Anthrax Bioterrorism and Current Vaccines

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Abstract

*Bacillus anthracis* a Category A agent with the potential to be used in a large-scale bioterrorism attack. The current vaccine, known as Anthrax Vaccine Adsorbed (AVA), consists of a culture filtrate from an attenuated strain adsorbed to aluminum salts as an adjuvant. Although considered to be safe and effective, it is difficult to produce large amounts within a short time frame. Thus, there exists a need to produce a new-generation vaccine against anthrax that can be produced quickly. In order for the new candidate vaccines to be effective, they must elicit a high titer of antibodies against protective antigen (PA). PA neutralization minimizes host susceptibility to anthrax toxemia. In addition, eliciting antibodies against additional virulence factors, such as capsule antigens of *B. anthracis*, may enhance clearance of pathogen from host. This review will discuss the history of bioterrorism and current vaccine development against anthrax. To date, there have been advances in vaccine design that utilize manipulated spores, modified protein subunits, conjugated vaccines and viral delivery vehicles. Utilizing one or more of these advances may provide a new, better vaccine against anthrax.

Keywords: Anthrax; Vaccine; Protective antigen; Lethal factor; Edema factor; Immunization

Introduction

*Bacillus anthracis*, a rod-shaped, Gram-positive, aerobic bacterium, is the etiologic agent of the acute disease anthrax. The bacterium can form endospores that survive in harsh conditions for decades. After endospore exposure to humans or animals, illness occurs after secretion of two toxins, lethal toxin (LeTx) and edema toxin (EdTx). Depending on the route of exposure, mortality can be over 50%, even with supportive care.

Because of the high virulence characteristic, anthrax is attractive as a potential bioweapon. Indeed, it has been used previously to incapacitate foreign economies or as fear tactics. In World War I, German and French agents used glanders and anthrax in order to infect foreign nations’ livestock; in World War II, Japan used anthrax, plague, and other diseases on populations to test bioweapon potential [1]. In the fall of 2001, *B. anthracis* spores were spread through letters mailed in the United States. Twenty two people were known to have been infected and five of those individuals died. A 92-page summary of evidence against the suspect, Bruce Ivins, was released by the FBI [2]. This most recent anthrax attack drew attention to the almost unlimited gaps in security and defense against bioterrorism. Research for anthrax vaccines plays a very important role in mitigating this threat.

In 1881, Louis Pasteur developed the first effective vaccine, a live attenuated *B. anthracis* strain, for anthrax [3]. Since the 1930s, the live attenuated Stern strain has been widely used to vaccinate domesticated animals. However, due to concerns about safety, live spore vaccines have not been adopted for general vaccination in the United States [4]. Anthrax Vaccine Adsorbed, otherwise known asAVA or BioThrax, is the only FDA licensed human anthrax vaccine available. This vaccine was licensed in 1970 based on safety studies conducted on 7000 participants [5]. In 1998, the Clinton administration required the immunization of all military members against anthrax. However, due to petitions and class-action lawsuits, mandatory immunization was halted for a period. Despite the conclusion that AVA is safe, AVA has shortcomings that warrant the development of new-generation vaccination strategies. Therefore, this review will focus on the current knowledge about anthrax pathogenesis and the requirements for development of an ideal vaccine for anthrax.

History of Bioterrorism and Bio warfare

Biological materials have been used as weapons throughout history. In 1346, the Tartar army, inflicted with plague, catapulted thousands of their own men, killed by plague, into the sieged city of Caffa. The resulting epidemic decimated the residents of the city [6]. The infected ones who escaped the siege likely transmitted the disease to other parts of Europe and contributed to the spread of the Black Death.

After World War I and the signing of the Geneva Protocol of 1925, many countries, including Germany, Great Britain, Japan, Belgium, Canada, France, Italy, the Netherlands, Poland, the Soviet Union and the United States conducted biological weapons research [7]. Although the Geneva Protocol prohibited the use in war of “asphyxiating, poisonous, or other gases and bacteriological methods of warfare,” there was no method to enforce compliance. One example of further bioweapons development during World War II includes Japan’s military Unit 731. This unit developed plague as a biological weapon by allowing laboratory fleas to feast on plague infected rats, and then the Japanese military released the fleas over Chinese cities to initiate plague epidemics. Unfortunately, the Japanese had not adequately prepared their military personnel for the effects of a released biological weapon. In addition to 10,000 casualties in the city of Changteh, 1700 deaths were reported among Japanese troops [8].

