The Search for a New-Generation Human Anthrax Vaccine

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THE SEARCH FOR A NEW-GENERATION HUMAN ANTHRAX VACCINE

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author do not purport to reflect the positions of the Army or the Department of Defense.

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Anthrax is a disease primarily of herbivores, but humans can become infected through contact with infected animals or animal products. The etiological agent, *Bacillus anthracis*, possesses two primary virulence factors: a poly-D-glutamic acid capsule and an exotoxic mixture of three proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF). None of the three proteins individually possess demonstrable toxic activity; however, intravenous injection of PA + LF kills mice, rats, and guinea pigs, whereas intradermal injection of PA + EF produces edematous lesions in the skin of guinea pigs and rabbits. The combination of PA + LF is termed "lethal toxin," whereas PA + EF is referred to as "edema-producing toxin." The conventional designation for mixtures of all three components is "anthrax toxin." The genes encoding PA, EF, and LF are located on a 174-kilobase (kb) plasmid, pX01, whereas the genes responsible for capsule synthesis are on a 91 kb plasmid, pX02.

The first anthrax vaccines were developed by Pasteur and consisted of cultures of virulent anthrax bacilli which had been attenuated by growth at 42 to 43°C (10). These vaccines were widely used in livestock, often with unpredictable results, during the late eighteenth and early nineteenth centuries. They were replaced in the early 1940s by viable spore suspensions of the toxigenic, non-encapsulated, Sterne strain of *B. anthracis* (13). In the United States, the currently licensed human vaccine against anthrax (designated here as MDPH-PA) consists of aluminum hydroxide-adsorbed, supernatant material, primarily PA (11), from fermenter cultures of a toxinogenic, nonencapsulated...
strain, V770-NPl-R. Immunization with MDPH-PA requires a series of six doses within 18 mo, followed by yearly boosters. Immunization occasionally results in local pain and inflammation, and there is some evidence indicating that MDPH-PA may have diminished efficacy against certain virulent strains of B. anthracis. The need for an improved human vaccine against anthrax is apparent. Such a vaccine should combine maximum safety and efficacy with minimum reactogenicity, and should require minimal injections to achieve and maintain maximum immunity.

Protective Immunogens

Of the various antigens produced by B. anthracis, only PA, LF, and EF have a demonstrated potential as protective immunogens (5, 9). The capsule, surface polysaccharide, and other somatic components are not protective (4, 12). In a recent series of experiments (5), attenuated strains of B. anthracis, lacking either the capsule plasmid pX02, the toxin plasmid pX01, or both, were tested for their efficacy as vaccines against aerosol or intramuscular challenge with virulent anthrax spores in Hartley guinea pigs. Only those animals immunized with toxigenic, nonencapsulated (pX01+, pX02−) strains survived and demonstrated post-immunization antibody titers to PA, LF, and EF.

In another set of experiments, guinea pigs were immunized with either MDPH-PA, Sterne spore vaccine, or purified toxin components adsorbed onto aluminum hydroxide. Animals then received an aerosol challenge with anthrax spores from a virulent strain. The results are presented in Table 1. The veterinary Sterne spore vaccine was significantly more effective than MDPH-PA (P<0.05). EF and LF alone were the least effective. Similar data were obtained when the challenge was intramuscular. These and previous studies have demonstrated that: i) all efficacious, anthrax vaccines contain or
produce PA; ii) neither LF nor EF are good protective immunogens by themselves; and iii) the live Sterne spore vaccine is more efficacious than MDPH-PA.

Among numerous, "New Generation," anthrax vaccine candidates are those listed in Table 2. Considerable progress has been made on the development and testing of several prototype vaccines. Although no single candidate has been selected for exclusive study, much valuable information has been obtained.

