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# Immunization Studies with Attenuated Strains of *Bacillus anthracis*

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**Live, attenuated strains of *Bacillus anthracis* lacking either the capsule plasmid pXO2, the toxin plasmid pXO1, or both were tested for their efficacy as vaccines against intravenous challenge with anthrax toxin in Fischer 344 rats and against aerosol or intramuscular challenge with virulent anthrax spores in Hartley guinea pigs. Animals immunized with toxigenic, nonencapsulated ($pXO1^+$, $pXO2^-$) strains survived toxin and spore challenge and demonstrated postimmunization antibody titers to the three components of anthrax toxin (protective antigen, lethal factor, and edema factor). Immunization with two nontoxigenic, encapsulated ($pXO1^-$, $pXO2^+$), Pasteur vaccine strains neither provided protection nor elicited titers to any of the toxin components. Therefore, to immunize successfully against anthrax toxin or spore challenge, attenuated, live strains of *B. anthracis* must produce the toxin components specified by the pXO1 plasmid.**

Virulent strains of *Bacillus anthracis* possess two virulence factors (22): a poly-D-glutamic acid capsule (11) and a tripartite exotoxin (1, 24), which consists of protective antigen (PA), edema factor (EF), and lethal factor (LF). Production of the toxin is controlled by a 110-megadalton plasmid, pXO1 (formerly called pBA1) (17), and synthesis of the capsule is dependent on a 60-megadalton plasmid, pXO2 (7, 27). Thus, fully virulent strains ($pXO1^+$, $pXO2^+$) produce both toxin and capsule; $pXO1^+$, $pXO2^-$ strains produce toxin only; $pXO1^-$, $pXO2^+$ strains produce capsule only; and $pXO1^-$, $pXO2^-$ strains produce neither toxin nor capsule (7).

In the United States, the currently licensed human vaccine against anthrax (in this report designated MDPH-PA) consists of aluminum hydroxide-adsorbed culture supernatant material, primarily PA (9), from a toxigenic, nonencapsulated strain of *B. anthracis*, V770-NP1-R (19). Immunization with this vaccine requires a series of six doses over an 18-month period, followed by boosters at yearly intervals. For immunization of livestock against anthrax, a suspension of viable spores of the toxigenic, nonencapsulated Sterne strain is commonly used (26).

The first anthrax vaccines that were widely used for livestock during the late 1800s and early 1900s were the Pasteur-type vaccines (17, 29, 30), which consisted of cultures of virulent anthrax bacilli attenuated by growth at 42 to 43°C. Unfortunately, these vaccines varied greatly in their degree of attenuation (29, 30). Some cultures were sufficiently virulent to kill the animals inoculated (13), while others were so attenuated that they conferred no immunity (29, 30). An explanation for the molecular mechanism of attenuation of Pasteur-type vaccines was recently proposed by Mikesell et al., who demonstrated that culturing *B. anthracis* at 42.5°C cures the bacillus of the toxin plasmid pXO1 (17) and thus converts fully virulent $pXO1^+$, $pXO2^+$ cells to avirulent $pXO1^-$, $pXO2^+$ cells.

The studies reported here are part of the continuing research toward the development of a more protective and less reactogenic human vaccine against anthrax. Their purpose was to compare *B. anthracis* strains lacking one or both primary virulence factors (and their respective plasmids) for their efficacy in immunizing experimental animals against anthrax toxin or spore challenge and to compare the effectiveness of various vaccine administration regimens. Additionally, these studies sought to elucidate the nature of Pasteur's early anthrax vaccines and to provide an explanation for their immunizing efficacy.

## MATERIALS AND METHODS

**Bacterial strains.** The *B. anthracis* strains used in this study are listed in Table 1.

**Experimental animals.** Male Fischer 344 rats weighing 200 to 300 g were used in the toxin challenge studies. Female Hartley guinea pigs weighing 275 to 325 g were used for the spore challenge studies.

