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Anthrax vaccines: Pasteur to the present[1]

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Abstract. Anthrax has been a major cause of death in grazing animals and an occasional cause of death in humans for thousands of years. Since the late 1800s there has been an exceptional international history of anthrax vaccine development. Due to animal vaccinations, the rate of infection has dropped dramatically. Anthrax vaccines have progressed from uncharacterized whole-cell vaccines in 1881, to pXO2-negative spores in the 1930s, to culture filtrates absorbed to aluminum hydroxide in 1970, and likely to recombinant protective antigen in the near future. Each of these refinements has increased safety without significant loss of efficacy. The threat of genetically engineered, antibiotic and vaccine resistant strains of *Bacillus anthracis* is fueling hypothesis-driven research and global techniques – including genomics, proteomics and transposon site hybridization – to facilitate the discovery of novel vaccine targets. This review highlights historical achievements and new developments in anthrax vaccine research.

Keywords. Anthrax, anthracis, AVA, vaccines, Pasteur, rPA, Sterne.

Introduction

Anthrax has been recognized since antiquity as a disease of humans and livestock. Some highlights of early research on anthrax include the confirmation of *Bacillus anthracis* as the cause of the disease by Robert Koch in 1876 [1]; the successful use of whole cell anthrax vaccines in 1880 and 1881 by Jean-Joseph Henri Toussaint, William Smith Greenfield and Louis Pasteur [2]; the discovery of a protective factor in anthrax edema fluid by Oskar Bail in 1904 [3]; the discovery of protective antigen (PA) in culture filtrates in 1946 by G. P. Gladstone [4]; and the discovery of the anthrax toxins by Harry Smith and colleagues in the 1950s [5]. Concern about anthrax has resulted from its impact on domestic animals, wildlife and people occupationally exposed to infected animals or their products (such as wool, hides/leather and bone meal) [6]. Vaccination efforts have reduced the threat and incidence of infections, but the disease persists in many areas of the world. Due to its exceptional viru-

lence, ease of preparation, and ability to form stable and environmentally resistant spores, *B. anthracis* has been developed as a biological weapon since World War I and, at least until recently, has been part of the offensive biological weapons programs of several nations [7, 8].

The potential of *B. anthracis* as a bioweapon is illustrated by several high-profile incidents occurring during the last 3 decades. In 1979, an apparent accidental release of spores at a military microbiology facility in Sverdlosk, Russian Republic, resulted in 96 reported cases of anthrax, including 68 deaths in people likely exposed downwind [9, 10]. In 1993, the Aum Shinrikyo doomsday cult sprayed *B. anthracis* from the top of a building in Tokyo, Japan [11]. This may be the first documented use of the bacterium as an aerosolized weapon by bioterrorists, but the strain used was subsequently found to be an attenuated variant surmised to be the Sterne vaccine strain [12]. In the autumn of 2001 the mailing of *B. anthracis* spores by an unknown culprit to at least seven locations in the United States resulted in 22 confirmed cases of anthrax (11 cutaneous and 11 inhalational, including five deaths) [13]. While none of the three incidents described above produced a sizeable medical impact, various projections have envisioned the potential of more devastating morbidity and mortality from a *B. anthracis* attack [14, 15].

The potential use of antibiotic and/or vaccine resistant strains by bioterrorists heightens the urgency to develop anthrax vaccines that augment existing PA-based vaccines. The advantage of multivalent vaccines is that they have the potential to neutralize multiple pathogen developmental stages or pathogenic processes. This may minimize pathogen replication in host tissues and expression of toxic moieties.

Anthrax pathogenesis

Anthrax is most often a disease of ruminants that can afflict a wide variety of mammals, including humans. Three forms of the disease are observed, each form dependent on the route of pathogen infection. Cutaneous anthrax, the most common form of anthrax, follows introduction of infectious *B. anthracis* spores into the dermis through a cut or abrasion; inhalational anthrax from introduction of the spores into the lungs; and gastrointestinal anthrax through ingestion of spores. After introduction of the spores into host tissues, the spores may germinate locally, such as in the dermis, or they may be transported to local lymph nodes before they germinate to form bacilli. The bacillus form of *B. anthracis* senses specific environmental cues and responds by expressing virulence factors that direct inactivation and evasion of elements of the host innate immune response. Bacilli then replicate to very high numbers, and kill the host through an incompletely determined mechanism that likely includes septic shock.