Transposon Mutagenesis in B. anthracis

Transposable genetic elements - transposons - have become important tools in microbiological research. They can insert into bacterial DNA, often at a variety of sites, and when insertion occurs in a structural or regulatory gene, mutations are produced. By using transposon Tn10 mutagenesis Hoiseth and Stocker (3) obtained aro- mutants of Salmonella typhimurium whose growth required the aromatic amino acids, phenylalanine, tyrosine, and tryptophan (as well as 2,3-dihydroxybenzoate [DHB] and p-aminobenzoate [PAB] under very stringent nutritional conditions). These aro- mutants were avirulent and effective as live vaccines in experimental animals. As a result of their inability to synthesize DHB or PAB or to obtain them in the host, infections by the strain were self-limiting and did not require a functioning host defense system for elimination. Since transposon mutagenesis has proven useful in generating mutants for prototype live vaccines (3), we developed a transposon mutagenesis system to produce aro- mutants of B. anthracis. We sought to develop B. anthracis strains which would produce PA and other anthrax antigens, but would be safer in the immunized host than the Sterne veterinary vaccine. We used Tn916 (1), a 15-kb, "conjugative" transposon originally detected in Streptococcus faecalis DS16, encoding tetracycline resistance, precise excision from DNA, and conjugative self-transfer from one
cell to another in the absence of plasmid DNA.

Tn916 was transferred from *S. faecalis* to *B. anthracis* by mating log-phase donor and recipient cells on filter membranes. One of the tetracycline-resistant (Tc<sup>R</sup>), *B. anthracis* recipients was then used to donate Tn916 to a streptomycin-resistant (Sm<sup>R</sup>), *B. anthracis* strain. From 3000 Tc<sup>R</sup>, Sm<sup>R</sup> transconjugants tested, two *aro*<sup>-</sup> mutants were isolated which can be tested as vaccine candidates in animals. These studies suggest that transposon mutagenesis may be a useful tool for obtaining specific *B. anthracis* mutants for study as prototype live vaccines.

**Cloning and Expression of the PA Gene in *Bacillus subtilis***

In another series of experiments, we demonstrated that PA produced in the complete absence of EF and LF is a protective immunogen against an anthrax spore challenge. (Previously, even highly purified preparations of PA were found to be contaminated with small amounts of EF and LF.) The gene encoding PA was cloned into *B. subtilis* (6), and two clones, designated PA1 and PA2, were identified which produced PA in broth culture at levels equal to or greater than those produced by *B. anthracis*. The PA cloned in *B. subtilis* was identical to *B. anthracis* PA with respect to migration on sodium dodecyl sulfate-polyacrylamide gels and to serological reactivity in Western blot assays. Addition of LF or EF to PA1 and PA2 culture supernatants generated biologically active, anthrax lethal toxin or edema-producing toxin, respectively. Guinea pigs injected with up to 10<sup>9</sup> CFU of PA1 or PA2 exhibited neither mortality nor morbidity, and they were protected against an intramuscular challenge of 20 LD<sub>50</sub>s of anthrax spores. In efficacy comparison tests, the PA-producing *B. subtilis* recombinants proved equal to the current human and veterinary vaccines in protecting against challenge with virulent *B. anthracis*. 
In contrast, the parental *B. subtilis* strain carrying the cloning vector but not the PA gene afforded no protection. Since the recombinants produced no *B. anthracis* antigens other than PA, it is apparent that PA is a necessary and sufficient immunogen for protecting against anthrax infection.

