to establish infection is to secrete protein factors that block intracellular signaling pathways essential for host defense.[17,18] Some of these proteins, like *Bacillus anthracis* secreted plasmid-encoded enzymes, also act as toxins directly causing a pathology associated with disease.[28,34]

B. anthracis is basically an extracellular pathogen. Its intracellular presence is likely to be a transient event that facilitates in vivo spreading. It relies on host cells for all aspects of its survival, from the initial attachment of its toxins to the membrane receptors to cellular invasion, acquisition of host cell metabolites, and intracellular replication. Critical steps in *B. anthracis* infection include spore entry and germination, and the interaction between the host and spore is vital to the development of the disease.[28] Destroying the spores either before or after they enter host cells is an attractive strategy for preventing disease development. However, such an approach aimed either at anthrax prevention or treatment has thus far not been developed. This is due to the inability of our immune system to disable anthrax spores and to our almost complete lack of molecular understanding of anthrax spores as well. Our knowledge of spores is essentially limited to descriptive electron−microscopic morphology dating back mostly to the 1960s,[18] (Figure 2).

The innate immune response is the first line of defense against invading *B. anthracis* spores and has a central role in acute infection.[11] Inhalational anthrax is initiated by dormant endospores gaining access to the alveolar spaces of the mammalian lungs, where they are efficiently engulfed by resident phagocytes (macrophages or dendritic cells). Human alveolar macrophages respond to *B. anthracis* spores through cell signal-mediated cytokine release.[36] Similarly, the pulmonary epithelium participates in the innate immune response through signal-mediated elaboration of cytokines and chemokines.[37] It is commonly believed that the host phagocytes then migrate across the alveolocapillary barrier transporting the intracellular bacteria into the lymphatic system. During this time the bacteria germinate, transforming from spores into vegetative bacilli, which begin to replicate within the phagocytes. Eventually, the bacteria kill the phagocytes and escape into the extracellular environment.[28] *Bacillus anthracis* causes high-level bacteremia, strongly suggesting paralysis of the innate immune system. Its toxins act on a variety of cell types, ultimately leading to death of the host.[19]

Lung dendritic cells (LDCs) constitute the main cellular population responsible for the early spread of anthrax spores. Although alveolar macrophages (AMs) are the first sentinels as they phagocytose most spores in the first 10 min in the alveoli, they are unable to transport them. In contrast, LDCs capture spores and then carry them rapidly to the thoracic lymph nodes. The passage across the epithelium or transepithelial crossing constitutes the first step of pathogen diffusion. The extreme rapidity of spore transport, between 30 min and 6 h after infection, is highly consistent with the fulminating form of inhalational anthrax. Therefore, an exposure to an anthrax aerosol must be considered a severe infection as early as 30 min after exposure, and the patient should be medically treated as soon as possible. These observations should be taken into account for medical management after exposure to an anthrax aerosol.[38]

A number of factors contribute to anthrax pathogenesis. LF causes release of TNF and IL-1, which were believed to be linked to the sudden deaths in cases of severe anthrax infection. However, growing evidence suggests that the LF-induced shock does not result from an excessive inflammatory response[34] but may be related to the direct injurious effects of LF on endothelial cell function. The effects of LT on the barrier function of primary human lung microvascular and large vessel endothelial cells suggest a possible role for LT-induced barrier dysfunction in the vascular permeability changes and hemorrhage that accompany a systemic infection.[35]

EF impairs neutrophil function in vivo and affects water homeostasis, leading to edema. By adding to the LF effect, EF makes a substantial contribution and demonstrates the common action of anthrax toxin components in increasing the host cell lethality.[39,40] Recently, it was shown that toxins are required for dissemination of bacteria and disease progression beyond the draining lymphoid tissue, leading to full virulence.[41]

LT mounts a broad-based attack on host immunity. It affects the adaptive immunity acting directly on T- and B-lymphocytes, blocking antigen receptor-dependent proliferation, cytokine

production, and Ig production.[42,43] It inhibits the chemotaxis of T-cells and macrophages through subverting signaling by chemokine receptors.[17] It markedly impairs PMNs actin assembly, and reductions in actin filament content are accompanied by a profound paralysis of PMN chemotaxis.[44] LT triggers the formation of a membrane-associated inflammasome complex in murine macrophages, resulting in the cleavage of cytosolic caspase-1 substrates and cell death.[45] In addition, while endogenous IFNs are essential for control of *B.anthracis* germination and lethality, administration of exogenous IFNs appears to increase the local inflammatory response, thereby increasing mortality.[46]

Despite the intensive biomedical research that has been conducted since 2001, the exact sequence of events that lead to toxin-induced death is still poorly understood. Moreover, it is not well understood why these bacteria cause a localized infection through the skin and a lethal disease through the lung. A clearer picture of pathogenesis in humans has yet to be drawn, which will facilitate the development of new modalities to eliminate the threat posed by this bioterrorism weapon.[47]

2.5. Clinical Manifestations. Anthrax can take three different disease forms: cutaneous, gastointestinal, and inhalational. Cutaneous anthrax is the most common, and inhalational anthrax, which is almost 100% fatal if untreated, is the most likely form in the context of a bioterrorist attack.

In inhalational anthrax, spores germinated in the tracheobronchial and mediastinal lymph nodes begin unimpeded extracellular multiplication within the lymphatic system, causing regional hemorrhagic lymphadenitis.[9] Vegetative bacilli then further spread via the bloodstream and lymphatics and continue rapid replication. Reaching as many as 10^9 organisms per milliliter of blood, the high level of secreted exotoxins intoxicates the host, causing septicemia. The disease begins after an incubation period varying from 1 to 6 days, presumably dependent upon the dose of inhaled spores. Onset is gradual and nonspecific, with fever, malaise, and fatigue, sometimes in association with a nonproductive cough and mild chest discomfort. In some cases, there may be a short period of improvement. The initial symptoms are followed in 2−3 days by the abrupt development of severe respiratory distress with dyspnea, stridor, and cyanosis. Key indicators of early anthrax cardiovascular-related pathogenesis include a dramatically widened mediastinum in association with pleural effusion and edema of the chest wall, whereas dyspnea, high fever, meningitis, and circulatory shock represent a terminal stage; treatment is often ineffective when started at that time.[48] Shock and death usually follow within 24−36 h of respiratory failure onset, with overwhelming bacteremia often associated with meningitis and subarachnoid hemorrhage.[9] Antibiotics are without therapeutic benefit from this point onward.[18]

Despite appropriate antibiotics and aggressive hemodynamic and pulmonary support, inhalational anthax is frequently associated with severe and often irreversible hypotensive shock. Hypotension with LT may not be a primary cause of lethality; rather, LT may cause direct cellular injury insensitive to vasopressors and neither increasing nor decreasing norepinephrine doses improves survival with LT.[49] In mice, it has been demonstrated that the anthrax morbidity and mortality are not cytokine-mediated but are due to a direct effect of the toxins on the cardiovascular system along with toxin-specific alterations in blood counts. PA/LF pathology matches that seen with acute cardiac failure, and PA/EF pathology coincides with direct vascular endothelial injury. These observations provide a rational basis for drug interventions to reduce the effect of these toxins on the heart and blood vessels.[50] It seems that during anthrax infection and shock, along with hemodynamic support, toxin-directed treatments may be necessary and therapies developed for treatment of cytokine-mediated septic shock will not be appropriate for the treatment of anthrax.[49]

3. The Medical "Arsenal" against Anthrax: Vaccines, Antibodies, Antibiotics, Antitoxin Agents, and Other Treatment Options

Naturally, vaccination seems the most effective form of mass protection against a biological attack.[7,14] Although safe and efficacious, the current vaccines have limitations that justify the widespread interest in developing improved treatment options for inhalational anthrax.

Theoretically, there are several ways to approach anthrax: (i) vaccination to prevent disease development, (ii) elimination of spores with antibodies against spore elements, (iii) antibiotics to kill vegetative bacilli before the disease reaches a systemic stage, and (iv) conjunctive antitoxin therapy against toxins.[9] This section of the paper gives an overview of the current and future medical countermeasures to protect humans from anthrax bioterrorism, including vaccines, antibodies, antibiotics, antitoxin agents, and other treatment options.

3.1. Vaccines. Inhalational anthrax is a complex infection, and fully effective accurate treatment may require complementary approaches such as antibiotics and antitoxic remedies. Because of nonspecific initial symptoms, however, the timing for administration is difficult. Therefore, a prophylactic vaccine that prevents infection or stops it at an early stage would be highly desirable.[8,9] There are several types of candidate vaccines in development, as indicated below.

3.1.1. Anticapsule Conjugate Vaccines. The capsule is an essential virulence factor that enables the bacterium to avoid demise in the host because a strong humoral immune response is not generated against the outer surface of the bacteria.[51] Currently, there is renewed interest in this capsule with regard to immunoprotection and it appears to be a promising antigen for a new anthrax vaccine.

The inherently weak capsule immunogenicity, similar to polysaccharides, can be significantly enhanced through conjugation to a strongly immunogenic protein carrier.[52] For example, a new-generation dually active anthrax vaccine (DAAV) was prepared by chemically conjugating γ-D-PGA and recombinant *B. anthracis* PA (rPA).[53] This vaccine was capable of inducing high levels of specific antibodies to both the capsule and the toxin and represented the paradigm of combining both antibacterial (i.e., prophylactic) and antitoxic (i.e., therapeutic) components into a single vaccine. Other carrier proteins that were bound to different lengths of synthetic γ-D-PGAs include bovine serum albumin, recombinant *Pseudomonas aeruginosa* exoprotein A, and tetanus toxoid. Tetanus toxoid and rPA were the best carriers, and the thioether bond was the optimal linkage type. Such a vaccine will be able to induce booster responses to glutamic acid.[51]

Another vaccine consists of a conjugate of γ-D-PGA with a dominant negative inhibitory (DNI) mutant of PA.

It significantly boosted the antibody response to γ-D-PGA, suggesting that DNI could serve as a strongly immunogenic protein carrier for poor immunogens.[54]

A powder vaccine, containing a combination of PA and capsule epitopes, was administered intranasally in rabbits. The immune protection that it provided against *B. anthracis* spores was superior compared with a single antigen (PA) vaccine.[55] Moreover, by inclusion of the pXO2 plasmid in a live anthrax spore vaccine expressing the PA, protection in a guinea pig model was increased dramatically.[56]

3.1.2. Existing and Future PA-Based Human Anthrax Vaccines. The central role of PA in the pathophysiology of anthrax makes it the predominant immunogen of the first-generation U.S. and U.K. licensed human anthrax vaccines and the principal target for both next-generation vaccine and antitoxin development. Anthrax vaccines have progressed from uncharacterized whole-cell vaccines in 1881 to pXO2-negative spores in the 1930s (the Sterne strain is still used as a live veterinary vaccine), to culture filtrates adsorbed to aluminum hydroxide in 1970, and likely to recombinant protective antigen in the near future.[5,10] Immunization of humans with live attenuated spores has been limited to the countries of the former Soviet Union and China. Western nations, such as the U.K. and the U.S., use nonliving subunit vaccines based primarily on PA because live attenuated spore vaccines have been considered unsuitable for use in humans owing to concerns over the possibility of residual virulence.[47]

3.1.2.1. First-Generation Licensed Vaccines. Two acellular vaccine formulations have been licensed for human use, one in the U.S. (AVA, now called BioThrax) and one in the U.K. Both vaccines are prepared by adsorbing filtered culture supernatants of an attenuated (nonencapsulated, toxin-producing) *B. anthracis* strain to aluminum hydroxide gel and alum precipitated as an adjuvant, respectively.[5]

AVA is the first modern acellular bacterial vaccine produced from a *Bacillus anthracis* strain, V770-NP1-R, a nonproteolytic variant of a bovine strain isolated in Florida in 1951.[16,47] AVA was approved for production in November 1970 at facilities owned by the state of Michigan. Those facilities were purchased by BioPort Corporation in 1998 and are now owned by Emergent BioSolutions. The development of AVA was based on the outcome of human trials conducted in the early 1950s, when purified components of *B. anthracis* were not available. It is the only vaccine licensed by the FDA to prevent anthrax, based on safety studies conducted in 7000 participants who received 16 000 doses of AVA.[16,57] More than two million men and women in the U.S. military have received the vaccine, and the DHHS has procured more than 28 million doses of BioThrax for the Strategic National Stockpile (a U.S. government reserve of medicines in the event of a public health emergency, such as a terrorist attack or flu outbreak). The principal antigenic component is PA with smaller amounts of EF and LF. While antibodies to PA are sufficient to neutralize toxin activity in animal models, AVA-induced LF and EF antibodies do not significantly contribute to anthrax toxin neutralization in humans.[58] In 1997 it was mandated that all U.S. military active- and reserve-duty personnel should receive it. The administration of AVA is burdensome. It comprises a priming course of six doses (subcutaneous injections at 0, 2, and 4 weeks and at 6, 12, and 18 months) followed by yearly booster shots for those with a continued risk of exposure.[11] The 6-month dose in the AVA primary series appears to be critical in sustaining IgG protective titers to PA in a substantial proportion of recipients because immunity is not long-lasting. Its ability to stimulate protective immunity in a range of animal models, including primates, has been repeatedly demonstrated. Although the establishment of rugged correlates of protection in humans is not feasible, the antibody profile to the vaccines currently licensed in the U.S. and the U.K. has been determined in human volunteers.[59−61] An enzyme-linked immunosorbent assay was used for the detection of PA-specific salivary IgG in AVA recipients. Results suggested that an oral fluid-based immunoassay might be a feasible alternative to routinely monitoring the serological response of vaccinated individuals. For field use, such a noninvasive test would be beneficial in evaluating the antibody response of anthrax-vaccinated individuals working within a high-risk area of possible exposure.[62]

In 1946, Gladstone demonstrated that the PA component of anthrax cultures was an effective vaccine, leading to the current culture supernatant-derived human vaccine, produced in the U.K. by the Defense Science and Technology Laboratory at Porton Down. The U.K. vaccine is produced from a static, aerobic culture of the *B. anthracis* Sterne strain, 34F2. The priming schedule comprises four doses followed by annual booster shots. In addition to containing large amounts of PA, the U.K. vaccine also comprises trace amounts of LF and other bacterially derived immunogenic antigens, which might also account for the transient reactogenicity seen in some individuals.[5,47]

The scientific basis for AVA safety and efficacy is sound; however, it has suffered an unusual amount of negative publicity and there are several concerns about it:[5,63] (a) AVA does not protect all animal hosts against different strains of *B. anthracis*, and its efficacy varies widely between species.[9] PA is antigenically complex, and vaccinated individuals have demonstrated considerable variability in their antibody responses to PA. The variability observed in the efficacy of elicited antibody responses (protective and nonprotective antibodies) is a result of host genetic background and implies a need to develop other antigens as vaccine candidates.[64] (b) The immunization schedule of AVA is not ideal, and it is based on a historical rather than an immunological rationale.[5] (c) The preparative processing of AVA is crude and lacks consistency. (d) There are relatively high rates of local and systemic adverse reactions, likely due to residual toxicity in AVA or other contaminants, and reactogenicity is reported in up to 35% of patients.[9] Mild problems include soreness, redness, a lump or itching where the shot was given, muscle or joint aches, headaches, chills or fever, and nausea. Moderate problems include large areas of redness where the shot was given. Severe problems include serious allergic reaction (very rare, less than once in 100 000 doses).[63] According to the National Academy of Sciences, anthrax vaccine has an adverse reaction profile that is similar to that of other adult vaccines. However, because of the adjuvanted aluminum hydroxide, given subcutaneously it causes an elevated rate of injection site pain (including a burning sensation lasting 1 min), with swelling and occasionally with peripheral neuropathy from pinching of the ulnar nerve. Therefore, anthrax vaccine should be administered over the deltoid region, not the triceps region. The Anthrax Vaccine Research Program Working Group enrolled 1005 people in a randomized clinical trial to assess the safety and serological outcomes of alternative schedules and routes of administration of the anthrax vaccine. By reduction of the number of anthrax vaccine doses (omission of the week

2 dose) and administration of it by intramuscular rather than subcutaneous injection, a three-dose intramuscular regimen elicited a noninferior serum antibody response compared with a four-dose regimen; moreover, it was associated with a significant reduction in injection site adverse events.[65]

AVA could complement the use of antibiotics in cases where anthrax spores persist in a person's lungs and germinate after the antibiotics are discontinued. The vaccine may also help to protect people who do not fully comply with the recommended 60-day course of antibiotics.[57] Vaccination could also be recommended for inhabitants who continue to live in a partially contaminated area, if full decontamination cannot be achieved.[8,57]

In 2005, the U.S. Department of Health and Human Services awarded a contract of $122.7 million to BioPort to buy 5 million additional doses of AVA.[66] On September 26, 2008, Emergent BioSolutions Inc. (Rockville, MD) signed a contract with NIAID, valued at up to $29.7 million, to fund the further development of AV7909 (http://www.emergentbiosolutions.com/NewsReleases.aspx?ReleaseID=1178612). This is a promising next-generation vaccine candidate comprising AVA in combination with the compound CPG 799, an oligodeoxynucleotide with immunostimulatory properties. It does not need refrigeration during storage, a key requirement of vaccine development, and it was successfully tested in preclinical studies.

3.1.2.2. Second-Generation PA-Based Vaccines (Vaccines under Development). Concerns over the suspicious role that AVA might have played in the Gulf War Syndrome in 1991 and the possibility of a future anthrax attack stimulated researchers to develop second-generation anthrax vaccines suitable for mass vaccination.[47,67]

Much of the current work on preparing a second-generation vaccine is based on the observation that antibodies to protective antigen (PA) are crucial in the protection against exposure to virulent anthrax spores. The most promising human vaccine candidate contains highly purified recombinant PA (rPA).[5,10] These vaccines are expected to differ from their predecessors in that their composition will be fully defined, they will be free from any adverse effects, and they will be produced from media with no animal-derived products. Furthermore, they will be amenable to large-scale production and storage at room temperature.[47] Numerous trials have been conducted showing the efficacy of the second-generation vaccines.[68−70]

For at least two rPA-based vaccines, one produced from an asporogenic variant of the Sterne strain by Vaxgen in the U.S. and the other from *E. coli* manufactured by Avecia in the U.K., the U.S. National Institutes of Health sponsored human safety and immunogenicity trials.[10,47] The Avecia vaccine, developed in collaboration with researchers at the Defense Science Technology Laboratory at Porton Down, comprises a nucleotide-codon-optimized version of the PA gene expressed from *E. coli*.[70] The VaxGen system, developed by researchers at the U.S. Army Medical Research Institute of Infectious Disease in Frederick, MD, comprises a recombinant multicopy plasmid-based expression system that expresses higher levels of PA than the Sterne strain from which it is derived.[71] This vaccine is produced using modern vaccine manufacturing techniques and may require fewer doses than the currently licensed vaccine. It was expected that the U.S. would stockpile at least one of these products to enable them to respond to a future emergency. Financed through Project BioShield, created in 2004 to allow development and acquisition of essential medical countermeasures for the Strategic National Stockpile, a contract of $878 million was awarded to VaxGen (Brisbane, CA) in November 2004 for the production of 75 million doses of a recombinant bioengineered anthrax vaccine, an amount capable of inoculating 25 million people. Although the preliminary results for immunogenicity and tolerance of the new VaxGen vaccine, rPA102, were encouraging, the government terminated the contract in December 2006 because the VaxGen vaccine decomposed, precluding stockpiling.[71,72]

Recently, a trivalent vaccine composed of an rPA mutant and inactive mutants of LF and EF was emulsified with mineral oils and evaluated in a rabbit model. Results indicated that this type of vaccine could be a good vaccine candidate for use in an emergency, with simultaneous long-acting antibiotics during an anthrax epidemic.[73]

In cases of mass protection of the human population, sufficient quantities of biologically active rPA must be provided. For this purpose, a scalable purification process for mass production of rPA protein has been developed that has exhibited the ability to generate multigram quantities of highly pure protein from recombinant *Escherichia coli* (yields in excess of 300 mg/L of fermentation culture).[74] Numerous other attempts to develop expression systems are based on a variety of organisms including attenuated strains of *B. anthracis, B.subtilis*, *B. brevis*, and *Salmonella typhimurium*.[47]

3.1.2.3. Third-Generation Anthrax Vaccines (Vaccines under Development). The fact that rPA vaccine has an estimated shelf- life of only 3 years highlights the importance of developing a third-generation anthrax vaccine with a longer shelf life, aiming to enhance the efficacy of the second-generation rPA-based vaccine.

Although preliminary animal testing has shown that the rPA products require fewer doses than the current licensed vaccines to achieve protection, they still suffer from the need to be given by needle, which requires the involvement of trained medical personnel.[70] In addition, the vaccines will have to be transported and stored at 4 °C, making it expensive to stockpile in remote regions and limiting their shelf life. The third-generation vaccines will have a shorter immunization schedule and a greater protective efficacy than before. Enhancements may include the use of antigens other than PA, novel adjuvants, and novel inoculation strategies.[75] Such vaccine formulations would ideally enable the needle-free self-delivery of rPA in a single dose to the mucosal surfaces of the gut, to the upper respiratory tract, or via the dermal route, facilitating stockpiling and mass vaccination programs.[47]

3.1.2.4. Oral Immunization. Efforts to develop a rPA vaccine for single-dose oral delivery have focused on two main approaches: optimizing delivery of rPA through expression in live attenuated bacterial vectors and use of plant-expressed, edible antigens.[5,10,47] To date, a number of live bacterial vector systems have been described for the delivery of rPA, including attenuated strains of *B. anthracis, B. subtilis, Salmonella*, *Lactobacillus*, and *vaccinia*.[47] A disadvantage of live systems is the need to culture and store the organism prior to use. Oral immunization of mice with a *Salmonella*-based DNA vaccine demonstrated its efficacy for mass vaccination against aerosolized *B. anthracis* spores.[76] An attenuated typhoid vaccine strain of *Salmonella enterica* serovar Typhi, licensed for human use, was genetically engineered for stable plasmid-based expression of PA.[77] Mice immunized with a vaccine delivered by *Salmonella enterica* serovar Typhi Ty21a were completely

protected from a lethal intranasal challenge with aerosolized spores.[78] Delivery of rPA through expression in attenuated recombinant live bacterial vectors may form the basis of third generation vaccines, possibly being developed over the next few years. The successful use of plant-based vaccines is particularly attractive, as they require little manufacturing capability, are free of animal pathogens, and stimulate immune responses in humans following oral dosing. Large quantities of economically purified edible PA protein were produced by expression of PA in transgenic chloroplasts *of Nicotiana benthamiana* plants.[79,80] One acre of transgenic tobacco plants expressing PA from a chloroplast-based system could yield 360 million doses of purified vaccine, which would be enough to immunize the entire population of the U.S. Although progress has been made in this area, such a licensed plant-based human anthrax vaccine will become available only in several years.