After host death, bacteria in the carcass exposed to air (through hemorrhages, opening of the carcass by scavengers, etc.) sporulate and return to the soil to await introduction into another host. Vaccines for preventing anthrax interfere with one or more of the bacterium's pathogenic processes; including inactivation of the anthrax toxins, opsonization of the anthrax capsule and inhibition of spore germination. This review will discuss the current understanding of how existing subunit and live attenuated vaccines function. The review will also discuss methods and insights provided by genomics studies that promise to identify additional antigens that could be added to existing vaccines to neutralize multiple pathogenic aspects of this potent and demonstrated biowarfare agent.

The *B. anthracis* genome

The genome of *B. anthracis* includes a single 5.2-megabase chromosome and two large virulence plasmids, pXO1 and pXO2 (182 and 95 kilobases, respectively). Altogether, the genome has 5838 predicted protein-coding genes [16]. The chromosomal sequence and gene organization is quite similar to that of the closely related bacteria *B. cereus* and *B. thuringiensis* [17]. *B. anthracis* likely evolved from a single clone of *B. cereus* that acquired pXO1 and pXO2 from the environment by lateral genetic transfer. Genes required for virulence factor expression and regulation are located on the plasmids [18]. pXO1 contains a large pathogenicity island which encodes lethal and edema toxins (LT and ET) [18, 19], while the biosynthetic enzymes of the poly-D-gamma glutamic acid capsule are encoded on a pathogenicity island on pXO2 [20, 21]. Loss of either plasmid significantly reduces virulence in most animal models [22–26].

B. anthracis toxins

The structure and mechanisms of action of the toxins have been intensely studied. The enzymatic effector proteins of the two toxins are called lethal factor (LF) and edema factor (EF). Both LF and EF can bind a third protein, PA. PA is cleaved by mammalian serum and/or cell surface proteases and can bind to at least two specific receptors (TEM8 and CMG2) located on host cell membranes [27, 28]. PA forms ring-shaped heptamers, and interacts with LF and EF [29], which then enter the host cell by endocytosis [30]. Upon acidification of the endocytic vacuole, the PA heptamer apparently forms a pore through which the EF and LF moieties are translocated. Molecular targets within mammalian cells have been clearly identified for both toxins. LF is a zinc metalloprotease capable of inhibiting signal transduction through the mitogen-activated protein kinase (MAPK) cascade by cleaving most MAPK kinases (MAPKKs or MEKs), preventing the phosphorylation of MAPKs such as p38, ERK and JNK [31–33]. EF is a calcium/calmodulin-dependent adenylate cyclase that increases intracellular levels of cyclic AMP [34], leading to massive edema. LT and ET appear to impair both the innate and adaptive immune systems, having effects on multiple cell types, including macrophages, dendritic cells and neutrophils.

B. anthracis capsule

The poly-glutamate capsule appears to be a fibrous structure in electron micrographs of the bacillus surface [35]. Early data show that capsule consists entirely of poly-D-γ-glutamate [36–39]. One-dimensional and two-dimensional nuclear magnetic resonance (NMR) data recently confirmed that capsule has γ-carboxyl peptide linkages, and gas chromatography data recently confirmed that capsule appears to contain glutamic acid only of the D configuration [40]. Capsule purified from autoclaved Ames bacilli appears to have an extended random coil structure, as determined by light scatter and circular dichroism data, and a heterologous size range averaging

about 700 kDa, as determined by light scatter analysis [40].

Capsule synthesis is dependent on four proteins (CapA, B, C and E) encoded by an operon in pXO2 [20, 21, 41, 43, 44]. CapD (or DepA), another protein encoded by the *cap* operon, can degrade capsule [45, 46], and the subsequent release of capsule fragments (low molecular weight capsule) has been linked to virulence [47]. A recent report not only confirms that CapD is required for full virulence in mice and that CapD can degrade capsule, but it also shows that CapD apparently covalently links capsule to cell wall peptidoglycan [48].