**Immunization and challenge studies.** Fischer 344 rats (five per group) and Hartley guinea pigs (seven to nine per group) were immunized intramuscularly (i.m.) either with attenuated-strain vegetative bacteria from late-log-phase R medium cultures (20) or with the anthrax PA vaccine MDPH-PA (2, 19), prepared for human use by the Michigan Department of Public Health. For anthrax toxin challenge studies, groups of rats were immunized with $2 \times 10^7$ CFU of *B. anthracis* Sterne, Pasteur 4229, ΔVollum-1B-1, ΔVollum-1B-3, ΔSterne-1, or ΔV770-NP1-R-1 cells twice weekly for 4 weeks and then given a final boost at 5.5 weeks. At 6 weeks, 1 ml of R medium culture supernatant containing 62 toxic units (8) of crude Vollum-1B anthrax lethal toxin (approximately 25 μg of PA and 3 μg of LF) (6) was injected into the dorsal penile veins of the rats, and time to death (TTD) in minutes was recorded.

For i.m. spore challenge studies, several vaccination regimens with various *B. anthracis* strains were used. Each group of guinea pigs was immunized on one of the following schedules: (i) two injections, spaced 2 weeks apart, of $10^6$ CFU of *B. anthracis* Sterne; (ii) two injections, spaced 2 weeks apart, of $10^7$ CFU of *B. anthracis* ΔVollum-1B-1, Pasteur 4229, or ΔSterne-1; (iii) four biweekly injections of $10^8$ CFU of *B. anthracis* ΔTexas-1 or ΔTexas-2; or (iv) four biweekly injections of *B. anthracis* Texas, with the first injection containing 500 CFU; the second 1,500 CFU, the third 5,000 CFU, and the fourth 10,000 CFU. Two weeks

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TABLE 1. *B. anthracis* strains

| Strain | Primary virulence factors | Plasmids[a] | Source |
|---|---|---|---|
| Vollum-1B | Toxin, capsule | pXO1, pXO2 | USAMRIID[b] |
| Sterne | Toxin | pXO1 | USAMRIID |
| Texas | Toxin | pXO1 | R. D. Welsh[c] |
| ΔVollum-1B-1 | Capsule | pXO2 | Derived from Vollum-1B[d] |
| ΔVollum-1B-3 | Capsule | pXO2 | Derived from Vollum-1B[d] |
| Pasteur 4229 | Capsule | pXO2 | ATCC[e] |
| Pasteur 6602 | Capsule | pXO2 | ATCC |
| ΔV770-NP1-R-1 | None | None | Derived from USAMRIID strain V770-NP1-R[d] |
| ΔSterne-1 | None | None | Derived from Sterne[d] |
| ΔTexas-1 | None | None | Derived from Texas[d] |
| ΔTexas-2 | None | None | Derived from Texas[d] |

[a] Plasmid analysis was done by Green et al. (7) and B. Ivins (unpublished observations).
[b] U.S. Army Medical Research Institute of Infectious Diseases.
[c] Bovine isolate, Texas A&M University, College Station.
[d] Passaged daily for 10 days at 42.5°C and then purified from isolated colonies twice.
[e] American Type Culture Collection, Rockville, Md.

after the final immunization, all animals received an i.m. injection of 1,500 (approximately 30 times the i.m. 50% lethal dose [$LD_{50}$]) Vollum-1B spores in phosphate-buffered saline (PBS). TTD in days was recorded, and harmonic mean TTD was calculated to provide a better indication of the overall survival of each group of guinea pigs (3, 4). For aerosol spore challenge studies, groups of guinea pigs were immunized with *B. anthracis* Sterne, ΔVollum-1B-3, ΔTexas-1, ΔSterne-1, Pasteur 4229, or Pasteur 6602 bacteria in four biweekly doses of $10^7$ CFU each or with MDPH-PA in three biweekly doses of 0.5 ml. Two weeks after the final immunization, the guinea pigs were placed in a modified Henderson aerosol apparatus (12, 16, 21) for 10 min and allowed to inhale either $1.58 \times 10^6$ (26 times the aerosol $LD_{50}$) or $2.09 \times 10^6$ (35 times the aerosol $LD_{50}$) virulent Vollum-1B spores. TTD in days was recorded, and the harmonic mean TTD was calculated.