3.1.2.5. Intranasal Immunization. Nasally administered rPA encapsulated in and bound to the surface of biodegradable poly L-lactide microspheres resulted in measurable serum IgG and protected mice against a lethal spore challenge.[47] Furthermore, mice nasally immunized with rPA adjuvanted with synthetic double-stranded RNA in the form of polyriboinosinic−polyribocytidylic acid (pI:C) developed strong systemic and mucosal anti-PA responses, indicating that this pI:C-adjuvanted rPA vaccine has the potential to be developed into an efficacious nasal vaccine.[81] Mucosal immunization with attenuated *Salmonella enterica* serovar Typhi expressing PA protected nonhuman primates from a spore challenge.[82] A triantigen nasal anthrax vaccine that contained a truncated PA (rPA63), the LF, the capsular poly-γ-D-glutamic acid as the antigens and the polyI:C as the adjuvant, protected immunized mice against a lethal LT challenge.[83] Moreover, mice nasally immunized with a nasal anthrax vaccine using PA protein carried by liposome−protamine−DNA (LPD) particles developed both systemic and mucosal anti-PA responses.[84] Intranasal delivery of powdered rPA formulations has offered complete protection to rabbits against inhalational anthrax. Spray freeze-dried formulations have displayed substantial improvement in storage stability over liquid formulations, suggesting their potential application for mass biodefense intranasal immunization.[85] Results of all these studies look promising, but as with the oral vaccination, they have yet to be confirmed in humans.

3.1.2.6. Transdermal Immunization. The effectiveness of a transcutaneous delivery system in inducing a protective response against rPA has been demonstrated in mice. The technology, called transcutaneous immunization (TCI), is a needle-free technique that delivers antigens and adjuvants to potent epidermal immune cells. It includes a dry adhesive skin patch containing rPA and *E. coli* heat-labile toxin, which acts as an adjuvant. The transdermal activity of the patch depended upon abrasion of the skin before immunization, which presumably allows penetration of large protein molecules through the stratum corneum.[86] Although promising, its utility as a means of protection against anthrax is yet to be demonstrated in humans and other primates.

3.1.2.7. Genetic Immunization. DNA-based vaccines would offer a number of advantages over conventional subunit vaccines. The gene encoding the vaccine antigen is introduced into the host and, expressed in vivo, stimulates a protective immune response. This is a major advantage because the need to develop expensive protein expression and purification systems is eliminated.[10] Delivery of rPA via vaccination with DNA plasmids could form the basis for multiagent anthrax vaccine development. Monovalent or bivalent plasmid DNA vaccines encoding genetically detoxified PA and LF proteins have provided an attractive technology platform against anthrax challenge.[87] VCL-AB01 is a novel cationic lipid-formulated plasmid DNA (pDNA)-based vaccine that contains genes encoding genetically detoxified PA and LF. Safety and immunogenicity clinical trials have been carried out in healthy adults and in nonhuman primates, and this vaccine was generally well tolerated in humans at a dose that provided immunity in monkeys.[88]

3.1.2.8. Live Viral Vectors. Expression of anthax antigens through attenuated viral vectors represents an attractive vaccine alternative, but safety problems remain a major concern.[89] Researchers have created recombinant adeno-associated virus type 1 (rAAV1) vectors containing synthetic genes derived from the PA or LF and tested them for immunogenicity and induction of toxin-neutralizing antibodies in rabbits. The development of robust neutralizing antibody responses after a single injection of these rAAV1-based vectors supports their further development as candidate anthrax vaccines.[90] Immunization of mice with T4 phage carrying three antigens (PA, LF, and EF) has elicited strong immune responses against all antigens, stronger than the phage displaying PA alone. The in vitro display of PA on phage-T4 particles offers a novel approach for the potential construction of multicomponent vaccines against anthrax threat.[91] The icosahedral insect Flock House virus has been used as a platform to display 180 copies of the high affinity, PA-binding von Willebrand A domain of the ANTXR2 cellular receptor. The chimeric virus-like particles (VLPs) correctly displayed the receptor von Willebrand A domain on their surface and protected rats from LT challenge. The recombinant VLP platform represents a novel and highly effective, dually acting reagent for treatment and protection against anthrax.[92]

3.1.3. LF-Based Active Immunization. Recently, much progress has been made in the search for specific inhibitors of LF and very little work has focused on immunization, active or passive. Immunization of mice with recombinant LF (rLF) has generated two monoclonal antibodies, 5B13B1 and 3C16C3, that showed neutralizing activity against LT in animal models.[93] The binding of short LF peptides by LF-specific neutralizing monoclonal antibodies suggests that generation of protective antibodies by LF peptide vaccination may be feasible and paves the way for a more effective anthrax vaccine by identifying discontinuous peptide epitopes of LF.[94]

3.1.4. EF-Based Active Immunization. EF-deficient *B. anthracis* strains are still toxic while those lacking LF are greatly attenuated, suggesting that LF is the dominant virulence factor of anthrax. Therefore, the study of inhibition of EF activity is of minor research interest.[95] EF could serve as a novel agent for inducing protective immunity to PA-based anthrax vaccines because it has been found that it acts as an adjuvant to nasally administered vaccine antigens.[96] The biological activities of EF can be studied using biologically active recombinant EF protein (rEF) which may feasibly be produced in large quantities from recombinant *E. coli*.[97] When it was evaluated as a candidate antigen in an anthrax viral vaccine, the N-terminal nontoxic fragment of this product elicited a protective immunity response.[98]

3.2. Antibodies. As recent bioterrorist attacks have highlighted, human anthrax infection cannot always be treated

successfully with antibiotics. Especially during the late stage of anthrax septicemia, in which significant amounts of toxin are being secreted, antibiotics are of less value because they have no effect on the toxins and it is too late for vaccination to offer protection.[8,99] Moreover, the emergence of antibiotic-resistant strains has prompted researchers to pursue additional therapeutic options. For these reasons, adjunct therapies for future anthrax infections are urgently needed and the principal approach involves passive immunization with therapeutic antibodies that inhibit the activity of the organisms' tripartite toxin system.[8,100] Although antitoxin antibodies are effective, they suffer from the same drawbacks as antibiotics: they have a significant clinical effect only if they are administered rapidly and should be given repeatedly to maintain an adequate level of protection.

The role of antibodies against toxins would be to protect mononuclear cells and any other sensitive cell types from toxin action. For example, antibodies to PA could inhibit binding to its cellular receptor, preventing toxin action, and antibodies to PA (expressed in the spore coat) may inhibit germination and control infection by binding to spores before their uptake by macrophages.

3.2.1. Anticapsule Antibodies. Although the structure of capsular γ-D-PGA units is relatively monotonous, research has revealed a striking diversity in the peculiar pattern with which anticapsular antibodies couple with the repeated γ-D-PGA units.[101] The γ-D-PGA capsule itself appears to be relatively nonimmunogenic. However, a number of investigators have shown that glutamic acid polymers conjugated to various carriers can generate an immune response to glutamic acid.[53,102]

As the role of antibodies to the capsule is to fix the complement and to lyse vegetative bacterial cells, it would be logical to think that anticapsule antibodies may be important in controlling the outgrowth phase of anthrax infection. There is renewed interest in the *B. anthracis* capsule with regard to immunoprotection. Investigators have produced mAbs specific for γ-D-PGA and have shown that their administration does indeed confer passive protection in naive mice against spores of the Ames strain by mediating opsonophagocytosis of *B. anthracis*, suggesting that some animals can be protected in the absence of antitoxin immunity.[101]

3.2.2. Anti-LF Monoclonal Antibodies. LF plays a pivotal role in the *Bacillus anthracis* cytotoxicity, and the most promising means for treating postexposure anthrax is based on inhibition of LF action. IQNLF is a fully human monoclonal antibody with specificity for LF. It recognizes domain I containing the PA binding region in LF. When combined with IQNPA (anti-PA), the antibodies had increased neutralization efficacy and enhanced endogenous immunity to anthrax.[103] LF8 is a neutralizing monoclonal antibody that protected macrophage J774A.1 cells and mice challenged with LF. Data suggested that LF8 binds LF near the PA binding domain.[104]

3.2.3. Anti-PA Monoclonal Antibodies. Antibodies to PA neutralize anthrax toxins by preventing adherence of PA to cell receptors or assembly of PA heptamers and subsequent binding of LF and EF (Figure 3). The levels of antibodies to PA must exceed a certain minimal threshold in order to induce and maintain protective immunity; however, it is not yet clear what levels of antibodies will be required to protect humans after vaccination or passive injection of protective antibodies. This consideration is very important, since challenge studies cannot be performed in humans and correlates of immunity have to be extrapolated from animal studies.[10,105]

Recently, the focus has been on the development of PA-specific toxin-neutralizing mAbs: two mAbs, W1 and W2, generated from immunized chimpanzees. They have inhibited the binding of PA molecules to their receptors, suggesting their potential use in the emergency treatment of anthrax.[106] PA-mAb is another mAb that reduced morbidity in a LT-infused rat model of anthrax sepsis, even when administered after the onset of septic shock symptoms.[107] Raxibacumab, now called ABthrax, is a PA monoclonal antibody produced by Human Genome Sciences. The U.S. Department of Health and Human Services contracted for 20 000 ABthrax treatment courses against future anthrax attacks.[8] AVP-21D9, AVP-22G12, and AVP-1C6 are three fully human anti-PA toxin-neutralizing antibodies. They have been isolated from blood lymphocytes from donors immunized with low doses of AVA. Fisher-344 rats challenged with LT were protected after their administration and thus represent attractive candidates for treatment and/or prophylaxis from anthrax toxins. In particular, the AVP-21D9 antibody has demonstrated very high affinity to the anthrax toxin in three animal models, alone or in combination with low ciprofloxacin levels, indicating that AVP-21D9 has the potential to become a life-saving medical countermeasure.[108] On September 3, 2008, the U.S. Department of Health and Human Services funded the further development of AVP-21D9, http://www.fiercebiotech.com/story/emergent-wins-24m-anthrax-contract/2008-09-04. An antibody phage display library was used for the isolation of three human single-chain variable fragments (scFvs) against PA. The antibodies were evaluated for their ability to bind to cell-bound heptameric PA. All antibodies competed with LF−PA cell surface interactions, further suggesting the potential application of human antibodies as passive immunization prophylactics against inhalational anthrax.[109]

3.2.4. Monoclonal versus Polyclonal Antibodies. The use of human-derived polyclonal anthrax antibodies would offer a number of advantages such as prolonged serum half-life, reduced adverse reactions, and the targeting of multiple epitopes. However, they should be maintained and sufficient stocks of them be constantly renewed to protect large numbers of individuals in the event of an attack.[47,80] For this reason, a number of research groups have been developing high-affinity, toxin-specific monoclonal antibodies with a significant half-life. They are nontoxic in humans, confer instant protection, and are equally effective against antibiotic-resistant strains. They can be produced on a commercial scale, enabling them to be stockpiled in sufficient quantities.[80] Given that the half-life of human antibodies is approximately 20 days, it raises the possibility that a single dose of a mAb might be sufficient to treat an infected individual during the late stage of anthrax septicemia, in which significant amounts of toxin are being secreted, and this property would offer considerable logistic advantages in a mass casualty setting.[47] An expert panel on monoclonal antibodies for anthrax has recommended that therapeutic antibodies should consist of a multitarget mixture (PA, LF and EF) of human mAbs (Expert Consultation on Monoclonal Antibodies for Anthrax rPA 2003, http://www.niaid.nih.gov/biodefense). Additionally, antibodies against the antiphagocytic capsule could be included in the mix. As monoclonal antibodies target only one epitope (antigenic specificity), in conventional therapy they may have advantages over polyclonal antibodies that neutralize several epitopes.

However, it is unclear whether monoclonal antibodies might be preferable to polyclonal antibodies. If bacteria could be reengineered to modify that single epitope, an elegant monoclonal antibody might be rendered ineffective.[47]

AIG is a polyclonal anthrax immunoglobulin product developed by Emergent BioSolutions (Rockville, MD) and derived from human plasma from individuals who have been vaccinated with BioThrax. It is a candidate for intravenous postexposure treatment for patients who display symptoms of anthrax disease.[47] In humans, the definitive efficacy of AIG has not yet been achieved; the only evidence comes from the successful treatment of inhalational anthrax in a 44-year-old man who naturally acquired anthrax after being exposed via African hides.[110]

3.3. Antibiotics. In the event of an attack with aerosolized anthrax spores over an unvaccinated metropolitan population, treatment would primarily consist of antibiotics. They may be used to treat people who are sick with anthrax, as well as to prevent disease in people who have been exposed to spores but are not yet sick.

Because inhalational anthrax may have a long incubation period, ranging from 2 to 46 days (11-day median), many people could start taking antibiotics in order to prevent the disease if they knew they had been exposed.[16] Regardless of the antibiotic chosen, it is important that chemotherapy should be given at the very early stage of infection, when symptoms are nonspecific and diagnosis is difficult.[47] Animal studies suggest that when the disease reaches the fulminant phase, even if the organism is sensitive to the agent, antibiotics are no longer effective owing to the accumulation of a lethal toxin level.[99,111,112]

Once the treatment is commenced, it is important to ensure that it is continued for prolonged periods. Antibiotics are active only against germinated spores. Experiments with rhesus monkeys, which serve as a good model for the study of inhalational anthrax, have shown that spores have the ability to persist in the lungs of primates for up to 60 days after exposure; retained spores germinated and caused disease after antibiotics were discontinued.[113] Moreover, in some monkeys fatal disease occurred up to 58 days and 98 days after exposure and viable spores were demonstrated in the mediastinal lymph nodes of one monkey 100 days after exposure.[28] These observations are the basis for recommending antibiotic therapy for at least 60 days, with oral therapy replacing intravenous therapy when the patient is clinically stable enough to take oral medication.[7,10] However, the duration of treatment for established anthrax is still controversial. Recently, researchers using rhesus macaques determined that the prolonged course of antibiotics required to achieve prophylaxis may not be necessary to prevent anthrax resulting from the germination of retained spores after the discontinuation of antibiotics.[114]

As there is evidence that postexposure vaccination can shorten the duration of antibiotic prophylaxis, infected individuals should be vaccinated at the commencement of treatment so that antibiotic administration could be discontinued once protective immunity has developed.[115] Given the attractiveness of anthrax as a biological weapon, the modification of wild-type *B. anthracis* to provide antibiotic resistant strains is a distinct possibility. Therefore, it is important to provide a postinfection anthrax treatment that is not prone to antibiotic resistance.[111]

3.3.1. Current Recommended Antimicrobials. On the basis of experiments conducted in primates, a course of aggressive antibiotic therapy has been adopted for postexposure prophylaxis. Most naturally occurring *Bacillus anthracis* strains are sensitive to penicillin, which historically has been the agent of choice for the treatment of anthrax. In vitro testing has shown that most strains of *Bacillus anthracis* are fortunately sensitive to a variety of antimicrobials, such as penicillins, fluoroquinolones, tetracyclines, chloramphenicol, aminoglycosides, macrolide antibiotics, imipenem, rifampicin, cefazolin, linezolid, clindamycin, and vancomycin. For patients allergic to penicillin, any of these agents offers an effective alternative.[47]

Antibacterials need to exhibit potent in vitro activity, intracellular bioactivity, and suitable locations in lymph nodes. In animal models, doxycycline and ciprofloxacin hydrochloride are the most active compounds for the most severe cases of postexposure inhalational anthrax, and although treatment of anthrax infection with ciprofloxacin has not been studied in humans, animal models suggest excellent efficacy. In vitro data suggest that other fluoroquinolone antibiotics would have equivalent efficacy. There are no controlled clinical studies for the treatment of inhalational anthrax in humans; thus, antibiotic regimens commonly recommended for empirical treatment of sepsis have not been studied. Ciprofloxacin, doxycycline, and penicillin G-procaine are approved by the FDA for postexposure prophylaxis of inhalational anthrax. For mild cases of cutaneous anthrax, the use of ciprofloxacin (500 mg twice daily), doxycycline (100 mg twice daily), or amoxicillin (500 mg 3 times daily) is recommended.[7]

The problem of increasing bacterial resistance to the current generation of antibiotics is well documented.[116] Engineered strains resistant to multiple antibiotics, including the front-line agents ciprofloxacin, doxycycline, and β-lactam antibiotics, have been constructed.[117] *Bacillus anthracis* strains are usually resistant to trimethoprim, sulfonamides, and many of the antibiotics used in empirical regimens for sepsis treatment, such as the extended-spectrum cephalosporins. It is recommended that antibiotic resistance to penicillin- and tetracycline-class antibiotics should be assumed following a terrorist attack until laboratory testing demonstrates otherwise. The current standard postexposure prophylaxis for adults with presumed inhalational anthrax is ciprofloxacin therapy twice daily for 60 days,[118] with doxycycline or amoxicillin used only as alternatives (doxycycline is the preferred option in the tetracycline group because of its proven efficacy in monkey studies and its ease of administration).[75] The same agents are recommended for the treatment of established inhalation or gastrointestinal anthrax with the addition of two or more antimicrobial agents (clindamycin and rifampicin are suggested for administration with ciprofloxacin or doxycycline).

As VaxGen was not able to meet the aggressive schedule for stockpiling 75 million doses of the new second-generation vaccine, the U.S. will depend on antibiotics to treat a largescale anthrax attack, a strategy that terrorists could overcome by creating antibiotic-resistant strains. The U.S. Department of Health and Human Services has purchased enough antibiotics to treat approximately 40 million Americans for 60 days.[16,119]

3.3.2. Other Antimicrobial Approaches. (a) PlyPH, PlyB, and PlyG are three bacteriophage hydrolases that lyse the peptidoglycan molecules of the bacterial host cell. Should infections occur with a strain of *B. anthracis* resistant to conventional antibiotics, these cell wall-cleaving enzymes might be considered as an alternative form of therapy.[120,121]

(b) The transcriptional regulator AtxA protein has major effects on the physiology of *B. anthracis*. At the first stages of germination, AtxA is the central orchestrator of spore responses to host signals; therefore, agents that could block AtxA activity would be a powerful class of antibiotics.[122]

(c) Various extracellular metalloproteases of *B. anthracis* affect host immune defenses and cause tissue damage during infection. Chemical protease inhibitors, such as phosphoramidon and 1,10-phenanthroline, have been used to treat mice challenged with Sterne spores and demonstrated a substantial protective efficacy in combination with ciprofloxacin.[123] Moreover, *B. anthracis* showed a high level of resistance to the human antimicrobial peptide LL-37 and its degradation by *B. anthracis* culture supernatant was blocked using the metalloprotease inhibitors EDTA and 1,10-phenanthroline.[124] These compounds constitute an interesting approach against strains resistant to approved antibiotics and could represent a promising class of new-generation antibiotics possessing a different mechanism of action.

(d) Thymidine monophosphate kinase is an enzyme of *Bacillus anthracis* essential in nucleotide biosynthetic pathways, and several lipophilic thymidine phosphate-mimicking compounds have been synthesized as potential enzyme inhibitors. Three of the tested compounds inhibited the activity of thymidine monophosphate and growth of the *B. anthracis* Sterne strain. The biological evaluation of this type of enzyme inhibitors suggests their potential use as novel antibiotics in anthrax treatment.[125]

(e) Dihydrofolate reductase (DHFR) is a biosynthetic enzyme necessary for anthrax pathogenicity. *B. anthracis* exhibits a natural resistance to trimethoprim (TMP), a clinically used antibacterial DHFR inhibitor, because of a lack of affinity between the enzyme and the inhibitor. It is a novel lead series of *B. anthracis* dihydrofolate reductase inhibitors characterized by an extended trimethoprim-like scaffold. The structure revealed several features that can be exploited for further development of narrow-spectrum antibiotics against anthrax.[126]

(f) Human secretory type-IIA phospholipase A2 (sPLA2-IIA) is an enzyme produced by macrophages, and its anthracidal activity is attributed to the ability to hydrolyze membrane phospholipids. *B. anthracis* evades the macrophage surveillance through the inhibitory action of LT on sPLA2-IIA secretion. This inhibition occurs through a process involving ET-mediated cAMP accumulation and represents a novel mechanism for evading the innate immune response of the host. The use of pharmacological approaches to inhibit the adenyl cyclase activity of invading bacteria may represent a therapeutic strategy for treating pulmonary anthrax.[127] This natural component of the immune system may serve as a new therapeutic agent that could be used complementary to current therapy, even against antibiotic-resistant strains of *B. anthracis*.[128]

(g) Oritavancin is a semisynthetic lipoglycopeptide, and its potent in vitro activity against *B. anthracis* prompted researchers to test its efficacy in a mouse aerosol-anthrax model. Efficacy in pre- and postexposure models of inhalation anthrax, together with a demonstrated low propensity to engender resistance, has encouraged the further study of oritavancin pharmacokinetics and its efficacy in nonhuman primate models.[118]

(h) Triclosan is an agent that provides a potential scaffold for the development of new broad-spectrum antibiotics. It exhibits a broad-spectrum antibacterial activity by targeting the fatty acid biosynthetic pathway through inhibition of enoyl-acyl carrier protein reductase (ENR). Researchers have developed novel aryl ether triclosan analogues that target ENR, and some of these compounds have exhibited improved potency against *Bacillus anthracis* ENR and increased efficacy against the Sterne strain. X-ray crystal structures suggest future rounds of optimization might be used to improve the potency of candidate compounds.[116]

(i) Germination of *Bacillus anthracis* spores into the vegetative form can be triggered in vitro by the common germinants inosine and alanine. Because inosine is a critical germinant in vitro, inosine analogues have been screened for the ability to block in vitro germination of *B. anthracis* spores. This led to the identification of 6-thioguanosine, which efficiently blocked spore germination in macrophages and prevented their destruction by *B. anthracis* spores. 6-Thioguanosine showed potential as an antianthrax therapeutic agent.[129]

3.4. Antitoxin Agents. The treatment of inhalation anthrax with antibiotics is usually ineffective, as the disease is rarely recognized before bacteremia and toxemia develop. There is an urgent need for antibiotics to provide improved therapy in combination with an anthrax antitoxin.[130] Toxin inhibitors, even though powerful, may suffer the same drawbacks as antibiotics and require rapid administration to have a significant clinical effect.