Roles of capsule in virulence

Remarkably, crude capsule preparations were shown to be antiphagocytic in 1907 [49], and *B. anthracis* capsule was associated with virulence in 1915 [50]. A 1963 report showed reduction of phagocytosis of strains NP and Sterne by guinea pig polymorphonuclear cells upon addition of exogenous capsule [51]. Addition of purified capsule also enhanced virulence of a Pasteur II strain in guinea pigs. Others have described capsule-mediated inhibition of phagocytosis in neutrophils and macrophages [44, 51, 52]. Conversely, antibody to capsule enhances phagocytosis [52–54].

Capsule may also camouflage bacilli from the immune system by binding host proteins. As far back as the 1930s, investigators showed that capsule binds to basic serum proteins, such as lysozyme [55, 56]. Recent evidence shows that it binds and deactivates antibacterial cationic peptides [57]. It has been suggested that capsule fragments might bind to mediators of innate immunity, acting as a sink that drains immune modulators [45]. Complement binding by capsule, perhaps in conjunction with S-layer proteins [35], and capsule-mediated inhibition of anthracidal activity of normal horse serum and guinea pig leukocyte extracts have also been reported [38].

Other virulence factors

In addition to anthrax toxins, capsule and their regulators, a number of genes/proteins that have a measurable contribution to virulence and survival in mice or guinea pig models of infection have been identified. A select few of these, such as specific proteases [58], may contribute directly to inflicting damage on the animal host; others, such as cell wall-modifying enzymes, may promote evasion of the innate immune system [59]. Most of the other genes/proteins known to affect virulence are not virulence factors per se, but appear to promote spore germination [60], acquisition of key nutrients [61–64], resistance to oxidative stress or coordination of an overall

stress response during replication in the host environment [62, 65].

Animal models for Anthrax vaccine studies

While a vaccine intended for human use would ultimately have to prove safe and efficacious in non-human primates, it is difficult to endorse a single animal model for early evaluation of experimental vaccines. Mice and guinea pigs are generally more difficult to protect from fully virulent *B. anthracis* strains [66]. Mice can be used to evaluate toxin-based vaccines if a Sterne-type (pXO2⁻ strain) challenge strain is used [67], and other vaccines if a pXO1⁻ challenge strain is used [25]. Fully virulent challenge strains are useful for evaluating vaccines containing PA in combination with other antigens and novel adjuvants [53]. The type of mouse used can be critical, as there is some variability among mouse lines. One study compared 10 types of mice and found all had low LD_{50}s (50% lethal dose) upon subcutaneous inoculation with *B. anthracis* Vollum 1B (5–30 spores), and their mean time of death after challenge with 60 spores varied from 3 days for DBA2J mice to 6.5 days for C57BR/cdJ mice [67]. LD_{50}s can vary significantly, however, with attenuated challenge strains. For example, some mice, such as A/J mice, are sensitive to Sterne ($LD_{50} = 1.1 \times 10^3$ spores), while other mice, such as C58/J, are resistant (Sterne $LD_{50} > 10^7$ spores) [67].

In laboratories for which *B. anthracis* is considered too dangerous, vaccines might be evaluated by their ability to protect against purified toxin challenge. Fischer 344 rats are often used, due to their remarkable sensitivity to LT [68, 69]. In one study, injecting 12 µg of PA plus 2.4 µg of LF (about $4 \times LD_{50}$) caused rats to die in less than 2 h, while mice challenged with $4 \times LD_{50}$ took up to 6 days to die [67]. It is intriguing that some animals, such as rats, are very sensitive to toxin but difficult to infect [70, 71], while other animals, such as guinea pigs, are more toxin resistant but can be killed with relatively few spores [51]. Reasons for these discrepancies are unknown but may speak to differences in host germinants and innate immunity, as well as direct toxin sensitivity [45, 67].