**Serological studies.** PA, EF, and LF were purified from R medium culture supernatants of *B. anthracis* Sterne as previously described (14). Antibody titers in postimmunization sera from rats and guinea pigs were determined for antibody against the three toxin components by enzyme-linked immunosorbent assay (ELISA). For the assays, microtiter plate (Linbro) wells were coated with PA, EF, or LF (100 μl of a 1-μg/ml solution in 0.05 M sodium borate, pH 9.5). After incubation for 4 h at 37°C or overnight at 4°C, 160 μl of either bovine serum albumin or gelatin (1 mg/ml in 5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]–0.15 M NaCl, pH 7.5) was added to each well, and the plates were reincubated for 30 min at 25°C. The wells were washed twice with 300 μl of PBST (0.85% sodium chloride, 10 mM sodium phosphate, 0.05% Tween 20). A 150-μl amount of PBST plus 5% (vol/vol) fetal calf serum (PBSTF) or 0.1% (wt/vol) gelatin (PBSTG) was added to each well. Fifty microliters of each serum sample to be tested was added to the first well of a row and then serially diluted up to 1:4 down the row. The plates were incubated overnight at 4°C and then washed twice with PBST. For guinea pig sera, each well received 100 μl of horseradish peroxidase protein A conjugate in PBST (final conjugate concentration, 1 μg/ml). For rat sera, each well received 100 μl of goat anti-rat horseradish peroxidase conjugate in PBSTG (final conjugate concentration, 1 μg/ml). Plates were incubated at 25°C for 60 min and then washed five times with PBST. Substrate [2,2'-azino-*bis*-(3-ethylbenzthiazadine-sulfonic acid)] (Sigma Chemical Co.) was dissolved in 0.1 M sodium citrate, pH 4.0, to a concentration of 1 mg/ml. Hydrogen peroxide (0.003%) was then added, and the substrate plus $H_2O_2$ solution was dispensed into the wells in 100-μl amounts. After incubation for 30 min, reactions were terminated by addition of 10% sodium dodecyl sulfate sulfate, 50 μl per well. Absorbance at 414 nm was determined with a Dynatech MR580 ELISA reader. Absorbance values which exceeded negative control values by more than 2.1-fold were scored as positive (28). The mean titers of sera from immunized animals compared with those from unimmunized controls were statistically analyzed by one-way analysis of variance and Fisher's least significant difference test (25).

To demonstrate that animals vaccinated with the various attenuated strains had received sufficient antigenic stimulus to engender an immune response, postvaccination sera from the guinea pigs were examined for activity in a *B. anthracis* whole-cell ELISA system. Experimental conditions were identical to those described above, with two exceptions: (i) $10^7$ log-phase Sterne strain cells in PBS were added to each well of a 96-well tissue culture plate (Costar) that was then dried overnight at 25°C and heated to 60°C for 30 min to fix the cells to the wells; and (ii) rather than serial dilutions, a single 1:4 dilution of each serum sample was assayed. ELISA absorbance values for serum samples from vaccinated animals were compared with values for sera from unvaccinated control animals. Statistical analyses were performed as described above.

## RESULTS

**Immunization of rats against toxin challenge.** Fischer 344 rats are the most anthrax toxin-sensitive animals known (1, 6, 8). When these rats were immunized with Sterne ($pXO1^+$, $pXO2^-$) strain bacteria, they were completely protected from lethal toxicity and exhibited significant titers to PA, LF, and EF ($P < 0.05$) (Table 2). Rats immunized with strains containing only pXO2 or with strains containing neither plasmid, however, showed no evidence of protection; they neither survived nor exhibited an extended TTD. Furthermore, no postvaccination titers to PA, LF, or EF could be demonstrated in these animals.

**Immunization of guinea pigs against i.m. spore challenge.** Since the toxigenic, nonencapsulated Sterne strain was an effective live vaccine against toxin challenge, we decided in the i.m. spore challenge studies to test another $pXO1^+$, $pXO2^-$, Texas, and two of its plasmid-cured derivatives, ΔTexas-1 and ΔTexas-2. Texas is an isolate of *B. anthracis* that, like the Sterne strain, produces all three toxin components but does not produce a capsule. Several different

TABLE 2. Immunization of rats against toxin challenge[a]

| Immunizing strain (plasmid content) | Serological response[a] | | | Response to toxin challenge | |
|---|---|---|---|---|---|
| | Anti-PA | Anti-LF | Anti-EF | Survival (%) | TTD (min)[b] |
| None | <4 | <4 | <4 | 0 | 76.0 ± 2.1 |
| Sterne (pXO1) | 6,400[c] | 2,016[c] | 635[c] | 100 | |
| Pasteur 4229 (pXO2) | <4 | <4 | <4 | 0 | 71.8 ± 2.2 |
| ΔVollum-1B-1 (pXO2) | <4 | <4 | <4 | 0 | 67.3 ± 2.1 |
| ΔVollum-1B-3 (pXO2) | <4 | <4 | <4 | 0 | 67.4 ± 1.5 |
| ΔSterne-1 (none) | <4 | <4 | <4 | 0 | 68.8 ± 2.9 |
| ΔV770-NP1-R-1 (none) | <4 | <4 | <4 | 0 | 67.2 ± 1.1 |