Targeting bacterial virulence is an alternative approach to antimicrobial therapy that offers promising opportunities to inhibit pathogenesis and its consequences without placing immediate life-or-death pressure on the target bacterium.[12] Disruption of toxin function is an obvious approach to inhibiting bacterial virulence which can occur either in a direct manner by inhibition of the toxin activity itself or in an indirect manner by modulating the host response to the toxin. Direct inhibition is the basis for the historical use of antibodies against toxins.[131]

Recently, much effort has been focused on inhibiting the effects of the three proteins that comprise anthrax toxins. Growing insights into the structure and function of PA, LF, and EF have led to the design and production of an increasing number of agents capable of neutralizing toxins or their effects and have revealed potential applications in medicine, including approaches to treating anthrax infections.[24,26,34] Such agents may be even more important than antibiotics because the latter, although quickly clearing bacteremia in patients during the 2001 outbreak, still did not prevent lethality in some patients. Current approaches are focused on agents that affect crucial steps in the intoxication process, including antitoxins−antibodies, receptor decoys, LF decoys, dominant-negative inhibitors of translocation, small molecule inhibitors, substrate analogues, furin inhibitors, etc. Moreover, the elucidation of the consequences of LF activity, downstream of proteolytic cleavage of MAPKKs, leading to the death of the host, would be of great interest for the design of therapeutics targeting the clinical effects of LT.

3.4.1. Targeting the Fundamental Steps in LT and ET Uptake by Host Cells (Figure 3). 3.4.1.1. PA-Based Antitoxin Approaches. Because of the principal role of PA in anthrax pathogenesis, PA is a potential target that could be inhibited in multiple ways, and great research efforts for the development of new anti-infective options have focused on this antigenic factor.[131] Promising anti-PA strategies include the blocking of PA receptors, the blocking of PA binding to its host receptors, the inhibition of PA processing by host

furin proteases, the prevention of PA oligomerization to form the heptameric PA63 prepore, and the blocking of PA63 heptamer pores with dominant-negative PA mutants to inhibit LF and EF trafficking and delivery within the cytosol.[23]

Each molecule of LF (or EF) binds stably only to PA_{63} dimers or higher order oligomers and not to monomers. Moreover, PA can bind LF and EF simultaneously, forming a greater variety of toxin complexes than were previously known.[23] The elucidation of the structural basis for LT and/or ET assembly may prove very useful in the near future in developing effective drugs against anthrax toxins.

3.4.1.1.1. Targeting PA-Host Receptors. As resistance of *B. anthracis* to antimicrobial therapeutics can be deliberately engineered, blocking host receptors used by the PA toxin subunit represents a powerful strategy for overcoming this problem because extensive alterations to the pathogen may be required to enable it to switch to a new receptor that can still support pathogenesis.

(a) Low-density lipoprotein receptor-related protein 6 (LRP6) is a co-receptor for the Wnt signaling pathway in *B. anthracis* toxicity. The extracellular domain of LRP6 interacts, either directly or indirectly, with TEM8 or CMG2 in a PA-independent manner. The formation of a multicomponent complex at the cell surface stimulates PA-binding and appears to play a very specific role in toxin entry. Recent studies have revealed the previously unsuspected biological role of LRP6 for prophylaxis and treatment of anthrax toxicity. LRP6-specific antibodies have protected cell cultures from destruction by LT, indicating that this newly identified co-receptor might be a viable target in providing protection against the effects of accumulated LT.[132]

(b) A novel inhibitor, designated "functionalized liposome," has been shown to block PA attachment to receptors on the surface of host cells. The liposome is a fatty bubble studded with small proteins that inhibit internalization of the toxin into the host cell by binding to multiple sites on the two host receptors. In animal studies, the new inhibitor has been shown to be many times more potent than current therapies and is especially promising as a countermeasure to antibiotic-resistant strains or as a potential adjunct to antibiotic therapy.[133]

3.4.1.1.2. Targeting $PA_{(83)7}$ Attachment to Host Cell Receptors. The three-dimensional structure of PA "docked" to one of two receptors has been mapped and has revealed the way that anthrax toxins use host receptors to enter human cells, providing new approaches for the discovery of anthrax antitoxins and the design of cancer therapeutics.[33] The passive transfer of antibodies against PA is one approach that mitigates the biological action of anthrax toxins.[103,134] Since the 2001 anthrax attacks, remarkable research efforts have been made for the production of PA-directed antibodies that contribute to the functional analysis of PA and offer immunotherapeutic perspectives for the treatment of anthrax infection (see also section 3.2):

(a) PAmAb is a fully human monoclonal antibody with high affinity for PA and has provided a survival advantage in rabbit and monkey models of inhalational anthrax. In phase 1 of the first clinical study since the 2001 anthrax attacks in the U.S., PAmAb was shown to be safe and well tolerated when administered to healthy adults volunteers.[135]

(b) mAb 7.5 is a monoclonal antibody against PA, which is able to neutralize the activities of anthrax toxins produced by *B. anthracis* in mice. It binds to domain 4 of PA and prevents the attachment of PA to its cellular receptors.[136]

(c) A fully human monoclonal antibody, IQNPA, with specificity for PA has been developed from individuals immunized with licensed anthrax vaccine. IQNPA binds to domain 4 of PA containing the host cell receptor binding site. It has been shown that it does not interfere with the establishment of endogenous immunity in mice treated with this antibody and infected with *B. anthracis* Sterne spores.[103]

(d) The anthrax toxin receptor (ATR) is a transmembrane protein of unknown physiological function, and a specific region, the VWA extracellular domain, has been identified on the surface of mammalian cells which is responsible for the binding of PA to the corresponding receptor.[32] The high affinity of the VWA domain of human sCMG2 to PA supports its potency in neutralizing anthrax toxins and demonstrates its potential utility for the design of toxin-blocking drugs based on the use of soluble forms of both receptors. Importantly, purified VWA protein responsible for the binding of PA to the TEM8 receptor has been produced as a fusion protein in *E. coli* and exhibited very high affinity to PA.[137] When soluble receptors were mixed with mammalian cells in the presence of anthrax toxin, they acted as decoys and competed with cellular receptors for binding and absorbed anthrax toxins before they could attach to target cells to produce their lethal effects.[32,138] sATR/CMG2 showed a stronger antitoxin potency than sATR/TEM8, suggesting that sCMG2 may be one of the most effective anthrax antitoxins.[138]

3.4.1.1.3. Targeting Assembly of the Heptameric PA63 Prepore. Recently, the crystal structure of the PA-ATR2 complex has been solved at 2.5 Å resolution, and modeling of the receptor-bound $(PA63)_7$ pore indicates that the receptor acts as a pH-sensitive brace to ensure accurate and timely membrane insertion. The structure revealed an extensive receptor−pathogen interaction and should provide new leads for the discovery of novel antitoxin agents against anthrax infection and aid the design of cancer therapeutics as well.[33] In addition to monoclonal antibodies, several other treatments directed against PA have also been developed and prevent the prepore assembly:

(a) One potentially promising strategy is the use of furin inhibitors. Furin is an endogenous membrane-associated endopeptidase that is used by *B. anthracis* to activate PA once delivered to target cells; it cleaves the precursor form of PA, PA(83), into the functionally active fragment of PA(63) and a soluble fragment known as PA(20). Inhibitors of furin have been shown to block the formation of functional PA-(63) heptameric units and to protect cells from the lethality of anthrax toxins. These compounds have a diverse chemical structure and may serve as valuable tools for studying furin action and potential therapeutic agents for anthrax and other furin-dependent diseases (Alzheimer's disease, cancer, viral and bacterial infections). A small stable compound, hexa-D-arginine amide (D6R), is a furin-inhibiting molecule that delays anthrax toxin-induced toxemia both in cells and in live animals.[139] Small furin inhibitor molecules, based on 2,5-dideoxystreptamine with nanomolar range potency against furin, have protected RAW 264.7 macrophage cells from toxicity caused by the furin-dependent processing of PA. Molecular modeling revealed that these inhibitors may target the active site of furin and behave as competing inhibitors of furin.[140] Inter-α-inhibitor protein (IαIp) is an endogenous serine protease inhibitor in human plasma, and

its levels decrease during acute inflammatory states. It has blocked furin activity in vitro and protected murine macrophages against LT-induced cytotoxicity. It seems that human IαIp may be an effective preventive or therapeutic agent against anthrax LT.[141] Polyarginine-containing peptides have inhibited furin action and represent promising compounds against *B. anthracis* toxemia.[142] The combined use of high-affinity antifurin drugs and weak bases such as chloroquine, which neutralizes acidic compartments, strongly augmented the inhibition of toxin-dependent lethality of murine macrophages; the combined use of these anti-infective agents may provide enhanced therapeutic benefits against inhalational anthrax.[143] The combined administration of inhibitors could represent an effective therapeutic approach: D6R or D9R (nona-D-arginine amide, both blocked the proteolytic activation of PA), combined in vitro with In-2-LF (an LF inhibitor, peptide hydroxamate), enhanced protection against anthrax lethal toxin.[144] Anthrax toxins require processing by host furin-like proprotein convertases (PCs) to enter host cells and to cause disease. Specific nanomolar inhibitors of PCs have confirmed that inhibiting PCs protected the host from anthrax infection and laid a foundation for novel therapies for furin-dependent bacterial toxins and viral pathogens.[145]

(b) The antitumor drug cisplatin has been shown to block the formation of heptameric prepore. Cisplatin-treated PA was unable to form heptameric PA63 oligomers required for LF binding and translocation; it acts by modifing the PA structure in a reversible noncovalent manner. The simultaneous administration of biologically relevant concentrations of cisplatin and a lethal dose of LT has been shown to be protective in rodent models.[146]

3.4.1.1.4. Targeting Toxin Translocation via the Pore and Delivery within the Cytosol. In addition to targeting toxin receptors and toxin activity, virulence could also be inhibited by interfering with the appropriate delivery of toxins to their site of action.[23] The $(PA_{63})_7$ pore apparatus is involved in delivering toxins into the host cell cytoplasm. It is believed that enzymatic toxin subunits are initially delivered into the lumen of intraluminal vesicles (within the endosomal pathway), which are then trafficked to late endosomes and fuse with the limiting endosomal membrane, releasing toxins into the cytosol. It seems that a phenylalanine clamp controls protein translocation through the heptameric PA pore, and this clamp can be targeted with small molecules that block the pore.[147] Therefore, toxin translocation could be inhibited either by blocking the PA toxin subunits or by altering the endosomal structure and function, resulting in impaired toxin delivery.[23,131]

(a) The screening of a phage-display library for mutant peptides that can bind the heptameric cell-binding subunit of anthrax toxin has identified a novel peptide sequence, TYWWLD. This could block toxin assembly and be used to develop potent polyvalent toxin inhibitors.[148]

(b) Dominant-negative inhibitors (DNI) are translocation-deficient point mutations of PA with abnormal loops that form dysfunctional heptamers when coassembled with wild-type PA protein. Mutations in DNI have been shown to inhibit the required conformational change of chimeric DNI/PA heptamers or $(DNI_{63})_7$ from a ring-shaped core to a β-barrel, thus preventing the heptamer from inserting into the endosomal membrane. Consequently, replacing a single residue in one subunit of the heptameric PA prepore can inhibit the translocation of LF or EF into the cytosol and prevent cytotoxicity transport activity of the oligomer almost completely, suggesting that DNI forms of PA could be used as novel antitoxins.[23,149] DNI of anthrax toxin may serve as both an anthrax vaccine and therapy. As antibiotic treatment cannot provide full protection against relapse or subsequent exposure to anthrax, conjunctive antibiotic treatment and vaccination with DNI would be an ideal option.[9]

(c) Clustering of the ATR with the $(PA_{63})_7$ complex causes its association to specialized cholesterol, glycosphingolipid-rich and detergent-resistant microdomains of the cell membrane, designated "lipid rafts". Although endocytosis of ATR is slow, clustering into rafts is necessary and sufficient to trigger efficient internalization of anthrax toxins and allow their delivery into the cytoplasm. Importantly, by use of drugs that altered raft integrity, LF delivery and cleavage of cytosolic MAPK kinases have been prevented, suggesting that lipid rafts could be therapeutic targets for drugs against anthrax.[23,150]

(d) Chloroquine is a commonly used antimalarial compound. Chemically it is a weak base that reduces the suppressive effect of LT on lymphocytes, possibly by interfering with intracellular acidification steps (neutralizes the acidic heptameric PA_{63} compartments).[143,151,152] It may augment current treatment and prophylaxis options for this otherwise lethal infection.

(e) ATP-activated macrophage P2X7 receptors are implicated in nucleotide-mediated macrophage lysis. It has been discovered that a potent P2X7 antagonist, oxidized ATP (o-ATP), can increase endosomal pH and completely protect BALB/cJ mice from LT-mediated cytolysis.[153]

(f) Two compounds, amiodarone and bepridil, used to treat cardiac arrhythmia or angina in humans, have been found to provide in vitro protection against LT. Both drugs appear to interfere with the insertion of the PA heptamer into the endosomal membrane via neutralization of the endosomal pH, thereby blocking toxin entry into the cytosol.[154]

(g) The chances of finding high affinity blocking agents increase if they have the same symmetry as the target PA pore. Guided by the shape and chemistry of the seven-sided PA pore, investigators have synthesized small cyclic molecules using β-cyclodextrin as the starting molecule. These derivatives of β-cyclodextrin were chemically modifidied to add seven positive charges to predominantly negatively charged PA pore lumen and inhibited in vitro LT action by blocking the ion conductance through the heptameric PA channel. This approach could serve as the basis for a structure-directed drug discovery program, with the goal of identifying a new class of drugs that block the pathway for toxin translocation into the cytosol.[155−157] β-Cyclodextrins have been shown to inhibit the toxicity of LT both in vivo and in vitro. When combined with LFn-Lip loaded with antianthrax drugs, β-cyclodextrins may be used against intracellular targets (LFn represents a complex of liposomes decorated with catalytically inactive fragments of LF).[158]

3.4.1.2. LF-Based Antitoxin Approaches. Since LF has been shown to be the dominant virulence factor in anthrax pathogenesis,[95] much work has focused on finding potent inhibitors of LF for treating exposure to *Bacillus anthracis* spores with small molecule drugs, particularly in the late stage of infection.[159,131,160] It is envisaged that a complementary combination of an antimicrobial mechanism and an LF-inhibiting mechanism has the potential to provide additional means of therapeutic intervention and substantially reduce mortality.

Though first suspected of being a metalloproteinase over 10 years ago, the first specific inhibitors of LF did not appear until 2002.[112] Since then, several classes of specific chemically distinct classes of inhibitory molecules have been identified, from generating conventional metal chelating substrate analogues to random screening of diverse compound libraries. They may present several advantages, including a potential increase in the survival rate of patients by reducing or preventing the damage to vascular circulation and the gaining of precious time for the antibiotics to clear the bacteria. Given prophylactically, they could prevent anthrax by helping the host innate immune system to act at an early stage.[112,159,161−163]

LF inhibitors act in very low concentrations (from low micromolar range to nanomolar range).[164] However, the need for these inhibitors to act intracellularly may mean that even higher affinity (subnanomolar) inhibitors will be needed to make viable therapeutics.[160]

(a) The aminoglycosides neamime and neomycin B have both been shown to inhibit LF protease activity. They bind to the LF in two different structural orientations and have important implications for the rational design of LF inhibitors. Guanidinylated derivatives of neamine have been found to represent a structural scaffold for the design of novel LF inhibitors. Besides aminoglycosides, several chemically distinct classes of molecules with LT-inhibitory action have been identified, including polyamines and cationic peptides. Spermine has been demonstrated for the first time to inhibit LF activity.[111,165,166]

(b) Rhodamine derivatives are small molecules with a strong and highly specific inhibition of LT protease activity of bacterial toxins, namely, the proteases anthrax lethal factor and the botulinum neurotoxin type A. The integration of results with structure−activity relationship studies has provided a framework for the development of drug candidates against anthrax and botulinum.[167]

(c) Catechins are compounds classified as polyphenols. They are contained in green tea, and some of them are powerful inhibitors of LF metalloproteolytic activity. The main catechin of green tea, (−)-epigallocatechin 3-gallate, has been shown to prevent the LF-induced death of macrophages and Fischer 344 rats.[168]

(d) In a search for small molecules that would protect the population from anthrax, Merck Co. has identified a hydroxymate that inhibits LF protease activity and promotes cellular survival in a macrophage cytotoxicity assay. This molecule bound the active site of LF and offered complete protection from spore infection when administered to mice in combination with ciprofloxacin 66 h postinfection.[169]

(e) Full-length, biologically inactive mutants of LF (and EF), when combined with PA, have competitively inhibited LT and ET-mediated activity in vitro and lethality in vivo, suggesting their potential use as prophylactic or therapeutic agents.[170]

(f) In the severe form of anthrax, bacteria spread throughout the body and the toxin produced remains active in the bloodstream for several days. Key structures have been identified in the LF molecule that could lead to the development of nontoxic compounds analogous to LF (decoys). These agents competed with native LF molecules for PA prepore binding and protected cells from anthrax LT.[24]

(g) High-throughput screening is currently a frequently used and promising approach in identifying novel LF inhibitors. A library of approximately 14 000 compounds has been screened using a fluorescence-based in vitro assay, resulting in the identification of new scaffolds. These agents inhibited LF in the low micromolar range and represent promising leads for the development of effective LF inhibitors.[162] Similarly, by the screening of a chemolibrary of 10 000 druglike molecules, 18 novel small molecules with important LF inhibitory activity have been identified. These compounds and the structural scaffolds could be further exploited for the development of potent and selective antitoxin agents.[171]

(h) A structure−activity relationship (SAR) of potential LF inhibitors has been presented in which the zinc-binding group (ZBG), linker, and backbone moieties for a series of hydroxypyrone-based compounds were systematically varied. Hydroxypyrothione ZBGs generated more potent inhibitors than hydroxypyrone ZBGs. The results highlighted the need for a better understanding of how the interplay between the ZBG, linker, and backbone can be optimized to procure improved LF inhibitors.[172]

(i) In regard to antibodies (see also section 3.2), in research conducted in France, an anti-LF single-chain variable fragment (scFv) originating from an immunized macaque was obtained by phage display. This antibody fragment inhibited the formation of the LF−PA complex, suggesting its suitability for prophylaxis and therapeutics.[173]

(j) In research conducted by Moayeri and colleagues, LT-treated mice, deficient in the enzyme neuronal nitric oxide synthase (nNOS), had striking architectural changes in heart morphology. Cardiac protective nitrite therapy and allopurinol therapy did not have any beneficial effect. The heart represents an early target of LT in mice, and nNOS has a protective role against LT-mediated cardiac damage.[174]

3.4.1.3. EF-Based Antitoxin Approaches. Because *B. anthracis* putatively produces all three toxin moieties in vivo, recent efforts have been made to shed light on the effects of ET on various cell types and to inhibit its toxicity.[175]

(a) Using the structure of the catalytic site of EF and screening a database of commercially available small molecular weight chemicals, researchers have identified one quinazoline compound (ethyl 5-aminopyrazolo[1,5-*a*]quinazoline-3-carboxylate) that specifically inhibits the adenylyl cyclase activity of EF. This compound is a competitive inhibitor because it binds to the adenine portion of the ATP binding site on EF; it neither affected the activity of host resident adenylyl cyclases nor the binding of calmodulin to EF. This compound could serve as a lead in the design of antitoxins combating inhalational anthrax and to address the role of EF in anthrax pathogenesis.[176]

(b) Adefovir dipivoxil is a nucleotide analogue, a clinically approved antihepatitis B virus drug that targets virus-specific DNA polymerase and mimics ATP, the natural endogenous substrate of EF. In murine macrophages it has electively blocked EF with great affinity and effectively prevented EF-induced increases in c-AMP, thus preventing the ET-mediated alteration of cellular functions.[177]

(c) Treatments against EF may be useful if combined with anti-LF treatment. It has been shown that purified rEF could compete in vitro with rLF over the binding regions of PA. The results of this study encourage the screening for new EF inhibitors and suggest that rEF and rLF can restrain each other competitively.[178]

(d) A structure-based method has been used to identify non-nucleotide inhibitors of EF. A library of small molecule fragments was docked to the EF-active site in existing crystal

structures. Nineteen diverse compounds with the best AutoDock binding/docking scores were assayed in a cell-based assay for their ability to reduce cAMP secretion induced by EF. This fragment-based pharmacophore method identified a small number of compounds from different structural groups that inhibited EF activity in the low micromolar range and could be used as lead compounds for combinatorial design.[179]