In vitro methods for evaluating vaccines include enzyme-linked immunosorbent assays (ELISAs) to measure antibody to toxins, capsule, killed spores and other antigens. Anti-LT response can also be evaluated using toxin neutralization assays (TNAs), in which mouse macrophages are protected from purified LT by antisera [72–74]. TNAs are generally regarded as the *in vitro* gold standard for evaluating PA-based vaccines, though one study found that TNA titers do not always correlate with protection [53]. In that study, immunization with crosslinked rPA (recombinant PA) improved protection of mice against Ames challenge but resulted in reduced serum TNA ti-

ters. Measurements of cellular immunity are generally not used to evaluate vaccines, as the pathogenesis of anthrax is probably mostly due to extracellular bacilli. However, a thorough evaluation of a new vaccine might include a combination of *in vitro* tests that measure antibody responses, toxin neutralization, killing of bacteria and cellular immunity.

Protective antigen-based vaccines: AVA and rPA

Anthrax vaccine adsorbed (AVA), also known as BioThrax since 2002, has been used to vaccinate against anthrax in the United States for over 30 years. AVA has been shown to be effective in preventing infection in several animal models, including non-human primates [66]. It consists of supernatant material from *B. anthracis* V770-NP1-R, which is pXO1$^+$ and pXO2$^-$, grown microaerophilically in a protein-free defined medium. This supernatant is filtered and adsorbed to aluminum hydroxide (Alhydrogel). The final product contains 1.2 mg/ml of aluminum, 25 µg/ml of benzethonium chloride and 100 µg/ml of formaldehyde. The current vaccination regimen consists of six subcutaneous injections over 18 months followed by yearly boosters. A vaccine similar to AVA is used in the United Kingdom, produced from culture supernatants of *B. anthracis* Sterne 34F2. It is well documented that PA is the primary effective component of AVA [75–77], and thus expression of PA is optimized for the vaccine manufacturing process [78].

While a vaccine preparation similar to AVA was shown to be safe and effective in a field trial [79], and occurrence of systemic adverse events associated with AVA vaccination is rare [80], concerns raised over manufacturing variability and reactogenicity have prompted development of a next-generation anthrax vaccine composed of rPA purified from an atoxigenic, asporogenic strain of *B. anthracis*. Comparative studies indicated that rPA with an aluminum-containing adjuvant conferred protection in a rhesus macaque aerosol model of anthrax [81]. In these studies, rhesus macaques were vaccinated once with either rPA or AVA and challenged 6 weeks later with spores of the virulent Ames strain. Both rPA formulated with Alhydrogel and AVA provided complete protection and elicited strong anti-PA immunoglobulin (Ig) G and IgM titers. Additionally, rPA-Alhydrogel and AVA elicited comparable toxin neutralization titers. In another study, rPA-Alhydrogel protected rhesus macaques against a target dose of 200 LD$_{50}$ Ames spores in an aerosol challenge model, and antisera from rPA-immunized rhesus macaques protected A/J mice in passive transfer studies [82]. A contract to manufacture the rPA vaccine was awarded to VaxGen Inc. (Brisbane, CA) by the Department of Health and Human Services in 2004, and clinical trials are currently being conducted to determine its safety and immunogenicity.

In addition to developing a next-generation anthrax vaccine, researchers are currently examining alternative vaccine delivery routes. AVA is administered by subcutaneous injection, but new research is underway to determine whether intramuscular delivery will be less reactogenic and equally efficacious with fewer doses. While both AVA and rPA elicit high IgG anti-PA titers in animal models, it may be that induction of both systemic and mucosal immunity would result in superior protection [83, 84]. Strategies to elicit mucosal immunity to anthrax include oral vaccination with *Salmonella* [85, 86] or *Lactobacillus* [87] expressing PA, nasal instillation with rPA [90, 91], nasal delivery of rPA associated with microspheres [90] or liposomes [91], and oral spore vaccination with attenuated *B. anthracis* expressing rPA [92]. Coulson et al. demonstrated that an attenuated strain of *Salmonella typhimurium* expressing PA could confer partial protection from challenge with *B. anthracis* Vollum 1B after oral vaccination, although PA-specific antibodies were not detected [85]. Galen et al. inoculated mice intranasally with *S. enterica* serovar Typhi CVD 908-htrA expressing domain 4 of PA and were able to detect serologic titers against PA [86]. Parenteral vaccination with a lysate of PA-expressing *Lactobacillus casei* resulted in a significant anti-PA response [87]. However, oral vaccination with the live strain failed to elicit a PA-specific response. These studies demonstrate the possibility of employing bacterial carriers to elicit PA immunity if technical hurdles can be overcome.