[a] Reciprocal geometric mean ELISA titers of prechallenge sera.
[b] Arithmetic mean ± standard deviation.
[c] Statistically significant versus unvaccinated controls ($P < 0.05$).

immunization regimens were used to determine whether the size or number of doses affected the guinea pigs' responses to spore challenge. Immunization with Sterne strain bacteria resulted in complete protection for the animals (Table 3). Immunization with the Texas strain was also completely protective. No animals vaccinated with *B. anthracis* bacteria lacking pXO1 survived challenge or had significant titers ($P < 0.05$) to PA, LF, or EF. Animals immunized with the Texas strain demonstrated titers to all three toxin components. Sterne strain-immunized guinea pigs had significant titers to PA and LF but not EF.

**Immunization of guinea pigs against aerosol spore challenge.** Although vaccination with *B. anthracis* strains lacking pXO1 did not provide protection against intravenous toxin challenge or i.m. spore challenge, it remained possible that vaccination with such strains might afford protection against an aerosol spore challenge. Although vaccination with either the MDPH-PA vaccine or live Sterne strain bacteria afforded substantial protection to aerosol-challenged guinea pigs, immunization with strains lacking pXO1 neither afforded survival nor extended the mean TTD (Table 4). Significant titers ($P < 0.05$) to the three toxin components were evident only in sera from animals vaccinated with Sterne cells or MDPH-PA.

**Serological response to immunization.** Postvaccination sera from all groups of guinea pigs immunized with the various *B. anthracis* strains were compared in the whole-cell ELISA system with sera from an unimmunized control group. Sera from vaccinated animals, regardless of the immunization regimen, demonstrated ELISA absorbance values significantly higher ($P < 0.01$) than did those of the unvaccinated control group (data not shown). Thus, the immunization regimens for the various strains were sufficient in all cases to stimulate an antibody response to cell surface antigens, although the response in animals receiving $pXO1^-$ strains was not protective.

## DISCUSSION

Since the report by Lincoln et al. (15) in 1967 that the susceptibility of an animal species to anthrax infection is inversely proportional to its susceptibility to anthrax toxin, it has been apparent that any successful anthrax vaccine must be able to protect against both toxin and spore challenge. From the data in this study that compared the relative immunizing efficacy of 10 attenuated strains of *B. anthracis* (Table 1), it is clear that vaccination with bacteria lacking the pXO1 plasmid confers no protection against challenge by either toxin or spores. This finding is in contrast to findings for immunization with nonencapsulated, toxin-producing ($pXO1^+$, $pXO2^-$) strains such as Sterne or Texas, which are fully protective under a variety of immunizing regimens, i.e., several large doses, a few small doses, or a series of graded doses. Also clear from these data and from previous observations (7) is that fully virulent *B. anthracis* strains must possess both the toxin plasmid pXO1 and the capsule plasmid pXO2. Furthermore, both the toxin and the capsule virulence factors must be expressed for any strain to be clinically significant as a nonopportunistic pathogen.

The finding that antibodies to PA, LF, and EF were elicited only in animals vaccinated with strains containing pXO1 provides further evidence that functional toxin component structural genes are not present on the *B. anthracis* chromosome, but only on pXO1. Immunization with the MDPH-PA human vaccine elicited antibodies to EF and LF as well as PA, indicating that the human "PA" vaccine in fact contains all three toxin components. The influence of

TABLE 3. Immunization of guinea pigs against i.m. spore challenge

| Immunizing strain (plasmid content) | Serological response[a] | | | Response to spore challenge | |
|---|---|---|---|---|---|
| | Anti-PA | Anti-LF | Anti-EF | Survival (%) | TTD (days)[b] |
| None | <4 | <4 | 4 | 0 | 2.24 |
| Sterne (pXO1) | 25,600[c] | 4,526[c] | 23 | 100 | |
| ΔVollum-1B-1 (pXO2) | 8 | <4 | 5 | 0 | 2.55 |
| Pasteur 4229 (pXO2) | <4 | <4 | 4 | 0 | 2.34 |
| ΔSterne-1 (none) | <4 | <4 | 4 | 0 | 2.22 |
| Texas (pXO1) | 33,779[c] | 6,400[c] | 8,444[c] | 100 | |
| ΔTexas-1 (none) | <4 | <4 | <4 | 0 | 2.94 |
| ΔTexas-2 (none) | <4 | <4 | 5 | 0 | 2.42 |