3.4.2. Targeting the Intracellular Toxin Effects. 3.4.2.1. Targeting the LF Enzymatic Activity. LT-induced cell death via cleavage of MAPKK remains questionable. Although it has been shown that small molecules that activate MAP kinase cascades protect mouse macrophages from LF-induced cell death, it is unclear how LF-mediated MAPKK cleavage is related to cytotoxicity. Rather than directly inhibiting toxin function, the downstream effects of LF could be blocked by targeting host intracellular proteins, e.g., targeting LF interactions with MAPKKs and MAPKKs substrate peptides.[131] Studies on these interactions have begun to explain the highly restricted specificity of LF and to provide a basis for design of protease inhibitors.[3,180−183]

(a) Proteins can be degraded by the proteasome via the N-end rule which is required for LT-mediated caspase-1 activation and cell death. Proteasome inhibitors block LT-mediated caspase-1 activation and can protect against cell death, indicating that the degradation of at least one cellular protein is required for LT-mediated cell death. It has been found that bestatin methyl ester, an aminopeptidase inhibitor, protects against LT challenge.[184]

(b) Celastrol, a triterpene compound derived from a plant extract, is a potential inhibitor of LT-induced macrophage lysis.This agent does not inhibit the cleavage of MAPKK-1 but the proteasome-dependent degradation of proteins. Celastrol could also rescue cells in the late stages of intoxication by conferring almost complete protection to them 1.5 h after intoxication.[185]

(c) α-Defensins form a group of compounds produced by human neutrophils. Human neutrophil α-defensins (HNP-1) have prevented deleterious effects of LT in vitro experiments by inhibiting the cleavage of MAPKKs and restoring their impaired signaling function in LT-challenged macrophages.[186]

(d) θ-Defensins are cyclic octadecapeptides encoded by the modified α-defensin genes of certain nonhuman primates. Retrocyclins are derivatives of θ-defensins that have the ability to bind the LF rapidly and with high affinity, preventing its deleterious effects. Certain defensins have not only exerted potent antibiotic activity against the spores and bacilli of *B. anthracis* but also inactivated the enzymatic activity of LF and protected murine RAW-264.7, providing molecular templates for structure-based improved drugs against *B. anthracis* and its toxins.[187]

(e) LT-Mediated necrosis of macrophages has been blocked by complete inhibition of caspase-1 activity. It seems that caspase-1-mediated macrophage necrosis is the source of the cytokine storm and inhibition of the caspase-1 activity could represent a potential anti-infective target.[188,189]

3.4.2.2. Targeting the EF Enzymatic Activity. The catalytic efficiency of EF is enhanced by approximately 1000-fold upon its binding to host protein calmodulin (CaM). The screening of a 10 000 member library has identified a molecule that inhibits the EF−CaM interaction and therefore the adenylyl cyclase activity. This compound represents an important reagent in the study of the role of EF in anthrax pathology and a drug lead against inhalational anthrax.[190] EF elicits edema in host tissues, but the target cells and events leading from EF-mediated c-AMP production to edema are unknown. It stimulates the release of multiple inflammatory mediators, specifically neurokinins, prostanoids, and histamine, which contribute to edema. These mediators increase vascular permeability, and interventions directed at these mediators may benefit hosts infected with *B. anthracis*.[191] EF has potent adenyl cyclase activity and increases host cell cytosolic cAMP levels. The production of cAMP is dependent on an influx of calcium, and cAMP accumulation is prevented by calcium channel antagonists or the absence of calcium.[34]

3.5. Other Anti-Infective Approaches. Alongside the classical plasmid-based virulence factors, there is much experimental evidence that supports the view that newly identified *B. anthracis* components, encoded by genes located either on the chromosome or on the virulence plasmids, could have immunogenic properties and contribute to the overall anthrax pathogenesis. These antigens may be additive ingredients for PA-based vaccines, resulting in products with a less demanding vaccination regimen.[192]

3.5.1. Other Approaches Targeting Germinated Spore Components. Analysis of the sequence of the chromosome of *B. anthracis* Ames has revealed several encoded proteins, including phospholipases, proteases, superoxide dismutases, anthrolysins, iron acquisition proteins, and surface proteins, etc. These molecules could be regarded as newer virulence factors contributing to the virulence of *B. anthracis*.[193]

(a) Sortase-B is an enzyme that contributes to heme iron scavenging from the host cells, an element that is vital for *B. anthracis* survival. Its location on the cell wall envelope of vegetative bacteria makes this enzyme an attractive target for anthrax therapy.[194]

(b) Anthrolysin (Anl-O) is a cholesterol-dependent cytolysin that is secreted by *B. anthracis* and is largely responsible for the hemolytic activity of *B. anthracis* culture supernates. The fact that Anl-O is lethal to human immune cells (macrophages, neutrophils, monocytes, and lymphocytes) supports the idea that it may represent a novel virulence factor and be a candidate target for the development of an antitoxin vaccine.[195,196]

(c) Purine nucleoside phosphorylase (PNP) is a key enzyme in the purine-salvage pathway of *B. anthracis* whose crystal structure has been solved. Because of its importance in *B. anthracis* survival, it might help in the development of a new generation of structure-based PNP anthrax inhibitors.[197]

(d) A novel protein required for the replication of the pXO1 virulence plasmid, designated "FtsZ-like protein", has been discovered. This replication agent may provide a novel biological target for the elimination of the pXO1 plasmid.[198]

(e) Sap and EA1 are two abundant S-layer proteins that have protected laboratory animals from anthrax challenge. They could serve as additional immunogenic targets for the development of new anthrax vaccines.[199]

(f) Two neutral zinc metalloproteases have been purified from the culture supernatant of a nonvirulent δ Ames strain (pXO1, pXO2) designated "Npr599" and "InhA" and belonging to the M4 and M9 thermolysin and collagenase families, respectively. Both proteases represent multifunctional pathogenic factors that may contribute to anthrax pathogenesis through direct degradation of host tissues. They could be regarded as effective biological targets in the development of novel approaches complementing anthrax therapy against LT.[200]

(g) Nowadays, it is possible to characterize host−pathogen interactions from a global proteomic viewpoint, aiming at improved therapeutic modalities. For this reason a network of seven biodefense proteomics research centers (PRCs) was recently established in the U.S. to support the development of innovative proteomic technologies. The screening for host−pathogen proteins identified 15 specific interacting host proteins for *B. anthracis*, seven for the PA and eight for LF. These proteins could serve as potential targets for novel therapeutics and/or diagnostics.[201]

(h) Ribonucleotide reductase provides deoxyribonucleotides for DNA synthesis needed for spore germination and the growth of pathogens. In combination therapies against *B. anthracis* infections, this key enzyme could represent a potential target of antiproliferative drugs such as hydroxyurea and the antioxidants hydroxylamine and *N*-methylhydroxylamine.[202]

(i) Macrophages generate large quantities of nitric oxide (NO) and other nitrogen and reactive oxygen species to combat infecting bacteria. Surprisingly, in vivo resistance of *Bacillus anthracis* to macrophage killing has been shown to depend on its own NO-synthase (bNOS) activity. Results showed *Bacillus anthracis* bNOS to be an essential new virulence factor and an attractive antimicrobial target for treatment of anthrax.[203]

(j) Three phospholipases C (PLCs) have been shown to contribute to the macrophage-associated growth of *B. anthracis* by aiding in the escape of the bacterium from phagocytic vacuoles. The functional redundancy between PLCs in the virulence of *B. anthracis* implies that their activities are important for anthrax pathogenesis and represent potential targets in anti-infective strategies.[204]

(k) *B. anthracis* synthesizes bacillibactin (BB) and petrobactin (PB), two small siderophore-based molecules to scavenge iron from its environment. Enzymes involved in siderophore biosynthesis for iron acquisition have become attractive targets for the discovery of new antibiotics.[205,206] Host cells synthesize siderocalin, a molecule that specifically reacts only with BB, to protect themselves from bacterial iron depletion. Surprisingly, *B. anthracis* synthesizes the alternative siderophore PB, whose structural variation precludes siderocalin binding, and in this way it stealthily evades the host immune surveillance.[207] Siderophores constitute potential therapeutic targets, and their structural determination is important for exploiting their therapeutic value.

3.5.2. Spore Components as Possible Anti-Infective Targets. A better understanding of the dynamic morphology of the dormant and germinating spore and its interaction with the host immune system could be important in developing an optimally efficacious anthrax vaccine.[208] PA-based anthrax vaccines acting on toxins are less effective than live attenuated vaccines, suggesting that additional antigens may contribute to protective immunity.[209] Recently, studies have focused on the recent wealth of genomic and proteomic information on *B. anthracis* spores to identify additional novel targets in order to augment the protective efficacy of PA-based vaccines.[193,210]

(a) *B. anthracis* exosporium contains 20 different protein species, and BclA has been characterized as the immunodominant protein of the exosporium. Vaccination with a combination of PA- and BclA-encoding pDNA has led to significantly better survival than immunization with plasmids encoding only PA or BclA.[211]

(b) Anthrose is a specific antigenic component of anthrax spores. Chemically it is a tetrasaccharide attached to BclA and could serve as an important agent in the development of anti-infective approaches targeting nonprotein *B. anthracis* spore structures.[212]

(c) Exosporially located components, ExsY, ExsF, ExsK, CotB, CotY, alanine racemace, and inosine hydrolase, especially the alanine racemace and inosine hydrolase, may influence spore germination within the macrophage and serve as potential biological targets for the development of novel anti-infective strategies.[213]

(d) The coat of *B. anthracis* spore contains at least 40 proteins. CotA is an abundant protein in the outer spore coat. It plays a key role in the spore resistance and could be an attractive antibacterial target.[214]

(e) Serological analysis of the extracellular and cytoplasmic proteomes of a nonvirulent *B. anthracis* strain has identified various in vivo expressed immunogenic proteins which were predicted to contribute to the establishment of anthrax infection. Such proteins include sulfatases, phosphatases, nucleotidases, chitinases, peptidases, and proteases. Among these, peptidases and proteases are considered to be important virulence factors and thus attractive antianthrax targets.[215,210]

(f) Spore opsonization-associated antigen A (SoaA) is a protein below the coat layer of the ungerminated spore that is involved in the interaction of spores with macrophages shortly after infection. In the presence of antispore antibodies, the SoaA protein in the Ames strain of *B. anthracis* has been seen to contribute to spore opsonization and increased the spore uptake by phagocytes, suggesting the potential use of SoaA as a vaccine antigen.[208]

3.5.3. Other Anticapsular Approaches. (a) The antiphagocytic action of the capsule can be neutralized by CapD depolymerase. CapD is a membrane-associated poly-γ-glutamate-specific depolymerase encoded on pX02 capsule plasmid. It contributes to *B. anthracis* virulence, first by anchoring capsular material to the *B. anthracis* envelope and second by degrading the high-molecular-weight capsule from the bacilli surface, resulting in the induction of macrophage phagocytosis of the encapsulated bacteria and killing them by human neutrophils. It has been shown that CapD variants failed to deposit the capsule into the envelope and displayed defects in anthrax pathogenesis. Similarly, the CapD inhibitor capsidin, 4-[(4-bromophenyl)thio]-3-(diacetylamino)benzoic acid blocked capsular assembly and enabled the phagocytic killing of nonencapsulated vegetative forms, suggesting the potential use of capsidin in anthax treatment.[216] It seems that the use of enzymes to degrade the capsule and enable the phagocytic killing of *B. anthracis* may lead to novel postexposure therapies.[217,218]

(b) Using poly-L-glutamic acid (L-PGA), scientists have synthesized biodegradable polyvalent peptide inhibitors at least 4 orders of magnitude more potent than the corresponding monovalent peptides. These synthetic peptides could represent attractive leads for designing biocompatible anthrax therapeutics.[219]

4. Treatment of Individuals Exposed to *B. anthracis* during the 2001 Anthrax Attacks

Subsequent to the outrage of September 11, 2001, in the U.S., after receiving a contaminated letter, a 63-year-old white man died of inhalation anthrax, the first person to develop this disease in the U.S. in 25 years. After the dispatch of probably

six such letters, as of January 26, 2002, a total of 11 cases of inhalation anthrax (five of these patients died) and 11 cases of cutaneous anthrax (seven confirmed and four suspected) had been identified. A letter sent to Senator Tom Daschle contained 2 g of powder with the Ames strain of *Bacillus anthracis* spores and an estimated spore quantity of >10 million LD_{50} doses.[2] Although the number of cases was relatively small to support a meaningful statistical conclusion, this experience brought bioterrorism and its potential into sharp focus as thousands of people in the U.S. began receiving prophylactic antibiotics after possible exposure to anthrax spores. Those events have resulted in a substantial impact on the health care system and public health planning.

Several clinical findings from the patients with inhalational anthrax deserve emphasis. Malaise and fever were symptoms in all cases. Cough, nausea, and vomiting were also prominent. Drenching sweats, dyspnea, chest pain, and headache were also seen in a majority of patients. Fever and tachycardia were seen in the majority of patients at presentation, as were hypoxemia and elevations in transaminases. Essential elements of care included rapid initiation of antibiotic therapy and supportive care (i.e., intravascular volume repletion, with vasopressor and ventilatory support as necessary), and drainage of the large pleural effusions, usually with chest tubes, was required in most of the cases. Therapy with corticosteroids had to be considered for patients with inhalational anthrax associated with meningitis or for patients who had severe mediastinal edema. The case-fatality rate among exposed individuals who received intensive care was 45%.[16,57]

The original recommendation from the CDC for postexposure prophylaxis was a 60-day course of antimicrobials. However, as anthrax spores persist in mediastinal nodes during antibiotic exposure and germinate to cause lethal disease after the withdrawal of antibiotics, recommendations were subsequently modified to permit 1 of 3 options: (1) a 60-day course of antibiotics followed by careful clinical observation, (2) extension of the course of antibiotics to 100 days, or (3) extension of antibiotic therapy to 100 days combined with administration of AVA in three doses at 2-week intervals. The AVA vaccination was not initiated immediately in persons believed to have been exposed to *B. anthracis*. Many Capitol Hill aides took AVA, but most postal workers refused, preferring to take their chances using antibiotics alone.[115]

An estimated 32 000 individuals initiated antimicrobial prophylaxis. Individuals were initially given ciprofloxacin, and when the antimicrobial sensitivities became available, they were encouraged to change to doxycycline. Amoxicillin was provided for pregnant women, breast-feeding mothers and children as a result of the concerns over the potential toxicity of the first two agents. Given the uncertainties regarding how many weeks or months spores may remain latent in the period following discontinuation of postexposure prophylaxis, individuals were instructed to report immediately flulike symptoms or febrile illness to their physicians who would then evaluate the need to initiate treatment for possible inhalational anthrax. While adverse events associated with antimicrobial prophylaxis were common (57%), serious events requiring hospitalization were rare (7%).[47]

Antibiotics were effective in the anthrax letters of 2001, but it will be recalled that ciprofloxacin, rather than penicillin, was recommended. This was due to concern that a bioengineered agent might have been used. If the anthrax strain had been engineered to be antibiotic-resistant, the aforementioned antibiotics might have been completely ineffective.[16] It is recommended that antibiotic resistance to penicillin- and tetracycline-class antibiotics should be assumed following a terrorist attack until laboratory testing demonstrates otherwise. Because the Ames strain causing these recent infections has shown the presence of constitutive and inducible β-lactamase, the treatment of systemic anthrax with penicillin or amoxicillin alone is no longer recommended. Moreover, although quickly clearing bacteremia in patients during the 2001 outbreak, antibiotics did not prevent lethality in some patients, suggesting that antitoxin agents may be even more important than antibiotics.[34]

5. The Sverdlovsk Anthrax Outbreak of 1979

The only large-scale outbreak of inhalational anthrax in the 20th century occurred in the former Soviet Union (FSU). The Sverdlovsk accident provides data on the only known aerosol release of *B. anthracis* spores resulting in an epidemic.

On April 2, 1979, an anthrax epidemic occurred among the citizens of Sverdlovsk (now Ekaterinburg), a city of 1.2 million people, 1400 km east of Moscow. Anthrax spores were accidentally released downwind from a military microbiology laboratory because of the failure to activate air filters.[7,8] At least 77 cases and 66 deaths occurred and livestock died, although U.S. intelligence sources claimed the toll might have reached 1000 victims.[9,14]

In 1986 Matthew Meselson (Department of Molecular and Cellular Biology, Harvard University) was invited to Moscow to discuss the incident with four Soviet physicians. The Soviet Union maintained that the anthrax outbreak was caused by consumption of contaminated meat that was purchased on the black market.[7,13] After the collapse of the Soviet Union, Boris Yeltsin, then the president of Russia, ordered an investigation into the causes of the epidemic in Sverdlovsk. In 1994 Meselson and his team returned to Russia to aid in these investigations. They reviewed the studies of pathologists at a local hospital in Sverdlovsk. One of the lead authors, Dr. Faina Abramova, made available her private records from a series of 42 autopsies.[220] In that accident, antibiotics, anti-anthrax globulin, corticosteroids, mechanical ventilation, and vaccine were used to treat some residents in the affected area after the accident. In fatal cases, the interval between onset of symptoms and death averaged 3 days. Post-mortem pathological analysis of specimens from 42 patients showed that all patients had necrotizing hemorrhagic thoracic mediastinitis, hemorrhagic lymphadenitis, pleural effusions and about half had hemorrhagic meningitis. The Sverdlovsk accident has shown that disease and death occurred from 2 to 43 days after exposure to the accidentally released anthrax spores.[28] The conclusion was that the pattern of these 42 cases of fatal anthrax bacteremia and toxemia was typical of inhalational anthrax as seen in experimentally infected nonhuman primates.[7]

6. Cost-Effectiveness Analysis of a Bioterrorism-Related Anthrax Attack

The small-scale terrorist anthrax attack that was perpetrated via the U.S. mail system raises the possibility of a similar future attack, employing the use of biological weapons of mass destruction on American soil and other countries, without warning or provocation.[15] In a large-scale attack the optimal future response may require strategies different from those required in a small-scale one. However, rapid screening

and identification of spores at the scene are extremely useful to prevent the costly interruption of services and potential referrals for medical evaluation.[221] An economic model developed by CDC suggests a cost of $26.2 billion per 100 000 persons exposed in a mass attack with anthrax.

A bioterrorism attack with anthrax will require rapid deployment of medical and pharmaceutical supplies to exposed individuals. A cost-effectiveness simulation analysis was conducted to determine the optimal response strategy for a small-scale attack perpetrated against U.S. Postal Service distribution centers in a large metropolitan area. Three strategies were compared: preattack vaccination of all U.S. distribution center postal workers, postattack antibiotic therapy followed by vaccination of exposed personnel, and postattack antibiotic therapy without vaccination of exposed personnel. Despite uncertainties about a future small-scale attack and exposure risk, postattack antibiotic therapy and vaccination of exposed postal workers seemed to be the most cost-effective response compared with the other strategies.[222] Similarly, another mathematical simulation of treatment approaches against anthrax in a genetically diverse population reached the same conclusion. It was shown that both vaccination and antibiotic administration should be the optimal strategy.[223] Regardless of which vaccination policy is adopted, vaccination before or after a large-scale attack, a rapid and effective postattack medical response has a large impact on the number of lives that can be saved.[224]

In September 2004 the CDC revised their recommendations on preventing anthrax disease to reflect the complementary roles of vaccine and antibiotics.[225] The CDC recommendations now include 60 days of oral antibiotics, along with a three-dose AVA vaccination regimen (0, 2, and 4 weeks). The difficulties of providing more than one vaccine dose to a large population, however, point to the need for a new anthrax vaccine that could be efficiently and rapidly delivered in a mass-vaccination campaign (one-dose vaccine, preferably delivered through a transcutaneous, nasal, or oral route).[57]

A critical question in planning an effective response to bioterrorism is how antibiotics and medical supplies should be stockpiled and dispensed. Researchers have modeled the regional and local supply chain for antibiotics and medical supplies as well as local dispensing capacity. For an attack exposing 250 000 people and requiring the prophylaxis of 5 million people, expected mortality fell from 243 000 to 145 000 as the dispensing capacity increased from 14 000 to 420 000 individuals per day. At low dispensing capacities ($< 14\,000$ individuals per day), nearly all exposed individuals died.[226] Apparently, the critical determinant of mortality following anthrax bioterrorism is local dispensing capacity, and for this reason bioterrorism preparedness efforts should be directed at improving local dispensing capacity.

A compartmental model has been developed to evaluate the costs and benefits of various strategies for preattack stockpiling and the postattack distribution and dispensing of medical and pharmaceutical supplies.[227] The results showed that improved surveillance systems can significantly reduce deaths from a large-scale bioterrorist attack only if the local community has sufficient antibiotic-dispensing capacity. Moreover, mortality from such an attack is significantly affected by the number of unexposed individuals seeking prophylaxis and treatment.