Alternative adjuvants, such as cholera toxin [89] and soya phosphatidyl choline [88] have been used with mucosal PA administration. These approaches led to significant anti-PA IgG and IgA titers and strong Th2 cytokine responses. Another approach to mucosal vaccination was demonstrated with intranasal delivery of microencapsulated PA. Intranasal vaccination of mice with PA microencapsulated in poly-L-lactide 100 kDa microspheres resulted in a protective immune response against aerosol challenge equal to that elicited by AVA vaccine [90]. Sloat et al. demonstrated that vaccinating mice with PA carried by liposome-protamine-DNA (LPD) particles resulted in IgG and IgA anti-PA responses [91]. Protective efficacy of intradermal rPA delivery and intranasal administration of a powder form of rPA has also been investigated [93]. In these studies, rabbits given intradermal injections of rPA with microneedles were fully protected from aerosol challenge of *B. anthracis*.

Finally, oral vaccination of guinea pigs with spores of a ΔSterne strain (pXO1$^-$, pXO2$^-$) transformed with a PA-expressing plasmid resulted in IgG and IgA anti-PA responses and partial protection against subcutaneous challenge of 20 LD$_{50}$ *B. anthracis* Vollum [92]. An advantage of an oral or nasal anthrax vaccine is the relative ease of delivery. With the current 6-dose initial vaccination regimen and yearly injections required for vaccinated individuals, such

a vaccine may prove to be a more efficient alternative to traditional parenteral vaccination and may provide stronger immunity and a higher level of compliance.

'Dual-protection' vaccines

As PA is a secreted toxin component, it has been suggested that rPA vaccines function merely by deactivating toxins, and that a more complete vaccine would also contain antigens such as capsule, on the surface of bacilli [52, 53, 94], and BclA, on the surface of spores [42, 95]. However, a small amount of PA has been detected on the surface of ungerminated spores, and it has been demonstrated that anti-PA can bind to spores, resulting in decreased spore germination and increased spore uptake and killing by cultured macrophages [96–99]. Thus, anti-PA not only deactivates toxin, but may also facilitate macrophage killing of spores. The threat of bioengineered or even natural isolates of *B. anthracis* that are vaccine resistant [100] has led to determined efforts to identify other vaccine targets that might be included with PA in a future generation vaccine. Indeed, superior protection has been observed in mice immunized with PA co-formulated with capsule [53] or formaldehyde-inactivated spores (FIS), demonstrating that additional immunogens can improve the protective efficacy of PA-based vaccines [101].

Spore antigens

Cohen et al. [102] reported that spore antigens might contribute to protection. They found that guinea pigs vaccinated with spores produced significant anti-spore antibody levels and were better protected against spore challenge than guinea pigs vaccinated with bacilli. Stepanov et al. reported similar findings with hamsters and rabbits [101]. Brossier et al. [103] showed that spore antigens can elicit a protective immune response. In this study, FIS injected twice with aluminum hydroxide into mice or guinea pigs partially protected against fully virulent strains and a PA knockout strain. Additionally, combining PA and FIS elicited better protection against fully virulent strains than either PA or FIS by itself. Glomski et al. found that FIS could protect mice against a pXO1-negative spore challenge, and that this immunity was antibody independent [105]. Finally, an exosporium glycoprotein, BclA, has proven to be highly immunogenic [97] and might contribute to protection [42].