In 1972, a “Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological and Toxic Weapons and on Their Destruction” was held. Known as the BWC, this treaty...
prohibits development, production and stockpiling of pathogens or toxins and requires parties to destroy stockpiles. In addition, signatories to the BWC were required to submit information about facilities where biological defense research was being conducted, scientific conferences at special facilities and disease outbreaks. Unfortunately, like the Geneva Protocol, the BWC did not provide guidelines for inspections, control of disarmament and compliance. And so, it was not surprising to find that by 1995, Iraq had produced 19,000 liters of concentrated botulinum toxin, 8,500 liters of concentrated anthrax and 2,200 liters of allatoxin to be used in modern warfare [9].

Organizations and individuals have also produced, stockpiled and released biological weapons on the general populace. In 1995, the Aum Shinrikyo, a Japanese religious group, released sarin nerve gas into five trains in the busy Tokyo subway killing 12 commuters and affecting hundreds more. Police raids discovered many biological weapon agents (anthrax and Ebola). The motives were unclear, but speculation ranged from hastening an apocalypse to bringing down the government. In the United States, the 2001 anthrax letters killed five individuals. Although no direct evidence of Bruce Ivins’ involvement was uncovered, US officials declared him the sole culprit [10]. It is clear that individuals have relatively easy access to bioweapons capable of killing hundreds and injuring thousands. The threat of bioterrorism in modern times comes from both foreign and domestic sources.

**Anthrax**

Natural *B. anthracis* infections are rare in developed countries and relatively few cases have been reported. When anthrax infection does occur, it primarily presents itself in three different ways: pulmonary, gastrointestinal, and cutaneous. A rare fourth syndrome involving soft tissue infection in injectional drug users is emerging due to unsanitary practices [11]. The deadliness of anthrax depends on the route of infection. Aerosolized endospores, targeting the inhalation route, are relatively easy to disseminate and were goals of bioweapons programs. Unfortunately, pulmonary anthrax infection is the most deadly form of anthrax. The aerosol LD₅₀ for rhesus macaques against the virulent anthrax Ames strain is about 5.5×10⁴ spores [12]. After inhalation, endospores may stay dormant within the lung for extended periods before the host begins to show symptoms. During an accidental release of anthrax spores in 1979 from a former Soviet biological warfare facility in Sverdlovsk, human anthrax disease appeared between 2 to 43 days after exposure [13]. The respiratory infection in humans presents flu-like symptoms for several days followed by severe respiratory collapse and major organ failures.

Gastrointestinal infection in humans is caused by eating anthrax-infected meat or plant matter. Symptoms may include vomiting of blood, severe diarrhea, inflammation and loss of appetite. It may spread into the bloodstream and thus, cause major organ failure leading to death. The cutaneous infection in humans presents as a boil-like skin lesion between 2 and 5 days after exposure. This route of infection is historically the most common due to anthrax spore contamination of cuts. If left untreated, cutaneous anthrax causes mortality in approximately 20% of cases [14]. However, with aggressive antibiotic treatment, the danger is lessened.

**Anthrax pXO1 and pXO2**

*B. anthracis* has two principal virulence determinants carried on two different plasmids, pXO1 and pXO2. The 181kb pXO1 plasmid, previously sequenced [15], encodes the genes (*pagA, cya* and *lef*) for secreted toxins PA, EF, and LF, respectively. The 90kb pXO2 plasmid encodes the genes for formation of an anti-phagocytic capsule. Both plasmids are required for *B. anthracis* to be fully virulent, such as the Ames strain. Strains such as the pXO2-Sterne strain cannot produce a capsule to resist macrophage phagocytosis.

**Protective antigen, edema factor and lethal factor**

Each individual anthrax toxin protein by itself is non-toxic. The proteins act as an A2B toxin complex. Protective antigen (PA or PA63) is a 735 amino acid protein (83 kDa) B toxin component that binds to either capillary morphogenesis gene 2 (CMG2) or tumor endothelial marker 8 (TEM8) on cells. CMG2 is widely distributed in human tissues, whereas TEM8 is highly expressed on macrophages, endothelial cells and tumor cells [16]. A furin-like protease [17] cleaves 20 kDa from PA83 to yield PA63, thus activating it. The PA63 forms a heptamer [18] to expose binding sites for three edema factor (EF) or lethal factor (LF), both A toxin components. The PA63 may also form an octamer that is able to bind four EF or LF which is more stable in bloodstream pH than the heptamer configuration [19]. When the toxin-receptor complex is internalized into an acidic endosome, the PA63 undergoes a conformational change to form a pore. LF completely translocates into the cytosol whereas EF remains membrane associated.