Fowler has produced a mathematical model, based on a 1% yearly risk of attack, arguing that combined postexposure antibiotics and vaccination would be safer and more cost-effective than mass preexposure vaccination.[16,228] His statistical analysis showed that during the 2001 anthrax attacks the preventative measure of the 60-day antibiotic prophylaxis may have saved many lives.[228]

In the event of a future terrorist attack, hospitals have been working vigorously to ensure they would be "ready" to provide appropriate medical care to victims. However, according to a recent U.S. General Accounting Office (GAO) nationwide survey, a number of hospitals throughout the U.S. are still not adequately prepared to manage mass causalties (hundreds or even thousands of lives) resulting from chemical or biological weapons.[15]

On January 28, 2003, President Bush announced the creation of Project BioShield. The Project BioShield Act was signed into law on July 21, 2004. BioShield serves as a procurement mechanism, allowing the government to finance the stockpiling of countermeasures for biological, chemical, nuclear, and radiological weapons.[57] However, biosecurity experts say that after 9 years and more than $50 billion spent since 9/11, the U.S. still has made only marginal progress in developing the security measures and new therapeutics needed to protect people from the next bioterrorist anthrax attack.[229]

7. Animal Models

The 2001 anthrax attacks precipitated a renewed interest in identifying both therapeutics and rapid diagnostic assays for inhalational anthrax. However, the scarcity and severity of human infection have resulted in the extensive use of animal models to determine the efficacy of medical countermeasures destined for human use.[230−232] The challenge has been to identify well-characterized animal models that closely resemble the human disease and respond in a similar manner to medical countermeasures, such as vaccines.[47] Patterns of antibody response to the currently licensed AVA in the U.K. and the U.S. have been determined in human volunteers.[59,233] Although there is a definite role for antitoxin antibodies in providing protection against anthrax, it is not clear what levels of antibodies will be required to protect humans after PA vaccination or passive immunization.[5] This consideration is important, since efficacy testing of the currently licensed and next-generation human vaccines will rely on the identification of rugged correlates of protection for humans extrapolated from animal models, for example, rabbits (they present a much better anthrax model compared with mice).[10,105,234,235] Researchers have developed a novel whole-cell vaccine utilizing a *B. anthracis* strain that is killed but metabolically active (KBMA). This vaccine was well-tolerated and elicited potent protective immune response, suggesting its potential application in the assessment of correlates of protective immunity against anthrax.[236]

Studies in rabbits and a guinea pig aerosol challenge model revealed a threshold-neutralizing titer, above which all animals survived a certain spore challenge dose.[237] It is from nonhuman primate studies that the FDA has concluded that any candidate anthrax vaccine will be licensed using well-defined correlates of protection from two relevant animal models.[238] It is important to remember that in the event of an aerosol attack, it is impossible to test the actual efficacy of any vaccine. As yet, the information on the pathogenesis of anthrax infection in humans is almost nonexistent and most available knowledge comes from experimental studies on primate animals, e.g., monkeys and other nonprimate animals.[230,234]

Some of the confusion regarding extrapolation of the information to the human situation also results from the current animal models and the *B. anthracis* strains being used. It is noted that the susceptibility or resistance to *B. anthracis* infection ranges widely in mammals, not only among species but also within a single species.[28] Moreover, the identification of antitoxic immunity as the most important means for protection against *B. anthracis* has been complicated by the observation that there is not yet an entirely well-accepted animal model for evaluating immunity to LT and to spores of different anthrax isolates, because of the varying susceptibility of animal models to spores of different origin.[5]

The availability of relevant and useful animal models is critical for progress in the development of effective vaccines and therapeutics. A review of the literature shows that although a wide range of animals have been employed, the principal focus has been on mice, guinea pigs, rabbits, and nonhuman primates. Following extensive characterization, the rabbit and rhesus macaque have been proposed as suitable models in which to test therapeutics destined for human use.[47,234] The enhancement of survival in vaccinated nonhuman primates and rabbits challenged with *B. anthracis* spores is clearly related to the prevention of mortality in exposed humans. This makes these animal models ideal for evaluating vaccine efficacy and determining a correlate of protection for human beings.[234] The rhesus macaque is considered the model most comparable to humans for predicting the ability of a vaccine to elicit protection against inhalational spore challenge but cannot be used in the experimental numbers needed to demonstrate the correlation of antibody titers with protection. Therefore, there is a critical need for an alternative nonhuman primate model because of the increasingly limited supply and cost of the current model. One report describes the pathology in 12 African green monkeys (AGMs) that succumbed after exposure to *Bacillus anthracis* Ames strain spores.[239] Pathologic changes in AGMs were remarkably similar to what has been reported in rhesus macaques and humans that succumbed to inhalational anthrax, demonstrating that the African green monkey could be a suitable animal model, exhibiting a disease course similar to that observed in the rhesus model and humans.[240]

The infection of rabbits and nonhuman primates with fully virulent *Bacillus anthracis* spores provides two excellent models of anthrax disease. However, the high cost of procuring and housing these animals and the specialized facilities required to deliver fully virulent spores limit their practical use in early stages of product development. Conversely, the small size and low cost associated with using mice make this animal model more practical for conducting experiments in which large numbers of animals are required. It has been shown that the murine aerosol challenge model is both useful and relevant to that described for other species, including rabbits and nonhuman primates, and provides a means for further investigating the host response and mechanisms of *B. anthracis* pathogenesis.[241] Furthermore, investigators have developed and standardized a new, relatively inexpensive mouse model for studying inhalational anthrax.[232] This is a major advance, since up until now the ability to conduct such studies was greatly limited by the short supply of nonhuman primates. Mice are also considered the best animal models to demonstrate a role for anticapsular protective immunity.[5]

8. Concluding Remarks and Future Perspectives

Although rare as a natural disease in humans, anthrax has recently gained substantial notoriety because of the capacity of its spores to be utilized as an agent of biological warfare and terrorism. *B. anthracis* spores have several characteristics that rank them as the leading threat among bioweapons, and their intentional release in the U.S. mail system in 2001 increased public vulnerability to anthrax bioterrorism. Compared to the large financial investments and technical knowledge required to develop nuclear weapons, biological weapons can be manufactured at a fraction of the cost and with relative ease.[224] However, most experts agree that the manufacture of an anthrax aerosol is beyond the capacity of individuals or groups and necessitates access to advanced biotechnology laboratories. Apparently, the subject of anthrax is complex, involving not only scientific but also political, social, military, and ethical issues.

The central role of PA in the pathophysiology of anthrax makes it an excellent target for vaccines and antitoxins development. PA is the principal protective immunogen of the current U.S.- and U.K.-licensed human anthrax vaccines. Although PA is clearly the most critical immunogen in protective immunity, there are additional immunogens in the spore or in the vegetative cells that may contribute to protective immunity against anthrax. The use of native PA as a vaccine is not optimal. If administered to people who have been freshly exposed to anthrax, PA may actually aid in anthrax toxin formation, thus posing a serious safety concern. A nonfunctional PA mutant may be a much safer alternative. Recombinant PA mutants, particularly DNI, hold great promise as better and safer antigens than wild-type PA for use in postexposure vaccination.[242]

Vaccination may be the most effective and economical strategy of mass immunization, and a considerable amount of time and money has been expended on developing effective vaccines. The available first-generation vaccines are considered to be safe and effective but have several drawbacks, most notably the local side effects and complicated dosing schedule. The development of second- and third-generation vaccines is based on purified preparations of rPA. Vaccine improvements include the use of recombinant bacterial, viral, or DNA vectors, the addition of new adjuvants, and the supplementing of the existing vaccines with other anthrax antigens, such as spore components, bacterial capsule, or antigens based on LF or EF. Ideally, a third-generation vaccine formulation would enable self-administration (needle-free) via the nasal, oral, or dermal routes to facilitate mass vaccination and storage at room temperature. In the next 5 years a subunit vaccine of purified rPA administered in combination with an aluminum-based adjuvant is the only candidate vaccine likely to receive licensing approval. Developing more effective anthrax countermeasures is expensive and technically difficult, especially because most countermeasures have a limited shelf-life.

A delay in antibiotic treatment may substantially lessen chances for survival. A combination of antibiotics and aggressive hospital supportive care may succeed in the prodromal stage, but in the fulminant stage antibiotics are no longer effective owing to the accumulation of a lethal toxin level and death generally occurs within 24 h. Because of nonspecific initial symptoms and given the difficulty of achieving rapid microbiologic diagnosis of anthrax, all persons in high-risk groups who develop fever or evidence of systemic disease should start receiving therapy for possible anthrax infection while awaiting the results of laboratory studies. Moreover, studies in rhesus monkeys have shown that spores persist in the lungs of primates for up to 60 days. To avoid recurrence,

infected individuals should be vaccinated on the commencement of antibiotic treatment and can be discontinued once protective immunity has developed. Sixty days of ciprofloxacin administration at 500 mg every 12 h is currently recommended for the prophylaxis of inhalational exposure to *Bacillus anthracis*. However, durations of 110 days have not achieved 99.9% eradication, irrespective of initial burden, because of variance in drug pharmacokinetics between patients. Given the absence of person-to-person transmission for *Bacillus anthracis*, adverse drug effects with long-term ciprofloxacin administration, and the possibility of engendering resistance in bodily flora, shorter prophylaxis duration should be given consideration, along with careful monitoring of all exposed individuals.[243]

The emergence of artificially engineered *B. anthracis* strains resistant to antibiotics has prompted researchers to pursue additional therapeutic options. Such alternatives include small molecules, e.g., LF inhibitors and antibodies against toxins (e.g., PA-specific antibodies). Passive immunization using a polyclonal or a high-affinity monoclonal antibody may offer adjunctive value to antibiotic therapy.

Because death is thought to be due to the persistence of circulating toxins, there is an urgent need for the discovery of antitoxin agents that would be effective at the end stage of anthrax. Growing insights into the structure and function of both LT and ET have led to the design and production of an increasing number of agents capable of neutralizing toxins or their effects and have also revealed potential applications in medicine. Much work has focused on finding potent LF inhibitors as complementary therapy to antibiotics. Research has resulted in the design of small molecules that can specifically interfere with the catalytic activity of LF. A low molecular weight LF inhibitor would be invaluable in combating the LT action by reducing or preventing the damage to vascular circulation, giving the antibiotics time to neutralize the bacterial infection.

In the event of an aerosolized spore attack on an unvaccinated metropolitan population, antibiotics (ciprofloxacin) and active immunization (AVA) represent the most effective and least expensive strategy for treatment of infected individuals who have been exposed but are not yet ill. Postexposure vaccination may shorten the duration of antibiotic administration. Yet until ample reserve stockpiles of vaccine are available, reliance must be placed only on antibiotic administration.

The development of new treatment modalities is hampered by the difficulty in demonstrating their effectiveness in humans. Animal models are extensively used to study the pathology of infection and to determine the efficacy of medical countermeasures. Currently, the challenge has been to identify well-defined animal models that recapitulate the detailed histopathology of systemic infection and responses observed in humans. Most of the available knowledge comes from monkeys and other nonprimate animals.

Since 2001, intensive biomedical research has been conducted and an enormous amount of money has been allocated to develop potent medical countermeasurements. In spite of this, there has been no real progress. The U.S. government has declared that an effective postexposure treatment of anthrax is a key national priority in the fight against bioterrorism, but it does not yet have the range of medical countermeasures needed to protect its citizens from a mass anthrax attack. It is expected that a next-generation anthrax vaccine and improved treatment modalities will be derived from a better understanding of the interaction of virulence factors with human and animal hosts and the role the host immune responses play in providing protective immunity.

Biography

Dimitrios G. Bouzianas obtained his degrees in Pharmacy (1978) and Biology (1993) at the Aristotle University of Thessaloniki (AUTH), Macedonia, Greece. He organized the Cell Culture Department in the Laboratory of Histology-Embryology, Faculty of Medicine, AUTH, where he completed his Ph.D in 1999, working on long-term bone marrow cultures. As a Visiting Scientist, he studied cell culture techniques at the Paterson Institute, Christie Hospital, Manchester, England. He is holder of a European Letters Patent for a new method of assessing the potential of hemopoietic stem cells. He is the author of a textbook of veterinary pharmacology for the students in the Department of Animal Production at the Athens Agricultural University. His research interests and experience are in the field of cell cultures and in anthrax bioterorrism.

References

(1) Webb, G. F. Being prepared: modeling the response to an anthrax attack. *Ann. Intern. Med.* **2005**, *142* (8), 667–668.
(2) Inglesby, T. V.; O'Toole, T.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Friedlander, A. M.; Gerberding, J.; Hauer, J.; Hughes, J.; McDade, J.; Osterholm, M. T.; Parker, G.; Perl, T. M.; Russell, P. K.; Tonat, K. Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA, J. Am. Med. Assoc.* **2002**, *287* (17), 2236–2252.
(3) Bromberg-White, J. L.; Duesbery, N. S. Biological and biochemical characterization of anthrax lethal factor, a proteolytic inhibitor of MEK signaling pathways. *Methods Enzymol.* **2008**, *438*, 355–365.
(4) Meropol, S. B.; Chan, K.; Chen, Z.; Finkelstein, J.; Hennessy, S.; Lautenbach, E.; Platt, R.; Schech, S. D.; Shatin, D.; Metlay, J. P. Adverse events associated with prolonged antibiotic use. *Pharmacoepidemiol. Drug Saf.* **2008**, *17* (5), 523–532.
(5) Brey, R. N. Molecular basis for improved anthrax vaccines. *Adv. Drug Delivery Rev.* **2005**, *57* (9), 1266–1292.
(6) Kuhn, J. F.; Hoerth, P.; Hoehn, S. P.; Preckel, T.; Tomer, K. B. Proteomics study of anthrax lethal toxin-treated murine macrophages. *Electrophoresis* **2006**, *27* (8), 1584–1597.
(7) Riedel, S. Anthrax: a continuing concern in the era of bioterrorism. *Baylor Univ. Med. Cent. Proc.* **2005**, *18* (3), 234–243.
(8) Grabenstein, J. D. Vaccines: countering anthrax: vaccines and immunoglobulins. *Clin. Infect. Dis.* **2008**, *46* (1), 129–1236.
(9) Wang, J. Y.; Roehrl, M. H. Anthrax vaccine design: strategies to achieve comprehensive protection against spore, bacillus, and toxin. *Med. Immunol.* **2005**, *4* (1), 4–11.
(10) Bouzianas, D. G. Potential biological targets of *Bacillus anthracis* in anti-infective approaches against the threat of bioterrorism. *Expert Rev. Anti-Infect. Ther.* **2007**, *5*, 665–684.
(11) Lew, D.; Garbino, J. *Bacillus anthracis* (anthrax). *Curr. Treat. Treat. Options Infect. Dis.* **2003**, *5*, 409–418.
(12) Cegelski, L.; Marshall, G. R.; Eldridge, G. R.; Hultgren, S. J. The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.* **2008**, *6* (1), 17–27.
(13) Meselson, M.; Guillemin, J.; Hugh-Jones, M.; Langmuir, A.; Popova, I.; Shelokov, A.; Yampolskaya, O. The Sverdlovsk anthrax outbreak of 1979. *Science* **1994**, *266*, 1202–1208.
(14) Spencer, R. C. *Bacillus anthracis*. *J. Clin. Pathol.* **2003**, *56* (3), 182–187.
(15) Bennett, R. L. Chemical or biological terrorist attacks: an analysis of the preparedness of hospitals for managing victims affected by chemical or biological weapons of mass destruction. *Int. J. Environ. Res. Public Health* **2006**, *3* (1), 67–75.
(16) Weiss, M. M.; Weiss, P. D.; Weiss, J. E. Anthrax vaccine and public health policy. *Am. J. Public Health* **2007**, *97* (11), 1945–1951.
(17) Rossi Paccani, S.; Tonello, F.; Patrussi, L.; Capitani, N.; Simonato, M.; Montecucco, C.; Baldari, C. T. Anthrax toxins inhibit immune cell chemotaxis by perturbing chemokine receptor signalling. *Cell. Microbiol.* **2007**, *9* (4), 924–929.
(18) Turk, B. E. Manipulation of host signalling pathways by anthrax toxins. *Biochem. J.* **2007**, *402* (3), 405–417.
(19) Baldari, C. T.; Tonello, F.; Paccani, S. R.; Montecucco, C. Anthrax toxins: a paradigm of bacterial immune suppression. *Trends Immunol.* **2006**, *27* (9), 434–440.

(20) Bergman, N. H.; Anderson, E. C.; Swenson, E. E.; Janes, B. K.; Fisher, N.; Niemeyer, M. M.; Miyoshi, A. D.; Hanna, P. C. Transcriptional profiling of *Bacillus anthracis* during infection of host macrophages. *Infect. Immun.* **2007**, *75* (7), 3434–3444.
(21) Li, Y.; Sherer, K.; Cui, X.; Eichacker, P. Q. New insights into the pathogenesis and treatment of anthrax toxin-induced shock. *Expert Opin. Biol. Ther.* **2007**, *7* (6), 843–854.
(22) Daubenspeck, J. M.; Zeng, H.; Chen, P.; Dong, S.; Steichen, C. T.; Krishna, N. R.; Pritchard, D. G.; Turnbough, C. L., Jr. Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the *Bacillus anthracis* exosporium. *J. Biol. Chem.* **2004**, *279* (30), 30945–30953.
(23) Young, J. A.; Collier, R. J. Anthrax toxin: receptor binding, internalization, pore formation, and translocation. *Annu. Rev. Biochem.* **2007**, *76*, 243–265.
(24) Pannifer, A. D.; Wong, T. Y.; Schwarzenbacher, R.; Renatus, M.; Petosa, C.; Bienkowska, J.; Lacy, D. B.; Collier, R. J.; Park, S.; Leppla, S. H.; Hanna, P.; Liddington, R. C. Crystal structure of the anthrax lethal factor. *Nature* **2001**, *414* (6860), 229–233.
(25) Leppla, S. H. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79* (10), 3162–3166.
(26) Drum, C. L.; Yan, S. Z.; Bard, J.; Shen, Y. Q.; Lu, D.; Soelaiman, S.; Grabarek, Z.; Bohm, A.; Tang, W. J. Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. *Nature* **2002**, *415* (6870), 396–402.
(27) Candela, T.; Mock, M.; Fouet, A. CapE, a 47-amino-acid peptide, is necessary for *Bacillus anthracis* polyglutamate capsule synthesis. *J. Bacteriol.* **2005**, *187* (22), 7765–7772.
(28) Passalacqua, K. D.; Bergman, N. H. *Bacillus anthracis*: interactions with the host and establishment of inhalational anthrax. *Future Microbiol.* **2006**, *1*, 397–415.
(29) Glomski, I. J.; Corre, J. P.; Mock, M.; Goossens, P. L. Noncapsulated toxinogenic *Bacillus anthracis* presents a specific growth and dissemination pattern in naive and protective antigen-immune mice. *Infect. Immun.* **2007**, *75* (10), 4754–4761.
(30) Ezzell, J. W.; Abshire, T. G.; Panchal, R.; Chabot, D.; Bavari, S.; Leffel, E. K.; Purcell, B.; Friedlander, A. M.; Ribot, W. J. Association of *Bacillus anthracis* capsule with lethal toxin during experimental infection. *Infect. Immun.* **2009**, *77* (2), 749–755.
(31) Bakhteeva, I. V.; Titareva, G. M.; Kravchenko, T.; Mironova, R. I.; Noskov, A. N. Anthrax: early steps of the intracellular stage of infection development. *Mol. Gen. Mikrobiol. Virusol.* **2005**, *4*, 3–9 (in Russian).
(32) Bradley, K. A.; Mogridge, J.; Mourez, M.; Collier, R.J.; Young, J. A. T. Identification of the cellular receptor for anthrax toxin. *Nature* **2001**, *414*, 225–229.
(33) Santelli, E.; Bankston, L. A.; Leppla, S. H.; Liddington, R. C. Crystal structure of a complex between anthrax toxin and its host cell receptor. *Nature* **2004**, *430* (7002), 905–908.
(34) Sherer, K.; Li, Y.; Cui, X.; Eichacker, P. Q. Lethal and edema toxins in the pathogenesis of *Bacillus anthracis* septic shock: implications for therapy. *Am. J. Respir. Crit. Care Med.* **2007**, *175* (3), 211–221.
(35) Warfel, J. M.; Steele, A. D.; D'Agnillo, F. Anthrax lethal toxin induces endothelial barrier dysfunction. *Am. J. Pathol.* **2005**, *166*, 1871–1881.
(36) Chakrabarty, K.; Wu, W.; Booth, J. L.; Duggan, E. S.; Coggeshall, K. M.; Metcalf, J. P. *Bacillus anthracis* spores stimulate cytokine and chemokine innate immune responses in human alveolar macrophages through multiple mitogen-activated protein kinase pathways. *Infect. Immun.* **2006**, *74* (8), 4430–4438.
(37) Chakrabarty, K.; Wu., W.; Booth, J.; Duggan, E. S.; Nagle, N. N.; Coggeshall, K. M.; Metcalf, J. P. Human lung innate immune response to *Bacillus anthracis* spore infection. *Infect. Immun.* **2007**, *75* (8), 3729–3738.
(38) Cleret, A.; Quesnel-Hellmann, A.; Vallon-Eberhard, A.; Verrier, B.; Jung, S.; Vidal, D.; Mathieu, J.; Tournier, J. N. Lung dendritic cells rapidly mediate anthrax spore entry through the pulmonary route. *J. Immunol.* **2007**, *178* (12), 7994–8001.
(39) Cui, X.; Li, Y.; Li, X.; Laird, M. W.; Subramanian, M.; Moayeri, M.; Leppla, S. H.; Fitz, Y.; Su, J.; Sherer, K.; Eichacker, P. Q. *Bacillus anthracis* edema and lethal toxin have different hemodynamic effects but function together to worsen shock and outcome in a rat model. *J. Infect. Dis.* **2007**, *195* (4), 572–580.
(40) Firoved, A. M.; Moayeri, M.; Wiggins, J. F.; Shen, Y.; Tang, W. J.; Leppla, S. H. Anthrax edema toxin sensitizes DBA/2J mice to lethal toxin. *Infect. Immun.* **2007**, *75* (5), 2120–2125.
(41) Loving, C. L.; Khurana, T.; Osorio, M.; Lee, G. M.; Kelly, V. K.; Stibitz, S.; Merkel, T. J. Role of anthrax toxins in dissemination, disease progression, and induction of protective adaptive immunity in the mouse aerosol challenge model. *Infect. Immun.* **2009**, *77* (1), 255–265.
(42) Fang, H.; Xu, L.; Chen, T. Y.; Cyr, J. M.; Frucht, D. M. Anthrax lethal toxin has direct and potent inhibitory effects on B cell proliferation and immunoglobulin production. *J. Immunol.* **2006**, *176* (10), 6155–6161.
(43) Comer, J. E.; Chopra, A. K.; Peterson, J. W.; Konig, R. Direct inhibition of T-lymphocyte activation by anthrax toxins in vivo. *Infect. Immun.* **2005**, *73* (12), 8275–8281.
(44) During, R. L.; Gibson, B. G.; Li, W.; Bishai, E. A.; Sidhu, G. S.; Landry, J.; Southwick, F. S. Anthrax lethal toxin paralyzes actin-based motility by blocking Hsp27 phosphorylation. *EMBO J.* **2007**, *26* (9), 2240–2250.
(45) Nour, A. M.; Yeung, Y. G.; Santambrogio, L.; Boyden, E. D.; Stanley, E. R.; Brojatsch, J. Anthrax lethal toxin triggers the formation of a membrane-associated inflammasome complex in murine macrophages. *Infect. Immun.* **2009**, *77* (3), 1262–1271.
(46) Gold, J. A.; Hoshino, Y.; Jones, M. B.; Hoshino, S.; Nolan, A.; Weiden, M. D. Exogenous interferon-alpha and interferon-gamma increase lethality of murine inhalational anthrax. *PLoS One* **2007**, *2* (1), No. e736.
(47) Baillie, L. W. Past, imminent and future human medical countermeasures for anthrax. *J. Appl. Microbiol.* **2006**, *101* (3), 594–606.
(48) Golden, H. B.; Watson, L. E.; Lal, H.; Verma, S. K.; Foster, D. M.; Kuo, S. R.; Sharma, A.; Frankel, A.; Dostal, D. E. Anthrax toxin: pathologic effects on the cardiovascular system. *Front. Biosci.* **2009**, *14*, 2335–2357.
(49) Li, Y.; Cui, X.; Su, J.; Haley, M.; Macarthur, H.; Sherer, K.; Moayeri, M.; Leppla, S. H.; Fitz, Y.; Eichacker, P. Q. Norepinephrine increases blood pressure but not survival with anthrax lethal toxin in rats. *Crit. Care Med.* **2009**, *37* (4), 1348–1354.
(50) Kuo, S. R.; Willingham, M. C.; Bour, S. H.; Andreas, E. A.; Park, S. K.; Jackson, C.; Duesbery, N. S.; Leppla, S. H.; Tang, W. J.; Frankel, A. E. Anthrax toxin-induced shock in rats is associated with pulmonary edema and hemorrhage. *Microb. Pathog.* **2008**, *44* (6), 467–472.
(51) Kubler-Kielb, J.; Liu, T. Y.; Mocca, C.; Majadly, F.; Robbins, J. B.; Schneerson, R. Additional conjugation methods and immunogenicity of *Bacillus anthracis* poly-gamma-D-glutamic acid−protein conjugates. *Infect. Immun.* **2006**, *74* (8), 4744–4749.
(52) Joyce, J.; Cook, J.; Chabot, D.; Hepler, R.; Shoop, W.; Xu, Q.; Stambaugh, T.; Aste-Amezaga, M.; Wang, S.; Indrawati, L.; Bruner, M.; Friedlander, A.; Keller, P.; Caulfield, M. Immunogenicity and protective efficacy of *Bacillus anthracis* poly-gamma-D-glutamic acid capsule covalently coupled to a protein carrier using a novel triazine-based conjugation strategy. *J. Biol. Chem.* **2006**, *281* (8), 4831–4843.
(53) Rhie, G. E.; Roehrl, M. H.; Mourez, M.; Collier, R. J.; Mekalanos, J. J.; Wang, J. Y. A dually active anthrax vaccine that confers protection against both bacilli and toxins. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (19), 10925–10930.
(54) Aulinger, B. A.; Roehrl, M. H.; Mekalanos, J. J.; Collier, R. J.; Wang, J. Y. Combining anthrax vaccine and therapy: a dominant-negative inhibitor of anthrax toxin is also a potent and safe immunogen for vaccines. *Infect. Immun.* **2005**, *73* (6), 3408–3414.
(55) Wimer-Mackin, S.; Hinchcliffe, M.; Petrie, C. R.; Warwood, S. J.; Tino, W. T.; Williams, M. S.; Stenz, J. P.; Cheff, A.; Richardson, C. An intranasal vaccine targeting both the *Bacillus anthracis* toxin and bacterium provides protection against aerosol spore challenge in rabbits. *Vaccine* **2006**, *24* (18), 3953–3963.
(56) Chabot, D. J.; Scorpio, A.; Tobery, S. A.; Little, S. F.; Norris, S. L.; Friedlander, A. M. Anthrax capsule vaccine protects against experimental infection. *Vaccine* **2004**, *23* (1), 43–47.
(57) Borio, L. L.; Gronvall, G. K. Anthrax countermeasures: current status and future needs. *Biosecur. Bioterrorism* **2005**, *3* (2), 102–112.
(58) Taft, S. C.; Weiss, A. A. Neutralizing activity of vaccine-induced antibodies to two *Bacillus anthracis* toxin components, lethal factor and edema factor. *Clin. Vaccine Immunol.* **2008**, *15* (1), 71–75.
(59) Pittman, P. R.; Norris, S. L.; Barrera Oro, J. G.; Bedwell, D.; Cannon, T. L.; McKee, K. T., Jr. Patterns of antibody response in humans to the anthrax vaccine adsorbed (AVA) primary (six-dose) series. *Vaccine* **2006**, *24* (17), 3654–3660.
(60) Grunow, R.; Porsch-Ozcurumez, M.; Splettstoesser, W.; Buckendahl, A.; Hahn, U.; Beyer, W.; Bohm, R.; Huber, M.; vd Esche, U.; Bessler, W.; Frangoulidis, D.; Finke, E. J. Monitoring of ELISA-reactive antibodies against anthrax protective antigen (PA), lethal factor (LF), and toxin-neutralising antibodies in serum of individuals vaccinated against anthrax with the PA-based UK anthrax vaccine. *Vaccine* **2007**, *25* (18), 3679–3683.