Capsule-based vaccines

When not crosslinked to a carrier protein, the anthrax capsule is considered a poor antigen [56, 94, 104]. This may be due to its repeating negative charge, its lack of L-amino acids and its unconventional peptide bonds [105]. Poor immunogenicity is consistent with the T-independent nature of bacterial polyanionic capsules [106, 107]. T-independence of *B. anthracis* capsule was demonstrated by Wang and Lucas [108], who showed that nude mice injected with killed bacteria could produce significant anti-capsule titers, while mice deficient in Brutons tyrosine kinase, a molecule required for T-independent signaling, did not produce detectable anti-capsule. Conversion to a T-dependent antigen by complexing with a carrier protein was first demonstrated by Goodman et al. [104], who showed enhanced immunogenicity when *B. anthracis* capsule was noncovalently linked to methylated BSA (bovine serum albumin) simply by allowing electrostatic interaction at pH 4. Leonard and Thorne were not able to detect antibody response to purified capsule injected into rabbits [56]. However, Chabot et al. showed capsule administered with Ribi two-component adjuvant was weakly immunogenic in mice [53]. Several recent reports have shown dramatically enhanced immunogenicity of *Bacillus* species capsules and poly D-γ-glutamic acid peptides after chemically crosslinking them to carrier proteins [52, 54, 94]. Several investigators have also reported strong anti-capsule response to killed *B. anthracis*, though these sometimes require several boosts [43, 108, 109].

Protective immune responses to capsule have been demonstrated. Two studies have shown passive protection of mice with antibody to capsule [40, 110]. Mice vaccinated with capsule complexed to Ribi two-component adjuvant were partially protected against a ΔAmes (pXO1⁻, pXO2⁺) challenge, and capsule enhanced PA protection against Ames challenge [53]. Surprisingly, the same study showed increased IgG anti-capsule but decreased protection when a carbodiimide was used to conjugate capsule to BSA. Decreased protection may have been due to carbodiimide cleavage of γ-peptidyl bonds [40]. A different conjugation method was later used in which capsule was activated with a triazine-based reagent, then derivatized with a sulfhydryl-reactive heterobifunctional moiety and finally coupled to thiolated *Neisseria* outer membrane protein [40]. Two or three doses of this conjugate complexed with aluminum hydroxide protected mice against Ames spore and bacillus challenges.

Global strategies to identify new vaccine targets

As previously discussed, several reports have suggested that vaccines composed of multiple antigens such as live attenuated strains and other multivalent vaccines confer enhanced protection compared with minimal subunit antigens such as rPA. This enhanced protection may be due to the ability of the multivalent vaccines to elicit

host responses that inactivate more than one pathogen developmental stages or pathogenic property. Thus, several efforts are underway to identify additional antigens that target and neutralize several *B. anthracis* pathogenic stage or mechanisms. Multivalent subunit vaccines may offer significant advantages over existing vaccines. For instance, future subunit vaccines may reduce pathogen replication within the host and confer protection against genetically engineered strains of anthrax designed to overcome existing vaccines while minimizing residual virulence and reactogenicity concerns associated with live attenuated strains.

The availability of tools for efficient genome, proteome and transcriptome analysis has facilitated research aimed at elucidating pathogenic mechanisms of several important microorganisms, including *B. anthracis* [111–114]. Mass spectrometric technology coupled with the recent availability of the complete *B. anthracis* genome sequence [16] allows rapid identification and annotation of the theoretical *B. anthracis* proteome. Indeed, a comprehensive proteomic map of *B. anthracis* is an important tool for the pursuit of alternative therapies and vaccines for anthrax. A proteomic analysis of vegetative cells of *B. anthracis* Sterne was reported by Francis et al. [112]. Using a combination of three pre-analytical separation techniques, they reported the detection of 1048 unique proteins. This represents approximately 19% of the predicted proteome and is similar to results obtained from analysis of the *B. anthracis* transcriptome in which 30% of the theoretical transcriptome was reported to be expressed [111]. Wang et al. generated a 2-DE reference map and database of *B. anthracis* A16R in which they identified 299 unique proteins [115]. Others have performed proteomics studies of proteins secreted by *B. anthracis* [114, 116, 117] as well as proteins associated with the endospore [118, 119], the spore coat [120], the germinating spore [119], the bacillus cell membrane [121] and germination [119]. Proteomics has also been used to identify immunoreactive antigens for possible testing as vaccine candidates. This was done by screening immune sera from previously infected or vaccinated animals or from vaccinated humans for antibodies that react with a subset of *B. anthracis* antigens [121–124]. Such efforts are leading to the identification of new virulence factors and antigens, such as MntA [62]