**Protective immunogenicity of PA and PA fragments.**

PA has a molecular weight of 85,000 (7). Although it is known to be a protective immunogen, the specific, protective epitopes on the molecule are unknown. The specific molecular interactions of PA with the EF and LF molecules and with the host cell also remain to be elucidated. Leppla and coworkers have investigated the structure-function relationships of the three toxin components (S. H. Leppla, personal communication). In studies to define functional domains of the PA molecule, they treated PA with trypsin, generating a 20-kilodalton (kDa) (N-terminus) and a 65-kDa (C-terminus) peptide, and then separated them by high pressure liquid chromatography. These two peptides, as well as uncleaved (intact) PA, and PA that had been treated with trypsin but not separated into the 20-kDa and 65-kDa fragments, were used to immunize guinea pigs. The various preparations were either adsorbed onto aluminum hydroxide or suspended in Freund's incomplete adjuvant. Intact PA, trypsin-treated PA, and the 65-kDa fragment protected the guinea pigs from spore challenge. The 20-kDa fragment was completely ineffective as a protective immunogen. Not surprisingly, animals receiving incomplete Freund's adjuvant had significantly higher anti-PA titers (as determined by enzyme-linked immunosorbent assay) than animals which had received the aluminum hydroxide adjuvant. As demonstrated by the data in Table 1, however, titers to PA do not always directly correlate with the level of immunity to anthrax.

**Future plans.**
We will continue developing and testing both live and chemical vaccine candidates. Further studies will be conducted on the 65-kDa fragment of PA, since it appears that this peptide possesses critical, immunogenic epitopes. The use of new adjuvants to increase immunogenicity and reduce the time to immunity will also be explored. Elucidation of the protective domains on the PA molecule may lead to a new, chemical vaccine, whereas inactivation of the LF and EF genes of the Sterne spore veterinary vaccine may make the live vaccine safe for human use. The investigations will demand considerable research effort, but the use of modern biotechnological tools will surely facilitate reaching the goal of a safe and more efficacious human vaccine against anthrax.

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References


Table 1

**Immunizing Efficacy of Various Vaccine Preparations**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Survivors/Total (%)</th>
<th>ELISA Antibody Titers To</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors/Total (%)</td>
<td>PA</td>
</tr>
<tr>
<td>Veterinary (Sterne Spore)</td>
<td>39/45 (87)</td>
<td>14,404</td>
</tr>
<tr>
<td>PA + EF</td>
<td>25/30 (83)</td>
<td>14,400</td>
</tr>
<tr>
<td>PA + LF</td>
<td>13/16 (81)</td>
<td>6,479</td>
</tr>
<tr>
<td>PA</td>
<td>25/36 (69)</td>
<td>7,759</td>
</tr>
<tr>
<td>Human (MDPH-PA)</td>
<td>20/30 (67)</td>
<td>58,710</td>
</tr>
<tr>
<td>PA + EF + LF</td>
<td>8/14 (57)</td>
<td>19,740</td>
</tr>
<tr>
<td>LF + EF</td>
<td>8/15 (53)</td>
<td>362</td>
</tr>
<tr>
<td>LF</td>
<td>3/15 (20)</td>
<td>35</td>
</tr>
<tr>
<td>EF</td>
<td>4/29 (14)</td>
<td>1,210</td>
</tr>
<tr>
<td>None</td>
<td>0/31 (0)</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

*Groups of 14 to 35 female, Hartley guinea pigs, 250 to 350 g, received three biweekly injections of the indicated vaccine preparation. Three wk after the final injection the animals received an aerosol challenge of 10 LD$_{50}$ doses of *B. anthracis* Vollum 1B spores. Animals were bled for determination of ELISA antibody titers one wk before challenge.*
Table 2

New Human Anthrax Vaccines - Possible Candidates

1. 

Bacterial agent other than \textit{B. anthracis} containing:

a) The toxin plasmid pX01 (encoding PA, EF, and LF)

b) The toxin plasmid pX01 containing one or more cross-reactive materials (CRM), which are serologically active, biologically inactive, toxin proteins

c) One or more cloned, toxin genes or CRM toxin genes

2. Viral agent (such as vaccinia) containing one or more cloned, toxin genes or CRM toxin genes

3. \textit{B. anthracis} Sterne mutant:

a) Which replicates only a few generations in the host (eg. \textit{aro}^-)

b) Which produces PA only (no EF or LF), PA + EF (or EF CRM), or PA + LF CRM + EF (or EF CRM)

4. Chemical vaccine (possibly with new adjuvant) containing:

a) PA + EF (or EF CRM)

b) PA + LF CRM

c) PA fragment