(61) Hewetson, J. F.; Little, S. F.; Ivins, B. E.; Johnson, W. M.; Pittman, P. R.; Brown, J. E.; Norris, S. L.; Nielsen, C. J. An in vivo passive protection assay for the evaluation of immunity in AVA-vaccinated individuals. *Vaccine* **2008**, *26* (33), 4262–4266.
(62) Bienek, D. R.; Chang, C. K.; Cohen, M. E. Detection of anti-protective antigen salivary IgG antibodies in recipients of the US licensed anthrax vaccine. *Vaccine* **2007**, *25* (32), 5978–5984.
(63) Wasserman, G. M.; Grabenstein, J. D.; Pittman, P. R.; Rubertone, M. V.; Gibbs, P. P.; Wang, L. Z.; Golder, L. G. Analysis of adverse events after anthrax immunization in US Army medical personnel. *J. Occup. Environ. Med.* **2003**, *45* (3), 222–233.
(64) Abboud, N.; Casadevall, A. Immunogenicity of *Bacillus anthracis* protective antigen domains and efficacy of elicited antibody responses depend on host genetic background. *Clin. Vaccine Immunol.* **2008**, *15* (7), 1115–1123.
(65) Marano, N.; Plikaytis, B. D.; Martin, S. W.; Rose, C.; Semenova, V. A.; Martin, S. K.; Freeman, A. E.; Li, H.; Mulligan, M. J.; Parker, S. D.; Babcock, J.; Keitel, W.; El Sahly, H.; Poland, G. A.; Jacobson, R. M.; Keyserling, H. L.; Soroka, S. D.; Fox, S. P.; Stamper, J. L.; McNeil, M. M.; Perkins, B. A.; Messonnier, N.; Quinn, C. P. Effects of a reduced dose schedule and intramuscular administration of anthrax vaccine adsorbed on immunogenicity and safety at 7 months: a randomized trial. *JAMA, J. Am. Med. Assoc.* **2008**, *300* (13), 1532–1543.
(66) HHS Awards BioShield Contract for AVA Anthrax Vaccine News. News Release. Department of Health and Human Resources. May 6, **2005**. http://www.os.dhhs.gov/news/press/2005pres/20050506.html (accessed Oct 7, 2005).
(67) Tournier, J. N.; Ulrich, R. G.; Quesnel-Hellmann, A.; Mohamadzadeh, M.; Stiles, B. G. Anthrax, toxins and vaccines: a 125-year journey targeting *Bacillus anthracis*. *Expert Rev. Anti-Infect. Ther.* **2009**, *7* (2), 219–236.
(68) Hepler, R. W.; Kelly, R.; McNeely, T. B.; Fan, H.; Losada, M. C.; George, H. A.; Woods, A.; Cope, L. D.; Bansal, A.; Cook, J. C.; Zang, G.; Cohen, S. L.; Wei, X.; Keller, P. M.; Leffel, E.; Joyce, J. G.; Pitt, L.; Schultz, L. D.; Jansen, K. U.; Kurtz, M. A recombinant 63-kDa form of *Bacillus anthracis* protective antigen produced in the yeast *Saccharomyces cerevisiae* provides protection in rabbit and primate inhalational challenge models of anthrax infection. *Vaccine* **2006**, *24* (10), 1501–1514.
(69) Duc le, H.; Hong, H. A.; Atkins, H. S.; Flick-Smith, H. C.; Durrani, Z.; Rijpkema, S.; Titball, R. W.; Cutting, S. M. Immunization against anthrax using *Bacillus subtilis* spores expressing the anthrax protective antigen. *Vaccine* **2007**, *25* (2), 346–355.
(70) Williamson, E. D.; Hodgson, I.; Walker, N. J.; Topping, A. W.; Duchars, M. G.; Mott, J. M.; Estep, J.; Lebutt, C.; Flick-Smith, H. C.; Jones, H. E.; Li, H.; Quinn, C. P. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. *Infect. Immun.* **2005**, *73* (9), 5978–5987.
(71) Gorse, G. J.; Keitel, W.; Keyserling, H.; Taylor, D. N.; Lock, M.; Alves, K.; Kenner, J.; Deans, L.; Gurwith, M. Immunogenicity and tolerance of ascending doses of a recombinant protective antigen (rPA102) anthrax vaccine: a randomized, double blinded, controlled, multicenter trial. *Vaccine* **2006**, *24* (33−34), 5950–5959.
(72) Lipton, E. U.S. cancels order for 75 million doses of anthrax vaccine. *N. Y. Times* **2006**, No. December 20, A23.
(73) Fasanella, A.; Tonello, F.; Garofolo, G.; Muraro, L.; Carattoli, A.; Adone, R.; Montecucco, C. Protective activity and immunogenicity of two recombinant anthrax vaccines for veterinary use. *Vaccine* **2008**, *26* (45), 5684–5688.
(74) Laird, M. W.; Zukauskas, D.; Johnson, K.; Sampey, G. C.; Olsen, H.; Garcia, A.; Karwoski, J. D.; Cooksey, B. A.; Choi, G. H.; Askins, J.; Tsai, A.; Pierre, J.; Gwinn, W. Production and purification of *Bacillus anthracis* protective antigen from *Escherichia coli*. *Protein Expression Purif.* **2004**, *38* (1), 145–152.
(75) Park, Y. S.; Lee, J. H.; Hung, C. F.; Wu, T. C.; Kim, T. W. Enhancement of antibody responses to *Bacillus anthracis* protective antigen domain IV by use of calreticulin as a chimeric molecular adjuvant. *Infect. Immun.* **2008**, *76* (5), 1952–1959.
(76) Stokes, M. G.; Titball, R. W.; Neeson, B. N.; Galen, J. E.; Walker, N. J.; Stagg, A. J.; Jenner, D. C.; Thwaite, J. E.; Nataro, J. P.; Baillie, L. W.; Atkins, H. S. Oral administration of a *Salmonella enterica*-based vaccine expressing *Bacillus anthracis* protective antigen confers protection against aerosolized *B. anthracis*. *Infect. Immun.* **2007**, *75* (4), 1827–1834.
(77) Baillie, L. W.; Rodriguez, A. L.; Moore, S.; Atkins, H. S.; Feng, C.; Nataro, J. P.; Pasetti, M. F. Towards a human oral vaccine for anthrax: the utility of a *Salmonella* Typhi Ty21a-based prime-boost immunization strategy. *Vaccine* **2008**, *26* (48), 6083–6091.
(78) Osorio, M.; Wu, Y.; Singh, S.; Merkel, T. J.; Bhattacharyya, S.; Blake, M. S.; Kopecko, D. J. Anthrax protective antigen delivered by *Salmonella enterica* serovar Typhi Ty21a protects mice from a lethal anthrax spore challenge. *Infect. Immun.* **2009**, *77* (4), 1475–1482.
(79) Streatfield, S. J. Engineered chloroplasts as vaccine factories to combat bioterrorism. *Trends Biotechnol* **2006**, *24* (8), 339–342.
(80) Hull, A. K.; Criscuolo, C. J.; Mett, V.; Groen, H.; Steeman, W.; Westra, H.; Chapman, G.; Legutki, B.; Baillie, L.; Yusibov, V. Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. *Vaccine* **2005**, *23* (17−18), 2082–2086.
(81) Sloat, B. R.; Cui, Z. Nasal immunization with anthrax protective antigen protein adjuvanted with polyriboinosinic−polyribocytidylic acid induced strong mucosal and systemic immunities. *Pharm. Res.* **2006**, *23* (6), 1217–1226.
(82) Galen, J. E.; Chinchilla, M.; Pasetti, M. F.; Wang, J. Y.; Zhao, L.; Arciniega-Martinez, I.; Silverman, D. J.; Levine, M. M. Mucosal immunization with attenuated *Salmonella enterica* serovar Typhi expressing protective antigen of anthrax toxin (PA83) primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine. *J. Infect. Dis.* **2009**, *199* (3), 326–335.
(83) Sloat, B. R.; Shaker, D. S.; Le, U. M.; Cui, Z. Nasal immunization with the mixture of PA63, LF, and a PGA conjugate induced strong antibody responses against all three antigens. *FEMS Immunol. Med. Microbiol.* **2008**, *52* (2), 169–179.
(84) Sloat, B. R.; Cui, Z. Strong mucosal and systemic immunities induced by nasal immunization with anthrax protective antigen protein incorporated in liposome−protamine−DNA particles. *Pharm. Res.* **2006**, *23* (2), 262–269.
(85) Jiang, G.; Joshi, S. B.; Peek, L. J.; Brandau, D. T.; Huang, J.; Ferriter, M. S.; Woodley, W. D.; Ford, B. M.; Mar, K. D.; Mikszta, J. A.; Hwang, C. R.; Ulrich, R.; Harvey, N. G.; Middaugh, C. R.; Sullivan, V. J. Anthrax vaccine powder formulations for nasal mucosal delivery. *J. Pharm. Sci.* **2006**, *95* (1), 80–96.
(86) Matyas, G. R.; Friedlander, A. M.; Glenn, G. M.; Little, S.; Yu, J.; Alving, C. R. Needle-free skin patch vaccination method for anthrax. *Infect. Immun.* **2004**, *72* (2), 1181–1183.
(87) McConnell, M. J.; Hanna, P. C.; Imperiale, M. J. Adenovirus-based prime-boost immunization for rapid vaccination against anthrax. *Mol. Ther.* **2007**, *15* (1), 203–210.
(88) Keitel, W. A.; Treanor, J. J.; El Sahly, H. M.; Evans, T. G.; Kopper, S.; Whitlow, V.; Selinsky, C.; Kaslow, D. C.; Rolland, A.; Smith, L. R.; Lalor, P. A. Evaluation of a plasmid DNA-based anthrax vaccine in rabbits, nonhuman primates and healthy adults. *Hum. Vaccines* **2009**, *5* (8), 536–544.
(89) Smith, M. E.; Koser, M.; Xiao, S.; Siler, C.; McGettigan, J. P.; Calkins, C.; Pomerantz, R. J.; Dietzschold, B.; Schnell, M. J. Rabies virus glycoprotein as a carrier for anthrax protective antigen. *Virology* **2006**, *353* (2), 344–356.
(90) Liu, T. H.; Oscherwitz, J.; Schnepp, B.; Jacobs, J.; Yu, F.; Cease, K. B.; Johnson, P. R. Genetic vaccines for anthrax based on recombinant adeno-associated virus vectors. *Mol. Ther.* **2009**, *17* (2), 373–379.
(91) Shivachandra, S. B.; Li, Q.; Peachman, K. K.; Matyas, G. R.; Leppla, S. H.; Alving, C. R.; Rao, M.; Rao, V. B. Multicomponent anthrax toxin display and delivery using bacteriophage T4. *Vaccine* **2007**, *25* (7), 1225–1235.
(92) Manayani, D. J.; Thomas, D.; Dryden, K. A.; Reddy, V.; Siladi, M. E.; Marlett, J. M.; Rainey, G. J.; Pique, M. E.; Scobie, H. M.; Yeager, M.; Young, J. A.; Manchester, M.; Schneemann, A. A viral nanoparticle with dual function as an anthrax antitoxin and vaccine. *PLoS Pathog.* **2007**, *3* (10), 1422–1431.
(93) Lim, N. K.; Kim, J. H.; Oh, M. S.; Lee, S.; Kim, S. Y.; Kim, K. S.; Kang, H. J.; Hong, H. J.; Inn, K. S. An anthrax lethal factor-neutralizing monoclonal antibody protects rats before and after challenge with anthrax toxin. *Infect. Immun.* **2005**, *73* (10), 6547–6551.
(94) Nguyen, M. L.; Crowe, S. R.; Kurella, S.; Teryzan, S.; Cao, B.; Ballard, J. D.; James, J. A.; Farris, A. D. Sequential B-cell epitopes of *Bacillus anthracis* lethal factor bind lethal toxin-neutralizing antibodies. *Infect. Immun.* **2009**, *77* (1), 162–169.
(95) Pezard, C.; Berche, P.; Mock, M. Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect. Immun.* **1991**, *59* (10), 3472–3477.
(96) Duverger, A.; Jackson, R. J.; van Ginkel, F. W.; Fischer, R.; Tafaro, A.; Leppla, S. H.; Fujihashi, K.; Kiyono, H.; McGhee, J. R.; Boyaka, P. N. *Bacillus anthracis* edema toxin acts as an adjuvant for mucosal immune responses to nasally administered vaccine antigens. *J. Immunol.* **2006**, *176* (3), 1776–1783.
(97) Cooksey, B. A.; Sampey, G. C.; Pierre, J. L.; Zhang, X.; Karwoski, J. D.; Choi, G. H.; Laird, M. W. Production of biologically active *Bacillus anthracis* edema factor in *Escherichia coli*. *Biotechnol. Prog.* **2004**, *20* (6), 1651–1659.