[a] Reciprocal geometric mean ELISA titers of prechallenge sera.
[b] Harmonic mean TTD.
[c] Statistically significant versus unvaccinated controls ($P < 0.05$).

TABLE 4. Immunization of guinea pigs against aerosol spore challenge

| Immunogen (plasmid content) | Serological response[a] | | | Challenge dose ($LD_{50}$) | Response to spore challenge | |
|---|---|---|---|---|---|---|
| | Anti-PA | Anti-LF | Anti-EF | | Survival (%) | TTD (days)[b] |
| None | <4 | <4 | <4 | 26 | 0 | 3.11 |
| MDPH-PA | 64,508[c] | 3,311[c] | 49[c] | 26 | 71 | 20.47 |
| Sterne (pXO1) | 16,127[c] | 8,064[c] | 504[c] | 26 | 100 | |
| ΔVollum-1B-3 (pXO2) | 4 | <4 | <4 | 26 | 0 | 3.00 |
| ΔTexas-1 (none) | 4 | 6 | 4 | 26 | 0 | 3.53 |
| ΔSterne-1 (none) | <4 | 4 | <4 | 26 | 0 | 3.86 |
| None | <4 | <4 | <4 | 35 | 0 | 2.80 |
| Pasteur 4229 (pXO2) | <4 | <4 | <4 | 35 | 0 | 2.33 |
| Pasteur 6602 (pXO2) | <4 | <4 | <4 | 35 | 0 | 2.80 |

[a] Geometric mean ELISA titers of prechallenge sera.
[b] Harmonic mean TTD.
[c] Statistically significant versus unvaccinated controls ($P < 0.05$).

the EF and LF components on the immunizing efficacy of the MDPH-PA vaccine as well as the efficacy of pure PA as an immunogen remains unknown. Clearly, however, these two questions must be resolved before any new human vaccine is developed. The protection and serological studies we report here indicate that to protect animals against a large anthrax toxin or spore challenge, one or more toxin components must be present in a chemical vaccine or produced by a live vaccine.

The finding that vaccination with $pXO1^-$, $pXO2^+$ *B. anthracis* strains, which produce capsule but not toxin, was not protective supports previous reports that the D-glutamyl polypeptide capsule of the organism is not an important immunogen (10, 23). The finding, however, does not explain how Pasteur's heat attenuation regimen, which causes the selective loss of pXO1 (7, 17), produced a successful live anthrax vaccine. It does emphasize the apparent paradox between Pasteur's original vaccine strains and the Pasteur-type strains available from the American Type Culture Collection, which are completely ineffective as immunogens. The current hypothesis to explain Louis Pasteur's mechanism of attenuation of *B. anthracis* is that by growing virulent cells at high temperatures, he induced the loss of the pXO1 toxin plasmid (17), resulting in an increased proportion of $pXO1^-$, $pXO2^+$ cells and a decreased proportion of virulent $pXO1^+$, $pXO2^+$ cells in the cultures. Since our studies have clearly demonstrated that pure cultures of $pXO1^-$, $pXO2^+$ *B. anthracis* cells are not effective live vaccines, we believe that the efficacy of Pasteur's vaccines can be attributed to the presence of small numbers of $pXO1^+$, $pXO2^+$ bacteria and that subclinical infection by these bacteria would have induced a protective immune response. Evidence that Pasteur's vaccine did indeed contain virulent cells was provided by Pasteur himself. He demonstrated that serial passage of the attenuated cultures through day-old guinea pigs restored virulence (5), apparently by selecting for the virulent bacilli present. The fact that the Pasteur-type strains from the American Type Culture Collection contain only the pXO2 plasmid (7), whereas those from the Japanese collection contain both plasmids (27), must reflect the different results obtained when pure culture technique was applied to Pasteur's mixed cultures without recognition that a mixture was present.

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