Edema toxin (EdTx) is an adenyl cyclase that acts to impair water homeostasis by catalyzing the conversion of ATP into cAMP [20]. In contrast to EdTx, LeTx is both more lethal and active in only certain cell lines and primary cultures of murine macrophages [21]. LeTx is a zinc-dependent metalloprotease that cleaves the N-terminus of several mitogen activated kinase (MAPKK) members. This cleavage allows for activation of many pathways, including upregulating or downregulating cytokines and leads to apoptosis.

**Macrophage secondary effects**

LeTx is also able to affect some cells by lysing them, thus leading to the hypothesis that rapid onset of shock-like death is attributable to the release of cytokines such as TNFa and IL-1β from macrophages [21-23]. However, there has been some controversy regarding this issue [24-27]. The sudden shock suggests a rapid change in body homeostasis. When mice were treated with antibodies against IL-1 during LeTx challenge, a protective effect was observed [22]. Antibodies against TNFa did not show the same effect. There are inconclusive findings and so, this area needs more research to elucidate the role of cytokines in anthrax pathogenesis.

LeTx is a primary virulence factor that causes clinical symptoms and specifically targets macrophages. Previous observations showed that when mice were specifically depleted of macrophages by silica injections, they became resistant to LeTx challenge [22]. However, the ubiquity of cellular receptors for PA demonstrates that there may be other human cell types can still be affected by LeTx without causing cellular lethal effects. Human umbilical vein endothelial cells undergo apoptosis when exposed to LeTx [28]. This may account for the vascular leakage demonstrated in some anthrax cases.

**Vaccination Strategies**

Current studies and advances for candidate vaccines are focused on targeting either the toxinemia (toxin) or septicemia (bacteria) aspects of anthrax disease. Efforts have been made to develop modified toxin subunits, characterize attenuated *B. anthracis* spores, encapsulate genetic information into viral delivery vectors and conjugate capsule antigens with proteins.

These candidate vaccines are tested in mouse models that are...
sensitive to anthrax LeTx. Although results may be promising, there are complications. Mechanisms underlying the susceptibility to anthrax infection are unknown. So, if a study reports a successful candidate vaccine in one strain of mouse, comparison strain studies and gender studies must be done to verify results. For example, BALB/c and C57BL/6j mice are much more resistant to LeTx compared to A/J mice. In both BALB/c and C57BL/6j mice, females required 4-fold more spores to attain 100% mortality compared with males of the same strain [29]. Different macrophage sensitivities have been attributed to genetic loci. Lethal toxin sensitivity 1 (Ltxs1) is mapped to a gene for kinesin-like protein (KiflC) on chromosome 11 [30,31]. Due to genetic differences, results from testing candidate anthrax vaccines should be carefully interpreted.

BioThrax

BioThrax, produced in the 1970s by Emergent BioDefense Corporation (formerly BioPort Corporation), is the only FDA licensed human anthrax vaccine in the United States. The vaccine is a protein subunit vaccine produced from culture filtrates of a non-virulent, non-capsule strain known as V770-NP1-R. The PA-containing sterile filtrate is adsorbed to aluminum hydroxide, an adjuvant. Human vaccination with BioThrax requires five intramuscular injections in the deltoid (0 and 4 weeks, then 6, 12, and 18 months) followed by annual boosters.

There are three main drawbacks to this vaccine: 1) the pain associated with five or more needle sticks, 2) the amount of doctor visits and 3) slow and expensive production costs. The pain caused by needle injections may diminish compliance for following the vaccine schedule. Therefore, a nasal spray method for this vaccine may reduce non-compliance. If the inhalation route generates the same safe and protective effect, perhaps the BioThrax immunization route can be modified. In one study, immunization with BioThrax via an intranasal route elicits PA specific antibodies and protects against challenge [32]. The high number of doctor visits may either reduce compliance, or, in a bioterror situation, overwhelm current medical facilities. A nasal spray requires little to no training compared to manipulating a needle, therefore, a mass-immunization effort could be implemented quickly and easily.

Subunit vaccines

Other subunit vaccines improve on the BioThrax paradigm by providing more purified and characterized proteins, reducing the number of doses needed to elicit the same protective effect and expedite a production process. Current high-yield recombinant protein techniques can yield substantial amounts of proteins, up to 0.5 g protein per liter of bacteria [33]. These techniques are attractive because the genetic codes for PA, EF, or LF can be modified to create more effective novel vaccines.