(98) Zeng, M.; Xu, Q.; Hesek, E. D.; Pichichero, M. E. N-Fragment of edema factor as a candidate antigen for immunization against anthrax. *Vaccine* **2006**, *24* (5), 662–670.
(99) Xiong, Y.; Wiltsie, J.; Woods, A.; Guo, J.; Pivnichny, J. V.; Tang, W.; Bansal, A.; Cummings, R. T.; Cunningham, B. R.; Friedlander, A. M.; Douglas, C. M.; Salowe, S. P.; Zaller, D. M.; Scolnick, E. M.; Schmatz, D. M.; Bartizal, K.; Hermes, J. D.; MacCoss, M.; Chapman, K. T. The discovery of a potent and selective lethal factor inhibitor for adjunct therapy of anthrax infection. *Bioorg. Med. Chem. Lett.* **2006**, *16* (4), 964–968.
(100) Chen, Z.; Moayeri, M.; Crown, D.; Emerson, S.; Gorshkova, I.; Schuck, P.; Leppla, S. H.; Purcell, R. H. Novel chimpanzee/human monoclonal antibodies that neutralize anthrax lethal factor: Evidence for possible synergy with anti-PA antibody. *Infect. Immun.* **2009**, *77* (9), 3902–3908.
(101) Kozel, T. R.; Thorkildson, P.; Brandt, S.; Welch, W. H.; Lovchik, J. A.; AuCoin, D. P.; Vilai, J.; Lyons, C. R. Protective and immunochemical activities of monoclonal antibodies reactive with the *Bacillus anthracis* polypeptide capsule. *Infect. Immun.* **2007**, *75* (1), 152–163.
(102) Wang, T. T.; Fellows, P. F.; Leighton, T. J.; Lucas, A. H. Induction of opsonic antibodies to the gamma-D-glutamic acid capsule of *Bacillus anthracis* by immunization with a synthetic peptide−carrier protein conjugate. *FEMS Immunol. Med. Microbiol.* **2004**, *40* (3), 231–237.
(103) Albrecht, M. T.; Li, H.; Williamson, E. D.; LeButt, C. S.; Flick-Smith, H. C.; Quinn, C. P.; Westra, H.; Galloway, D.; Mateczun, A.; Goldman, S.; Groen, H.; Baillie, L. W. Human monoclonal antibodies against anthrax lethal factor and protective antigen act independently to protect against *Bacillus anthracis* infection and enhance endogenous immunity to anthrax. *Infect. Immun.* **2007**, *75* (11), 5425–5433.
(104) Zhao, P.; Liang, X.; Kalbfleisch, J.; Koo, H. M.; Cao, B. Neutralizing monoclonal antibody against anthrax lethal factor inhibits intoxication in a mouse model. *Hum. Antibodies* **2003**, *12* (4), 129–135.
(105) Rossi, C. A.; Ulrich, M.; Norris, S.; Reed, D. S.; Pitt, L. M.; Leffel, E. K. Identification of a surrogate marker for infection in the African green monkey model of inhalation anthrax. *Infect. Immun.* **2008**, *76* (12), 5790–5801.
(106) Chen, Z.; Moayeri, M.; Zhou, Y. H.; Leppla, S.; Emerson, S.; Sebrell, A.; Yu, F.; Svitel, J.; Schuck, P.; St Claire, M.; Purcell, R. Efficient neutralization of anthrax toxin by chimpanzee monoclonal antibodies against protective antigen. *J. Infect. Dis.* **2006**, *193* (5), 625–633.
(107) Cui, X.; Li, Y.; Moayeri, M.; Choi, G.; Subramanian, G. M.; Li, X.; Haley, M.; Fitz, Y.; Feng, J.; Banks, S.; Leppla, S.; Eichacker, P. Q. Late treatment with a protective antigen-directed monoclonal antibody improves hemodynamic function and survival in a lethal toxin-infused rat model of anthrax sepsis. *J. Infect. Dis.* **2005**, *191* (3), 422–434.
(108) Peterson, J. W.; Comer, J. E.; Noffsinger, D. M.; Wenglikowski, A.; Walberg, K. G.; Chatuev, B. M.; Chopra, A. K.; Stanberry, L. R.; Kang, A. S.; Scholz, W. W.; Sircar, J. Human monoclonal anti-protective antigen antibody completely protects rabbits and is synergistic with ciprofloxacin in protecting mice and guinea pigs against inhalation anthrax. *Infect. Immun.* **2006**, *74* (2), 1016–1024.
(109) Steiniger, S. C.; Altobell, L. J., 3rd; Zhou, B.; Janda, K. D. Selection of human antibodies against cell surface-associated oligomeric anthrax protective antigen. *Mol. Immunol.* **2007**, *44* (10), 2749–2755.
(110) Walsh, J. J.; Pesik, N.; Quinn, C. P.; Urdaneta, V.; Dykewicz, C. A.; Boyer, A. E.; Guarner, J.; Wilkins, P.; Norville, K. J.; Barr, J. R.; Zaki, S. R.; Patel, J. B.; Reagan, S. P.; Pirkle, J. L.; Treadwell, T. A.; Messonnier, N. R.; Rotz, L. D.; Meyer, R. F.; Stephens, D. S. A case of naturally acquired inhalation anthrax: clinical care and analyses of anti-protective antigen immunoglobulin G and lethal factor. *Clin. Infect. Dis.* **2007**, *44* (7), 968–971.
(111) Holty, J. E.; Bravata, D. M.; Liu, H.; Olshen, R.; McDonald, K. M.; Owens, D. K. Systematic review: a century of inhalational anthrax cases from 1900 to 2005. *Ann. Intern. Med.* **2006**, *144* (4), 270–280.
(112) Turk, B. E. Discovery and development of anthrax lethal factor metalloproteinase inhibitors. *Curr. Pharm. Biotechnol.* **2008**, *9* (1), 24–33.
(113) Friedlander, A. M.; Welkos, S.; Pitt, M. L.; Ezzell, J. W.; Worsham, P. L.; Rose, K. J.; Ivins, B. E.; Lowe, J. R.; Howe, G. B.; Mikesell, P. Postexposure prophylaxis against experimental inhalation anthrax. *J. Infect. Dis.* **1993**, *167*, 1239–1243.
(114) Vietri, N. J.; Purcell, B. K.; Tobery, S. A.; Rasmussen, S. L.; Leffel, E. K.; Twenhafel, N. A.; Ivins, B. E.; Kellogg, M. D.; Webster, W. M.; Wright, M. E.; Friedlander, A. M. A short course of antibiotic treatment is effective in preventing death from experimental inhalational anthrax after discontinuing antibiotics. *J. Infect. Dis.* **2009**, *199* (3), 336–341.
(115) Vietri, N. J.; Purcell, B. K.; Lawler, J. V.; Leffel, E. K.; Rico, P.; Gamble, C. S.; Twenhafel, N. A.; Ivins, B. E.; Heine, H. S.; Sheeler, R.; Wright, M. E.; Friedlander, A. M. Short-course postexposure antibiotic prophylaxis combined with vaccination protects against experimental inhalational anthrax. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (20), 7813–7816.
(116) Tipparaju, S. K.; Mulhearn, D. C.; Klein, G. M.; Chen, Y.; Tapadar, S.; Bishop, M. H.; Yang, S.; Chen, J.; Ghassemi, M.; Santarsiero, B. D.; Cook, J. L.; Johlfs, M.; Mesecar, A. D.; Johnson, M. E.; Kozikowski, A. P. Design and synthesis of aryl ether inhibitors of the *Bacillus anthracis* enoyl-ACP reductase. *ChemMedChem* **2008**, *3* (8), 1250–1268.
(117) Athamna, A.; Athamna, M.; Abu-Rashed, N.; Medlej, B.; Bast, D. J.; Rubinstein, E. Selection of *Bacillus anthracis* isolates resistant to antibiotics. *J. Antimicrob. Chemother.* **2004**, *54* (2), 424–428.
(118) Heine, H. S.; Bassett, J.; Miller, L.; Bassett, A.; Ivins, B. E.; Lehoux, D.; Arhin, F. F.; Parr, T. R., Jr.; Moeck, G. Efficacy of oritavancin in a murine model of *Bacillus anthracis* spore inhalation anthrax. *Antimicrob. Agents Chemother.* **2008**, *52* (9), 3350–3357.
(119) Lipton, E. U.S. cancels order for 75 million doses of anthrax vaccine. *N. Y. Times* **2006**, No. December 20, A23.
(120) Yoong, P.; Schuch, R.; Nelson, D.; Fischetti, V. PlyPH, a bacteriolytic enzyme with a broad pH range of activity and lytic action against *Bacillus anthracis*. *J. Bacteriol.* **2006**, *188* (7), 2711–2714.
(121) Porter, C. J.; Schuch, R.; Pelzek, A. J.; Buckle, A. M.; McGowan, S.; Wilce, M. C.; Rossjohn, J.; Russell, R.; Nelson, D.; Fischetti, V. A.; Whisstock, J. C. The 1.6 Å crystal structure of the catalytic domain of PlyB, a bacteriophage lysin active against *Bacillus anthracis*. *J. Mol. Biol.* **2007**, *366* (2), 540–550.
(122) Stülke, J. Regulation of virulence in *Bacillus anthracis*: the phosphotransferase system transmits the signals. *Mol. Microbiol.* **2007**, *63* (3), 626–628.
(123) Popov, S. G.; Popova, T. G.; Hopkins, S.; Weinstein, R. S.; MacAfee, R.; Fryxell, K. J.; Chandhoke, V.; Bailey, C.; Alibek, K. Effective antiprotease-antibiotic treatment of experimental anthrax. *BMC Infect. Dis.* **2005**, *5* (1), 25.
(124) Thwaite, J. E.; Hibbs, S.; Titball, R. W.; Atkins, T. P. Proteolytic degradation of human antimicrobial peptide LL-37 by *Bacillus anthracis* may contribute to virulence. *Antimicrob. Agents Chemother.* **2006**, *50* (7), 2316–2322.
(125) Byun, Y.; Vogel, S. R.; Phipps, A. J.; Carnrot, C.; Eriksson, S.; Tiwari, R.; Tjarks, W. Synthesis and biological evaluation of inhibitors of thymidine monophosphate kinase from *Bacillus anthracis*. *Nucleosides, Nucleotides Nucleic Acids* **2008**, *27* (3), 244–260.
(126) Beierlein, J. M.; Frey, K. M.; Bolstad, D. B.; Pelphrey, P. M.; Joska, T. M.; Smith, A. E.; Priestley, N. D.; Wright, D. L.; Anderson, A. C. Synthetic and crystallographic studies of a new inhibitor series targeting *Bacillus anthracis* dihydrofolate reductase. *J. Med. Chem.* **2008**, *51* (23), 7532–7540.
(127) Raymond, B.; Leduc, D.; Ravaux, L.; Le Goffic, R.; Candela, T.; Raymondjean, M.; Goossens, P. L.; Touqui, L. Edema toxin impairs anthracidal phospholipase A2 expression by alveolar macrophages. *PLoS Pathog.* **2007**, *3* (12), e187.
(128) Piris-Gimenez, A.; Paya, M.; Lambeau, G.; Chignard, M.; Mock, M.; Touqui, L.; Goossens, P. L. In vivo protective role of human group IIa phospholipase A2 against experimental anthrax. *J. Immunol.* **2005**, *175* (10), 6786–6791.
(129) Akoachere, M.; Squires, R. C.; Nour, A. M.; Angelov, L.; Brojatsch, J.; Abel-Santos, E. Identification of an in vivo inhibitor of *Bacillus anthracis* spore germination. *J. Biol. Chem.* **2007**, *282* (16), 12112–12118.
(130) Hanna, M. L.; Tarasow, T. M.; Perkins, J. Mechanistic differences between in vitro assays for hydrazone-based small molecule inhibitors of anthrax lethal factor. *Bioorg. Chem.* **2007**, *35* (1), 50–58.
(131) Clatworthy, A. E.; Pierson, E.; Hung, D. T. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat. Chem. Biol.* **2007**, *3* (9), 541–548.
(132) Ding, Z.; Bradley, K. A.; Amin, H.; Arnaout, M.; Xiong, J. P. Expression and purification of functional human anthrax toxin receptor (ATR/TEM8) binding domain from *Escherichia coli*. *Protein Expression Purif.* **2006**, *49* (1), 121–128.
(133) Bann, J. G.; Cegelski, L.; Hultgren, S. J. LRP6 holds the key to the entry of anthrax toxin. *Cell* **2006**, *124* (6), 1119–1121.

(134) Rai, P.; Padala, C.; Poon, V.; Saraph, A.; Basha, S.; Kate, S.; Tao, K.; Mogridge, J.; Kane, R. S. Statistical pattern matching facilitates the design of polyvalent inhibitors of anthrax and cholera toxins. *Nat. Biotechnol.* **2006**, *24* (5), 582–586.
(135) Staats, H. F.; Alam, S. M.; Scearce, R. M.; Kirwan, S. M.; Zhang, J. X.; Gwinn, W. M.; Haynes, B. F. In vitro and in vivo characterization of anthrax anti-protective antigen and anti-lethal factor monoclonal antibodies after passive transfer in a mouse lethal toxin challenge model to define correlates of immunity. *Infect. Immun.* **2007**, *75* (11), 5443–5452.
(136) Subramanian, G. M.; Cronin, P. W.; Poley, G.; Weinstein, A.; Stoughton, S. M.; Zong, J.; Ou, Y.; Zmuda, J. F.; Osborn, B. L.; Freimuth, W. W. A phase-1 study of PamAb, a fully human monoclonal antibody against *Bacillus anthracis* protective antigen, in healthy volunteers. *Clin. Infect. Dis.* **2005**, *41*, 12–20.
(137) Brossier, F.; Levy, M.; Landier, A.; Lafaye, P.; Mock, M. Functional analysis of *Bacillus anthracis* protective antigen by using neutralizing monoclonal antibodies. *Infect. Immun.* **2004**, *72* (11), 6313–6317.
(138) Scobie, H. M.; Thomas, D.; Marlett, J.; Destito, G.; Wigelsworth, D. J.; Collier, R. J.; Young, J.; Manchester, M. A soluble receptor decoy protects rats against anthrax lethal toxin challenge. *J. Infect. Dis.* **2005**, *192* (6), 1047–1051.
(139) Sarac, M. S.; Peinado, J,R.; Leppla, S.; Lindberg, I. Protection against anthrax toxemia by hexa-D-arginine in vitro and in vivo. *Infect. Immun.* **2004**, *72* (1), 602–605.
(140) Jiao, G. S.; Cregar, L.; Wang, J.; Millis, S. Z.; Tang, C.; O'Malley, S.; Johnson, A. T.; Sareth, S.; Larson, J.; Thomas, G. Synthetic small molecule furin inhibitors derived from 2,5-dideoxystreptamine. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (52), 19707–19712.
(141) Opal, S. M.; Artenstein, A. W.; Cristofaro, P. A.; Jhung, J. W.; Palardy, J. E.; Parejo, N. A.; Lim, Y. P. Inter-α-inhibitor proteins are endogenous furin inhibitors and provide protection against experimental anthrax intoxication. *Infect. Immun.* **2005**, *73* (8), 5101–5105.
(142) Kacprzak, M. M.; Peinado, J. R.; Than, M. E.; Appel, J.; Henrich, S.; Lipkind, G.; Houghten, R. A.; Bode, W.; Lindberg, I. Inhibition of furin by polyarginine-containing peptides: nanomolar inhibition by nona-D-arginine. *J. Biol. Chem.* **2004**, *279* (35), 36788–36794.
(143) Komiyama, T.; Swanson, J. A.; Fuller, R. S. Protection from anthrax toxin-mediated killing of macrophages by the combined effects of furin inhibitors and chloroquine. *Antimicrob. Agents Chemother.* **2005**, *49* (9), 3875–3882.
(144) Peinado, J. R.; Kacprzak, M. M.; Leppla, S. H.; Lindberg, I. Cross-inhibition between furin and lethal factor inhibitors. *Biochem. Biophys. Res. Commun.* **2004**, *321* (3), 601–605.
(145) Shiryaev, S. A.; Remacle, A. G.; Ratnikov, B. I.; Nelson, N. A.; Savinov, A. Y.; Wei, G.; Bottini, M.; Rega, M. F.; Parent, A.; Desjardins, R.; Fugere, M.; Day, R.; Sabet, M.; Pellecchia, M.; Liddington, R. C.; Smith, J. W.; Mustelin, T.; Guiney, D. G.; Lebl, M.; Strongin, A. Y. Targeting host cell furin proprotein convertases as a therapeutic strategy against bacterial toxins and viral pathogens. *J. Biol. Chem.* **2007**, *282* (29), 20847–20853.
(146) Moayeri, M.; Wiggins, J. F.; Lindeman, R. E.; Leppla, S. H. Cisplatin inhibition of anthrax lethal toxin. *Antimicrob. Agents Chemother.* **2006**, *50* (8), 2658–2665.
(147) Krantz, B. A.; Melnyk, R. A.; Zhang, S.; Juris, S. J.; Lacy, D. B.; Wu, Z.; Finkelstein, A.; Collier, R. J. A phenylalanine clamp catalyzes protein translocation through the anthrax toxin pore. *Science* **2005**, *309* (5735), 777–781.
(148) Gujraty, K.; Sadacharan, S.; Frost, M.; Poon, V.; Kane, R.; Mogridge, J. Functional characterization of peptide-based anthrax toxin inhibitors. *Mol. Pharmaceutics* **2005**, *2* (5), 367–372.
(149) Janowiak, B. E.; Finkelstein, A.; Collier, R. J. An approach to characterizing single-subunit mutations in multimeric prepores and pores of anthrax protective antigen. *Protein Sci.* **2009**, *18* (2), 348–358.
(150) Abrami, L.; Leppla, S. H.; van der Goot, F. G. Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. *J. Cell Biol.* **2006**, *172* (2), 309–320.
(151) Hirsh, M. I.; Manov, I.; Cohen-Kaplan, V.; Iancu, T. C. Ultrastructural features of lymphocyte suppression induced by anthrax lethal toxin and treated with chloroquine. *Lab. Invest.* **2007**, *87* (2), 182–188.
(152) Hirsh, M. I.; Cohen, V. Chloroquine prevents T lymphocyte suppression induced by anthrax lethal toxin. *J. Infect. Dis.* **2006**, *194* (7), 1003–1007.
(153) Moayeri, M.; Wickliffe, K. E.; Wiggins, J. F.; Leppla, S. H. Oxidized ATP protection against anthrax lethal toxin. *Infect. Immun.* **2006**, *74* (7), 3707–3714.
(154) Sanchez, A. M.; Thomas, D.; Gillespie, E. J.; Damoiseaux, R.; Rogersm, J.; Saxe, J. P.; Huang, J.; Manchester, M.; Bradley, K. A. Amiodarone and bepridil inhibit anthrax toxin entry into host cells. *Antimicrob. Agents Chemother.* **2007**, *51* (7), 2403–2411.
(155) Karginov, V. A.; Nestorovich, E. M.; Schmidtmann, F.; Robinson, T. M.; Yohannes, A.; Fahmi, N. E.; Bezrukov, S. M.; Hecht, S. M. Inhibition of *S. aureus* alpha-hemolysin and *Bacillus anthracis* lethal toxin by beta-cyclodextrin derivatives. *Bioorg. Med. Chem.* **2007**, *15* (16), 5424–5431.
(156) Karginov, V. A.; Yohannes, A.; Robinson, T. M.; Fahmi, N. E.; Alibek, K.; Hecht, S. M. Beta-cyclodextrin derivatives that inhibit anthrax lethal toxin. *Bioorg. Med. Chem.* **2006**, *14* (1), 33–40.
(157) Karginov, V. A.; Nestorovich, E. M.; Yohannes, A.; Robinson, T. M.; Fahmi, N. E.; Schmidtmann, F.; Hecht, S. M.; Bezrukov, S. M. Search for cyclodextrin-based inhibitors of anthrax toxins: synthesis, structural features, and relative activities. *Antimicrob. Agents Chemother.* **2006**, *50* (11), 3740–3753.
(158) Backer, M. V.; Patel, V.; Jehning, B. T.; Claffey, K. P.; Karginov, V. A.; Backer, J. M. Inhibition of anthrax protective antigen outside and inside the cell. *Antimicrob. Agents Chemother.* **2007**, *51* (1), 245–251.
(159) Hanna, M. L.; Tarasow, T. M.; Perkins, J. Mechanistic differences between in vitro assays for hydrazone-based small molecule inhibitors of anthrax lethal factor. *Bioorg. Chem.* **2007**, *35* (1), 50–58.
(160) Gaddis, B. D.; Avramova, L. V.; Chmielewski, J. Inhibitors of anthrax lethal factor. *Bioorg. Med. Chem. Lett.* **2007**, *17* (16), 4575–4578.
(161) Turk, B. E.; Wong, T. Y.; Schwarzenbacher, R.; Jarrell, E. T.; Leppla, S. H.; Collier, R. J.; Liddington, R. C.; Cantley, L. C. The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor. *Nat. Struct. Mol. Biol.* **2004**, *11* (1), 60–66.
(162) Johnson, S. L.; Chen, L. H.; Pellecchia, M. A high-throughput screening approach to anthrax lethal factor inhibition. *Bioorg. Chem.* **2007**, *35* (4), 306–312.
(163) Verma, R. P.; Hansch, C. Combating the threat of anthrax: a quantitative structure−activity relationship approach. *Mol. Pharmaceutics* **2008**, *5* (5), 745–759.
(164) Dalkas, G. A.; Papakyriakou, A.; Vlamis-Gardikas, A.; Spyroulias, G. A. Low molecular weight inhibitors of the protease anthrax lethal factor. *Mini-Rev. Med. Chem.* **2008**, *8* (3), 290–306.
(165) Kuzmic, P.; Cregar, L.; Millis, S. Z.; Goldman, M. Mixed-type noncompetitive inhibition of anthrax lethal factor protease by aminoglycosides. *FEBS J.* **2006**, *273* (13), 3054–3062.
(166) Goldman, M. E.; Cregar, L.; Nguyen, D.; Simo, O.; O'Malley, S; Humphreys, T. Cationic polyamines inhibit anthrax lethal factor protease. *BMC Pharmacol.* **2006**, *6*, 8.
(167) Johnson, S. L.; Chen, L. H.; Harbach, R.; Sabet, M.; Savinov, A.; Cotton, N. J.; Strongin, A.; Guiney, D.; Pellecchia, M. Rhodanine derivatives as selective protease inhibitors against bacterial toxins. *Chem. Biol. Drug Des.* **2008**, *71* (2), 131–139.
(168) Dell'Aica, I.; Dona, M.; Tonello, F.; Piris, A.; Mock, M.; Montecucco, C.; Garbisa, S. Potent inhibitors of anthrax lethal factor from green tea. *EMBO Rep.* **2004**, *5* (4), 418–422.
(169) Shoop, W. L.; Xiong, Y.; Wiltsie, J.; Woods, A.; Guo, J.; Pivnichny, J. V.; Felcetto, T.; Michael, B. F.; Bansal, A.; Cummings, R. T.; Cunningham, B. R.; Friedlander, A. M.; Douglas, C. M.; Patel, S. B.; Wisniewski, D.; Scapin, G.; Salowe, S. P.; Zaller, D. M.; Chapman, K. T.; Scolnick, E. M.; Schmatz, D. M.; Bartizal, K.; MacCoss, M.; Hermes, J. D. Anthrax lethal factor inhibition. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (22), 7958–7963.
(170) Gupta, M.; Alam, S.; Bhatnagar, R. Catalytically inactive anthrax toxin(s) are potential prophylactic agents. *Vaccine* **2007**, *25* (50), 8410–8419.
(171) Schepetkin, I. A.; Khlebnikov, A. I.; Kirpotina, L. N.; Quinn, M. T. Novel small-molecule inhibitors of anthrax lethal factor identified by high-throughput screening. *J. Med. Chem.* **2006**, *49* (17), 5232–5244.
(172) Agrawal, A.; de Oliveira, C. A.; Cheng, Y.; Jacobsen, J. A.; McCammon, J. A.; Cohen, S. M. Thioamide hydroxypyrothiones supersede amide hydroxypyrothiones in potency against anthrax lethal factor. *J. Med. Chem.* **2009**, *52* (4), 1063–1074.
(173) Pelat, T.; Hust, M.; Laffly, E.; Condemine, F.; Bottex, C.; Vidal, D.; Lefranc, M.; Dübel, S.; Thullier, P. High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of *Bacillus anthracis* by inhibiting protective antigen−LF complex formation. *Antimicrob. Agents Chemother.* **2007**, *51* (8), 2758–2764.
(174) Moayeri, M.; Crown, D.; Dorward, D. W.; Gardner, D.; Ward, J. M.; Li, Y.; Cui, X.; Eichacker, P.; Leppla, S. H. The heart is an early target of anthrax lethal toxin in mice: a protective role for