Identifying *B. anthracis* proteins specifically or preferentially expressed during infection or that can be shown to contribute to virulence may lead to discovery of additional potential vaccine or therapeutic targets. While several studies have been conducted to examine the proteome of *B. anthracis* and other pathogens grown *in vitro*, there is relatively little proteomic data from *in vivo* isolated organisms. In recent findings on the proteome of *B. anthracis* isolated from infected animals, approximately 50% of the predicted proteome was detected from protein preparations of whole cell, membrane and secreted fractions of *B. anthracis* bacilli grown in laboratory culture as well as whole cell and membrane components from bacilli isolated from infected guinea pigs [125]. Genomics studies aimed at directly identifying factors required for *B. anthracis* virulence include transposon site hybridization (TraSH). TraSH can be used to determine the site of transposon insertion mutations in a population using microarray technology. *B. anthracis* populations containing transposon insertions in approximately 4000 of the pathogen's approximately 5700 genes were used to identify 290 genes required for anthrax virulence [126].

Live vaccines vs. subunit vaccines

Comparison of vaccines is somewhat subjective, as success can depend on experimental design. However, several studies have shown that live Sterne spores offer better protection than subunit vaccines even when subunit vaccines elicit stronger anti-PA responses [64, 127–129]. Little and Knudson [128] found over 80% protection against 1000 spores of 11 different *B. anthracis* strains in guinea pigs vaccinated with three doses of Sterne, while three doses of AVA gave better than 50% protection only against two of the strains, Vollum and Vollum 1B. However, AVA is very effective in rabbits and other higher animals [66, 127], while Sterne-type vaccines are toxigenic and can cause injury at injection sites and rare animal deaths [130]. In one study Sterne protected several strains of mice from Vollum 1B only at doses at least $0.1 \times LD_{50}$ [131]. Another study showed Sterne as an effective vaccine in guinea pigs, but about one out of three guinea pigs vaccinated intramuscularly with 10^7 Sterne spores died from the vaccine [64]. Yet another report showed 10^7 Sterne spores delivered subcutaneously killed most guinea pigs [102]. In most countries Sterne is not considered safe enough for use in humans [132]. The goal in developing new live vaccines is to maintain protection while reducing reactogenicity.

Live vaccines: Pasteur through Sterne and ST1

Commercial use of vaccines to prevent anthrax in animals began in 1881 with Pasteur's heat-attenuated strains [132]. The toxin plasmid pXO1 is heat-unstable [19], so it was proposed that Pasteur's vaccines were attenuated due to the loss of toxin expression. This seems unlikely, as pXO1⁻ strains are generally not protective [22, 24], and there are some Pasteur strains that contain both plasmids. It is now widely believed that Pasteur's vaccines were mixed cultures containing some bacteria that produced toxin [22, 133]. A mixed culture, possibly containing a very small percent of fully virulent bacteria, may have

improved protection, while occasionally causing disease. In fact it was not unexpected for an occasional livestock animal to die from the vaccine in return for herd immunity. However flawed they were, variations on Pasteur's two-dose bacillus vaccine were effectively used in European and South American livestock for about 50 years [134].

Pasteur-type vaccines were largely supplanted in the 1930s, when Sterne-type vaccines, consisting of spores of pXO2-cured strains, were introduced. Sterne-type strains (pXO1$^+$, pXO2$^-$) have shown dramatically better protection than pXO1$^-$, pXO2$^+$ strains. For example, in one study bacilli from two pXO2$^-$ strains protected guinea pigs from Vollum 1B challenge, while none of three pXO1$^-$ strains were protective [22], indicating pXO1 might be sufficient for protection, while pXO2 is not.

In 1940 the Soviet Union introduced a Sterne-like vaccine, ST1 [135], which has since been used in millions of people. Turnbull et al. [129] found Sterne and ST1 provided guinea pigs similar anti-PA titers and protection against anthrax. Several epidemiological studies indicate STI provides humans with partial protection against cutaneous anthrax. However, ST1, like Sterne, produces toxins and may be reactogenic. One study found fewer than 1% vaccinated people have side effects [135], while others report that general and local responses are common [99]. One study found local reactions in 87% when administered by jet injector, 72% when administered by scarification and 14% when injected subcutaneously [136].