In one study, A/J mice were vaccinated with three doses of a combination of non-toxic mutant LF and PA63 to elicit high antibody titers and induced a 100% protective effect against challenge with the Sterne strain [34]. Another study demonstrated that domain 2 of PA, or the loop neutralizing determinant (LND), is an important target for an epitope-specific vaccine for anthrax. The n-terminus portion of LND provided 100% protection against spore challenge in rabbits [35]. A different study found that a novel fusion protein comprising domain 1 of LF and domain 4 of PA provided complete protection against spore challenge in mice [36]. Although we see complete protection, the amount of spores varies between experiments. The antibody titers generated may not be as high as those produced from live attenuated vaccines.

Spore vaccines

In the Soviet Union, live attenuated vaccines based on B. anthracis spores have been widely used for human immunization [37]. However, due to safety concerns regarding pathogenicity, it was not used for humans in the United States. Generally, an attenuated spore vaccine would present a full complement of bacterial antigens to the host immune system. It is thought that spores or other live attenuated vaccines may elicit better neutralizing antibodies against multiple targets, thus conferring higher degrees of efficacy than subunit vaccines such as BioThrax. So, the main concern in developing a spore vaccine is the safety profile.

Strains have been developed that express PA, LF and EF with point mutations. A strain expressing PA with mutations in the furin binding site or translocation site were more attenuated. It has been observed that spores producing only EF or LF do not elicit high antibody titers against EF or LF. However, when spores produce both PA and EF or PA and LF, anti-EF and anti-LF antibodies were increased [38]. Other methods combine inactivated spores with PA to elicit protective effects. The addition of formaldehyde-inactivated spores to PA (PA-FIS) elicits total protection against cutaneous anthrax [39]. Unfortunately, production of the live attenuated vaccine takes time to produce. In the event of a vaccine shortage and bioterror event, production may not be enough to cover demand.

Genetic vaccines

Many novel delivery systems are being developed for use as the next-generation of vaccines. These delivery systems utilize viral vectors to deliver target genetic information to the host to confer host immunity. The vectors also act as stimulants for the immune system. A vaccine using viral vectors may also be delivered intranasally or orally, thus eliciting a mucosal immune response, which may be more relevant to combating pulmonary anthrax. In one study, three times intramuscular immunization with an adenoviral vectored vaccine coding the n-fragment of EF (Ad/EFn) elicited antibodies that were against both LF and EF and induced a 57% protective effect against challenge with the Sterne strain in A/J mice [40]. DNA can also be directly used in immunization strategies. A plasmid containing a fragment of either PA for LF genes has been studied. Mice immunized with a plasmid encoding PA63 were protected against challenge with LeTx [41].

The adenoviral vectors can be mass produced quickly and efficiently by infecting tissue culture cells. If preexisting immunity exists to the vector, a different vector, if effective and safe, can be used as an alternative. So, there is a disadvantage in producing these types of vaccines. DNA plasmid vaccines can also be produced quickly, but it requires trained personnel to administer by needle sticks.

Conjugate vaccines

The previous vaccines have focused on the toxemia portion of anthrax because targeting the bacterium itself is difficult because anthrax capsule proteins are poorly immunogenic. Conjugating the capsule with immunogenic proteins can solve this issue. Antibodies to the capsule exist when patients are recovering from anthrax infection [42]. Passive immunization in mice results in protection from pulmonary anthrax [43]. Since the capsule portion is a weak immunogen, the poly-gamma-d-glutamic acid (PGA) portion of the capsule was conjugated to PA to create a potent immunogen [44]. The immunized mice were protected against LeTx challenge. And so, this reveals an important secondary approach to immunize against B. anthracis.
Summary

There have been advances in different approaches to vaccine technology. A new anthrax vaccine that can elicit high antibody titers to both PA and capsule proteins, has a good safety profile, is easily produced in mass quantities and encourages compliance is the ideal vaccine. Animal studies can vary due to the nature of anthrax toxins, but upon successful replication of results in different strains, there should be a push to advance the vaccine to higher organisms.

Advances in anthrax vaccines may also be translated to other vaccine efforts. Methods applied to the poorly immunogenic capsule proteins can be applied to other pathogens' poorly immunogenic proteins. Any pathogen's gene can be encoded into a viral vector as a delivery vehicle. Since anthrax can be genetically manipulated and easily grown, perhaps even the anthrax spore itself can become a delivery vehicle to produce target antigens for immunization of other diseases.

Due to the ease of anthrax spore production, availability of virulent spores and the history of organizations utilizing anthrax as a bioweapon, there is a high potential for bioterrorist activity. Security measures may be put into place, but they may also be easily circumvented. Vaccinations are the key to mitigating damage that may be done by bioterrorists. And so, there is an urgent need to develop a new-generation safe and effective FDA approved anthrax vaccine.

References