neuronal nitric oxide synthase (nNOS). *PLoS Pathog*. **2009**, *5* (5), No. e1000456.
(175) Chen, D.; Menche, G.; Power, T. D.; Sower, L.; Peterson, J. W.; Schein, C. H. Accounting for ligand-bound metal ions in docking small molecules on adenylyl cyclase toxins. *Proteins* **2007**, *67* (3), 593–605.
(176) Soelaiman, S.; Wei, B. Q.; Bergson, P.; Lee, Y. S.; Shen, Y.; Mrksich, M.; Shoichet, B. K.; Tang, W. J. Structure-based inhibitor discovery against adenylyl cyclase toxins from pathogenic bacteria that cause anthrax and whooping cough. *J. Biol. Chem*. **2003**, *278* (28), 25990–25997.
(177) Shen, Y.; Zhukovskaya, N. L.; Zimmer, M. I.; Soelaiman, S.; Bergson, P.; Wang, C. R.; Gibbs, C. S.; Tang, W. J. Selective inhibition of anthrax edema factor by adefovir, a drug for chronic hepatitis B virus infection. *Proc. Natl. Acad. Sci. U.S.A*. **2004**, *101* (9), 3242–3247.
(178) Dong, D. Y.; Xu, J. J.; Song, X. H.; Fu, L.; Chen, W. Expression and analysis of biological activity of the recombination anthrax edema factor. *Weishengwu Xuebao* **2005**, *45* (3), 459–462.
(179) Chen, D.; Misra, M.; Sower, L.; Peterson, J. W.; Kellogg, G. E.; Schein, C. H. Novel inhibitors of anthrax edema factor. *Bioorg. Med. Chem*. **2008**, *16* (15), 7225–33.
(180) Joshi, M.; Ebalunode, J. O.; Briggs, J. M. Computational insights into the interaction of the anthrax lethal factor with the N-terminal region of its substrates. *Proteins* **2009**, *75* (2), 323–335.
(181) Zakharova, M. Y.; Dubiley, S. A.; Chudakov, D. M.; Gabibov, A. G.; Shemyakin, I. G.; Kolesnikov, A. V. Substrate specificity of the anthrax lethal factor. *Dokl. Biochem. Biophys*. **2008**, *418*, 14–17.
(182) Zakharova, M. Y.; Kuznetsov, N. A.; Dubiley, S. A.; Kozyr, A. V.; Fedorova, O. S.; Chudakov, D. M.; Knorre, D. G.; Shemyakin, I. G.; Gabibov, A. G.; Kolesnikov, A. V. Substrate recognition of anthrax lethal factor examined by combinatorial and pre-steady-state kinetic approaches. *J. Biol. Chem*. **2009**, *284* (27), 17902–17913.
(183) Gupta, P. K.; Moayeri, M.; Crown, D.; Fattah, R. J.; Leppla, S. H. Role of N-terminal amino acids in the potency of anthrax lethal factor. *PLoS One* **2008**, *3* (9), No. e3130.
(184) Wickliffe, K. E.; Leppla, S. H.; Moayeri, M. Killing of macrophages by anthrax lethal toxin: involvement of the N-end rule pathway. *Cell. Microbiol*. **2008**, *10* (6), 1352–1362.
(185) Chapelsky, S.; Batty, S.; Frost, M.; Mogridge, J. Inhibition of anthrax lethal toxin-induced cytolysis of RAW264.7 cells by celastrol. *PLoS One* **2008**, *3* (1), No. e1421.
(186) Kim, C.; Gajendran, N.; Mittrucker, H. W.; Weiwad, M.; Song, Y. H.; Hurwitz, R.; Wilmanns, M.; Fischer, G.; Kaufmann, S. H. Human alpha-defensins neutralize anthrax lethal toxin and protect against its fatal consequences. *Proc. Natl. Acad. Sci. U.S.A*. **2005**, *102* (13), 4830–4835.
(187) Wang, W.; Mulakala, C.; Ward, S. C.; Jung, G.; Luong, H.; Pham, D.; Waring, A. J.; Kaznessis, Y.; Lu, W.; Bradley, K. A.; Lehrer, R. I. Retrocyclins kill bacilli and germinating spores of *Bacillus anthracis* and inactivate anthrax lethal toxin. *J. Biol. Chem*. **2006**, *281* (43), 32755–32764.
(188) Muehlbauer, S. M.; Evering, T. H.; Bonuccelli, G.; Squires, R. C.; Ashton, A. W.; Porcelli, S. A.; Lisanti, M. P.; Brojatsch, J. Anthrax lethal toxin kills macrophages in a strain-specific manner by apoptosis or caspase-1-mediated necrosis. *Cell Cycle* **2007**, *6* (6), 758–766.
(189) Wickliffe, K. E.; Leppla, S. H.; Moayeri, M. Anthrax lethal toxin-induced inflammasome formation and caspase-1 activation are late events dependent on ion fluxes and the proteasome. *Cell. Microbiol*. **2008**, *10* (2), 332–343.
(190) Lee, Y. S.; Bergson, P.; He, W. S.; Mrksich, M.; Tang, W. J. Discovery of a small molecule that inhibits the interaction of anthrax edema factor with its cellular activator, calmodulin. *Chem. Biol*. **2004**, *11* (8), 1139–1146.
(191) Tessier, J.; Green, C.; Padgett, D.; Zhao, W.; Schwartz, L.; Hughes, M.; Hewlett, E. Contributions of histamine, prostanoids, and neurokinins to edema elicited by edema toxin from *Bacillus anthracis*. *Infect. Immun*. **2007**, *75* (4), 1895–1903.
(192) Gat, O.; Grosfeld, H.; Ariel, N.; Inbar, I.; Zaide, G.; Broder, Y.; Zvi, A.; Chitlaru, T.; Altboum, Z.; Stein, D.; Cohen, S.; Shafferman, A. Search for *Bacillus anthracis* potential vaccine candidates by a functional genomic-serologic screen. *Infect. Immun*. **2006**, *74* (7), 3987–4001.
(193) Read, T. D.; Peterson, S. N.; Tourasse, N.; Baillie, L. W.; Paulsen, I. T.; Nelson, K. E.; Tettelin, H.; Fouts, D. E.; Eisen, J. A.; Gill, S. R.; Holtzapple, E. K.; Okstad, O. A.; Helgason, E.; Rilstone, J.; Wu, M.; Kolonay, J. F.; Beanan, M. J.; Dodson, R. J.; Brinkac, L. M.; Gwinn, M.; DeBoy, R. T.; Madpu, R.; Daugherty, S. C.; Durkin, A. S.; Haft, D. H.; Nelson, W. C.; Peterson, J. D.; Pop, M.; Khouri, H. M.; Radune, D.; Benton, J. L.; Mahamoud, Y.; Jiang, L.; Hance, I. R.; Weidman, J. F.; Berry, K. J.; Plaut, R. D.; Wolf, A. M.; Watkins, K. L.; Nierman, W. C.; Hazen, A.; Cline, R.; Redmond, C.; Thwaite, J. E.; White, O.; Salzberg, S. L.; Thomason, B.; Friedlander, A. M.; Koehler, T. M.; Hanna, P. C.; Kolsto, A. B.; Fraser, C. M. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **2003**, *423* (6935), 81–86.
(194) Marraffini, L. A.; Schneewind, O. Sortase C-mediated anchoring of BasI to the cell wall envelope of *Bacillus anthracis*. *J. Bacteriol*. **2007**, *189* (17), 6425–6436.
(195) Mosser, E. M.; Rest, R. F. The *Bacillus anthracis* cholesterol-dependent cytolysin, anthrolysin O, kills human neutrophils, monocytes and macrophages. *BMC Microbiol*. **2006**, *6*, 56.
(196) Cowan, G. J.; Atkins, H. S.; Johnson, L. K.; Titball, R. W.; Mitchell, T. J. Immunisation with anthrolysin O or a genetic toxoid protects against challenge with the toxin but not against *Bacillus anthracis*. *Vaccine* **2007**, *25* (41), 7197–7205.
(197) Grenha, R.; Levdikov, V. M.; Fogg, M. J.; Blagova, E. V.; Brannigan, J. A.; Wilkinson, A. J.; Wilson, K. S. Structure of purine nucleoside phosphorylase (DeoD) from *Bacillus anthracis*. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun*. **2005**, *61* (Part 5), 459–462.
(198) Tinsley, E.; Khan, S. A. A novel FtsZ-like protein is involved in replication of the anthrax toxin-encoding pXO1 plasmid in *Bacillus anthracis*. *J. Bacteriol*. **2006**, *188* (8), 2829–2835.
(199) Mikshis, N. I.; Korsakova, A.; Bolotnikova, M. F.; Novikova, L. V.; Popov Iu, A. Role of the components of the S-layer in immunogenicity of *Bacillus anthracis*. *Zh. Mikrobiol., Epidemiol. Immunobiol*. **2006**, *1*, 29–32.
(200) Chung, M. C.; Popova, T. G.; Millis, B. A.; Mukherjee, D. V.; Zhou, W.; Liotta, L. A.; Petricoin, E. F.; Chandhoke, V.; Bailey, C.; Popov, S. G. Secreted neutral metalloproteases of *Bacillus anthracis* as candidate pathogenic factors. *J. Biol. Chem*. **2006**, *281* (42), 31408–31418.
(201) Zhang, C. G.; Chromy, B. A.; McCutchen-Maloney, S. L. Host−pathogen interactions: a proteomic view. *Expert Rev. Proteomics* **2005**, *2* (2), 187–202.
(202) Torrents, E.; Sahlin, M.; Biglino, D.; Graslund, A.; Sjoberg, B. M. Efficient growth inhibition of *Bacillus anthracis* by knocking out the ribonucleotide reductase tyrosyl radical. *Proc. Natl. Acad. Sci. U.S.A*. **2005**, *102* (50), 17946–17951.
(203) Shatalin, K.; Gusarov, I.; Avetissova, E.; Shatalina, Y.; McQuade, L. E.; Lippard, S. J.; Nudler, E. *Bacillus anthracis*-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proc. Natl. Acad. Sci. U.S.A*. **2008**, *105* (3), 1009–1013.
(204) Heffernan, B. J.; Thomason, B.; Herring-Palmer, A.; Hanna, P. *Bacillus anthracis* anthrolysin O and three phospholipases C are functionally redundant in a murine model of inhalation anthrax. *FEMS Microbiol. Lett*. **2007**, *271* (1), 98–105.
(205) Pfleger, B. F.; Lee, J. Y.; Somu, R. V.; Aldrich, C. C.; Hanna, P. C.; Sherman, D. H. Characterization and analysis of early enzymes for petrobactin biosynthesis in *Bacillus anthracis*. *Biochemistry* **2007**, *46* (13), 4147–4157.
(206) Lee, J. Y.; Janes, B. K.; Passalacqua, K. D.; Pfleger, B. F.; Bergman, N. H.; Liu, H.; Hakansson, K.; Somu, R. V.; Aldrich, C. C.; Cendrowski, S.; Hanna, P. C.; Sherman, D. H. Biosynthetic analysis of the petrobactin siderophore pathway from *Bacillus anthracis*. *J. Bacteriol*. **2007**, *189* (5), 1698–1710.
(207) Abergel, R. J.; Wilson, M. K.; Arceneaux, J. E.; Hoette, T. M.; Strong, R. K.; Byers, B. R.; Raymond, K. N. Anthrax pathogen evades the mammalian immune system through stealth siderophore production. *Proc. Natl. Acad. Sci. U.S.A*. **2006**, *103* (49), 18499–18503.
(208) Cote, C. K.; Bozue, J.; Moody, K. L.; DiMezzo, T. L.; Chapman, C. E.; Welkos, S. L. Analysis of a novel spore antigen in *Bacillus anthracis* that contributes to spore opsonization. *Microbiology* **2008**, *154* (Part 2), 619–632.
(209) Gauthier, Y. P.; Tournier, J. N.; Paucod, J. C.; Corre, J. P.; Mock, M.; Goossens, P. L.; Vidal, D. R. Efficacy of a vaccine based on protective antigen and killed spores against experimental inhalational anthrax. *Infect. Immun*. **2009**, *77* (3), 1197–1207.
(210) Delvecchio, V. G.; Connolly, J. P.; Alefantis, T. G.; Walz, A.; Quan, M. A.; Patra, G.; Ashton, J. M.; Whittington, J. T.; Chafin, R. D.; Liang, X.; Grewal, P.; Khan, A. S.; Mujer, C. V. Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. *Appl. Environ. Microbiol*. **2006**, *72* (9), 6355–6363.
(211) Hahn, U. K.; Boehm, R.; Beyer, W. DNA vaccination against anthrax in mice—combination of anti-spore and anti-toxin components. *Vaccine* **2006**, *24* (21), 4569–4571.

(212) Mehta, A. S.; Saile, E.; Zhong, W.; Buskas, T.; Carlson, R.; Kannenberg, E.; Reed, Y.; Quinn, C. P.; Boons, G. J. Synthesis and antigenic analysis of the BclA glycoprotein oligosaccharide from the *Bacillus anthracis* exosporium. *Chemistry* **2006**, *12* (36), 9136–9149.
(213) Redmond, C.; Baillie, L. W.; Hibbs, S.; Moir, A.; Moir, A. Identification of proteins in the exosporium of *Bacillus anthracis*. *Microbiology* **2004**, *150* (Part 2), 355–363.
(214) Kim, H. S.; Sherman, D.; Johnson, F.; Aronson, A. I. Characterization of a major *Bacillus anthracis* spore coat protein and its role in spore inactivation. *J. Bacteriol.* **2004**, *186* (8), 2413–2417.
(215) Chitlaru, T.; Gat, O.; Grosfeld, H.; Inbar, I.; Gozlan, Y.; Shafferman, A. Identification of in vivo-expressed immunogenic proteins by serological proteome analysis of the *Bacillus anthracis* secretome. *Infect. Immun.* **2007**, *75* (6), 2841–2852.
(216) Richter, S.; Anderson, V. J.; Garufi, G.; Lu, L.; Budzik, J. M.; Joachimiak, A.; He, C.; Schneewind, O.; Missiakas, D. Capsule anchoring in *Bacillus anthracis* occurs by a transpeptidation reaction that is inhibited by capsidin. *Mol. Microbiol.* **2009**, *71* (2), 404–420.
(217) Scorpio, A.; Chabot, D. J.; Day, W. A.; O'Brien, D, K.; Vietri, N. J.; Itoh, Y.; Mohamadzadeh, M.; Friedlander, A. M. Poly-gamma-glutamate capsule-degrading enzyme treatment enhances phagocytosis and killing of encapsulated *Bacillus anthracis*. *Antimicrob. Agents Chemother.* **2007**, *51* (1), 215–222.
(218) Scorpio, A.; Tobery, S. A.; Ribot, W. J.; Friedlander, A. M. Treatment of experimental anthrax with recombinant capsule depolymerase. *Antimicrob. Agents Chemother.* **2008**, *52* (3), 1014–1020.
(219) Joshi, A.; Saraph, A.; Poon, V.; Mogridge, J.; Kane, R. S. Synthesis of potent inhibitors of anthrax toxin based on poly-L-glutamic acid. *Bioconjugate Chem.* **2006**, *17* (5), 1265–1269.
(220) Abramova, F. A.; Grinberg, L. M.; Yampolskaya, O. V.; Walker, D. H. Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90* (6), 2291–2294.
(221) Wills, B.; Leikin, J.; Rhee, J.; Saeedi, B. Analysis of suspicious powders following the post 9/11 anthrax scare. *J. Med. Toxicol.* **2008**, *4* (2), 93–95.
(222) Schmitt, B.; Dobrez, D.; Parada, J. P.; Kyriacou, D. N.; Golub, R. M.; Sharma, R.; Bennett, C. Responding to a small-scale bioterrorist anthrax attack: cost-effectiveness analysis comparing preattack vaccination with postattack antibiotic treatment and vaccination. *Arch. Intern. Med.* **2007**, *167* (7), 655–662.
(223) Kumar, R.; Chow, C. C.; Bartels, J. D.; Clermont, G.; Vodovotz, Y. A mathematical simulation of the inflammatory response to anthrax infection. *Shock* **2008**, *29* (1), 104–111.
(224) Baccam, P.; Boechler, M. Public health response to an anthrax attack: an evaluation of vaccination policy options. *Biosecur. Bioterrorism* **2007**, *5* (1), 26–34.
(225) Centers for Disease Control and Prevention. Emergency Preparedness and Response, Anthrax Q & A: Preventive Therapy. Updated March 25, **2005**. http://www.bt.cdc.gov/agent/anthrax/faq/preventive.asp (accessed May 10, 2005).
(226) Bravata, D. M.; Zaric, G. S.; Holty, J. E.; Brandeau, M. L.; Wilhelm, E. R.; McDonald, K. M.; Owens, D. K. Reducing mortality from anthrax bioterrorism: strategies for stockpiling and dispensing medical and pharmaceutical supplies. *Biosecur. Bioterrorism* **2006**, *4* (3), 244–262.
(227) Zaric, G. S.; Bravata, D. M.; Cleophas Holty, J. E.; McDonald, K. M.; Owens, D. K.; Brandeau, M. L. Modeling the logistics of response to anthrax bioterrorism. *Med. Decis. Making* **2008**, *28* (3), 332–350.
(228) Fowler, R. A.; Sanders, G. D.; Bravata, D. M.; Nouri, B.; Gastwirth, J. M.; Peterson, D.; Broker, A. G.; Garber, A. M.; Owens, D. K. Cost-effectiveness of defending against bioterrorism: a comparison of vaccination and antibiotic prophylaxis against anthrax. *Ann. Intern. Med.* **2005**, *142* (8), 601–610.
(229) Bouzianas, D. G. Medical countermeasures to protect humans from anthrax bioterrorism. *Trends Microbiol.* **2009**, *17* (11), 522–528.
(230) Stearns-Kurosawa, D. J.; Lupu, F.; Taylor, F. B., Jr.; Kinasewitz, G.; Kurosawa, S. Sepsis and pathophysiology of anthrax in a nonhuman primate model. *Am. J. Pathol.* **2006**, *169* (2), 433–444.
(231) Duong, S.; Chiaraviglio, L.; Kirby, J. E. Histopathology in a murine model of anthrax. *Int. J. Exp. Pathol.* **2006**, *87* (2), 131–137.
(232) Lyons, C. R.; Lovchik, J.; Hutt, J.; Lipscomb, M. F.; Wang, E.; Heninger, S.; Berliba, L.; Garrison, K. Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. *Infect. Immun.* **2004**, *72* (8), 4801–4809.
(233) Grunow, R.; Porsch-Ozcurumez, M.; Splettstoesser, W.; Buckendahl, A.; Hahn, U.; Beyer, W.; Bohm, R.; Huber, M.; vd Esche, U.; Bessler, W.; Frangoulidis, D.; Finke, E. J. Monitoring of ELISA-reactive antibodies against anthrax protective antigen (PA), lethal factor (LF), and toxin-neutralising antibodies in serum of individuals vaccinated against anthrax with the PA-based UK anthrax vaccine. *Vaccine* **2007**, *25* (18), 3679–3683.
(234) Phipps, A. J.; Premanandan, C.; Barnewall, R. E.; Lairmore, M. D. Rabbit and nonhuman primate models of toxin-targeting human anthrax vaccines. *Microbiol. Mol. Biol. Rev.* **2004**, *68* (4), 617–629.
(235) Weiss, S.; Kobiler, D.; Levy, H.; Marcus, H.; Pass, A.; Rothschild, N.; Altboum, Z. Immunological correlates for protection against intranasal challenge of *Bacillus anthracis* spores conferred by a protective antigen-based vaccine in rabbits. *Infect. Immun.* **2006**, *74* (1), 394–398.
(236) Skoble, J.; Beaber, J. W.; Gao, Y.; Lovchik, J. A.; Sower, L. E.; Liu, W.; Luckett, W.; Peterson, J. W.; Calendar, R.; Portnoy, D. A.; Lyons, C. R.; Dubensky, T. W., Jr. Killed but metabolically active *Bacillus anthracis* vaccines induce broad and protective immunity against anthrax. *Infect. Immun.* **2009**, *77* (4), 1649–1663.
(237) Little, S. F.; Ivins, B. E.; Fellows, P. F.; Pitt, M. L.; Norris, S. L.; Andrews, G. P. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. *Vaccine* **2004**, *22* (3−4), 422–430.
(238) New Drug and Biological Drug Products. Evidence Needed To Demonstrate Effectiveness of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible1; 21CFR 314 and 601; U.S. Food and Drug Administration and U.S. Department of Health and Human Services: Rockville, MD, 2002.
(239) Twenhafel, N. A.; Leffel, E.; Pitt, M. L. Pathology of inhalational anthrax infection in the african green monkey. *Vet. Pathol.* **2007**, *44* (5), 716–721.
(240) Rossi, C. A.; Ulrich, M.; Norris, S.; Reed, D. S.; Pitt, L. M.; Leffel, E. K. Identification of a surrogate marker for infection in the African green monkey model of inhalation anthrax. *Infect. Immun.* **2008**, *76* (12), 5790–5801.
(241) Loving, C. L.; Kennett, M.; Lee, G. M.; Grippe, V. K.; Merkel, T. J. Murine aerosol challenge model of anthrax. *Infect. Immun.* **2007**, *75* (6), 2689–2698.
(242) Yan, M.; Roehrl, M. H.; Basar, E.; Wang, J. Y. Selection and evaluation of the immunogenicity of protective antigen mutants as anthrax vaccine candidates. *Vaccine* **2008**, *26* (7), 947–955.
(243) Drusano, G. L.; Okusanya, O. O.; Okusanya, A.; Van Scoy, B.; Brown, D. L.; Kulawy, R.; Sorgel, F.; Heine, H. S.; Louie, A. Is 60 days of ciprofloxacin administration necessary for postexposure prophylaxis for *Bacillus anthracis*? *Antimicrob. Agents Chemother.* **2008**, *52* (11), 3973–3979.
(244) Hammamieh, R.; Ribot, W. J.; Abshire, T. G.; Jett, M.; Ezzell, J. Activity of the *Bacillus anthracis* 20 kDa protective antigen component. *BMC Infect. Dis.* **2008**, *8*, 124.
(245) Nablo, B. J.; Halverson, K. M.; Robertson, J. W.; Nguyen, T. L.; Panchal, R. G.; Gussio, R.; Bavari, S.; Krasilnikov, O. V.; Kasianowicz, J. J. Sizing the *Bacillus anthracis* PA63 channel with nonelectrolyte poly(ethylene glycols). *Biophys. J.* **2008**, *95* (3), 1157–1164.