In addition to Pasteur-type and Sterne-type vaccine strains, there are live vaccines that have both plasmids but might be attenuated by chromosomal mutations. These include some Pasteur strains [24, 47, 137], the Italian Carbosap vaccine strain [138], the Argentinian strain A [137], and the Russian Tsenkovsky strains. Understanding how these strains are attenuated may help to more fully understand anthrax pathogenesis and lead to identification of new vaccine and therapy targets.

Next-generation live vaccines

There are other avenues to explore with live attenuated vaccines. One is to express toxin components from relatively harmless *Bacillus* species. *B. subtilis* transformed with a PA expression plasmid protected guinea pigs from a Vollum 1B spore challenge and Fischer 344 rats from toxin challenge (50 µg of PA plus 10 µg of LF) [139]. A single injection of PA-expressing *B. subtilis* protected 9 of 9 CBA/J mice against Vollum 1B, though it failed to protect A/J mice, which have a complement factor 5 defect [131]. This record is still relatively impressive, considering that mice are difficult to protect against wild-type *B. anthracis* challenge; in the same study three doses of AVA

failed to protect either type of mouse, despite eliciting higher anti-PA titers.

Another live vaccine consists of Sterne attenuated by replacement of pXO1 with a plasmid that constitutively expressed PA [140]. Intramuscular injection of a single dose of this strain conferred partial protection to guinea pigs against a stringent intramuscular challenge of 2000 LD$_{50}$ Ames spores. Other PA plasmids, which contained the natural PA promoter regulatory elements, produced less PA, induced lower guinea pig anti-PA titers and resulted in less protection. Another study showed PA expression could be dramatically increased when transcribed from *B. amyloliquefaciens* amylase promoter [102]. PA expressed from this promoter, rather than the native PA promoter, in a live vaccine provided guinea pigs superior protection against Vollum spore challenge. Barnard et al. [140] found that spores of a *B. anthracis* strain cured of both virulence plasmids and transformed with plasmid encoding PA could protect some guinea pigs against intramuscular Ames challenge, but better protection and anti-PA titers were achieved if pXO2 was also present in the vaccine. A single intramuscular dose of this vaccine was fully protective in guinea pigs challenged intramuscularly and rabbits challenged by aerosol route with the Ames strain [127].

A disadvantage of replacing pXO1 with a PA-expression plasmid is that other immunogens on pXO1 are absent. Immune responses to LF and EF can contribute to protection [141]. One study showed that deleting just *pag* or *lef* completely attenuated Sterne in a mouse model, and deleting *cya* reduced virulence [23]. Subsequently, it was discovered that simply putting deactivating point mutations in the toxin genes could attenuate without affecting toxin immunogenicity [142]. Guinea pigs vaccinated with spores of a strain cured of both virulence plasmids and transformed with a plasmid encoding wild-type or deactivated PA were protected against a 20 LD$_{50}$ Vollum challenge [143]. These vaccines fully protected against challenge even 50 weeks post-vaccination. Replacing *pag* with mutated *lef* resulted in partial protection. When injected intramuscularly, vaccine strains were gradually eliminated but could still be detected at the vaccine site 60 days post-vaccination and in the spleen at 30 days, consistent with other reports that spores can persist in lymphoid organs without germinating [99].

Another direction to take with live vaccines is to introduce mutations that affect bacterial fitness in the host. Two of 3000 Tn*916*-transposed UM23-1 (Sterne-like) isolates were deficient in synthesis of all three aromatic amino acids [144]. A single dose of one of these Aro$^-$ mutant bacilli protected guinea pigs against 16 LD$_{50}$ Ames [64]. Aro$^-$ mutants occasionally reverted to wild type *in vitro*, probably due to transposon excision. This instability and the presence of the transposon kanamycin resistance gene may have dampened enthusiasm for these Aro$^-$ mutants

as live vaccines. However, new sequence data and mutagenesis techniques would make it much easier to create defined stable Aro⁻ mutants without resistance antibiotic resistance. As new information comes to light, it should be possible to further attenuate live *B. anthracis* vaccines without reducing efficacy.

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