Vaccination against Anthrax with Attenuated Recombinant Strains of Bacillus anthracis That Produce Protective Antigen

JOHN P. BARNARD† AND ARTHUR M. FRIEDLANDER*

Division of Bacteriology, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701-5011

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The protective efficacy of several live, recombinant anthrax vaccines given in a single-dose regimen was assessed with Hartley guinea pigs. These live vaccines were created by transforming AANR and ASterne, two nonencapsulated, nontoxicogenic strains of Bacillus anthracis, with four different recombinant plasmids that express the anthrax protective antigen (PA) protein to various degrees. This enabled us to assess the effect of the chromosomal background of the strain, as well as the amount of PA produced, on protective efficacy. There were no significant strain-related effects on PA production in vitro, plasmid stability in vivo, survival of the immunizing strain in the host, or protective efficacy of the immunizing infection. The protective efficacy of the live, recombinant anthrax vaccine strains correlated with the anti-PA antibody titers they elicited in vivo and the level of PA they produced in vitro.

A major factor in the virulence of Bacillus anthracis is its secretion of two binary toxins, lethal toxin and edema toxin (35, 46). These toxins possess a common cell receptor-binding (B) component but have distinct biochemically active (A) components. Lethal toxin consists of the cell-binding component, protective antigen (PA) (9), plus an A protein, lethal factor (LF) (28). Likewise, edema toxin is comprised of the same B protein, PA, plus a second A protein, edema factor (EF) (28). All three of these toxin proteins are encoded on a naturally occurring, 184-kb plasmid known as pXO1 (28, 34, 35, 46). A third virulence factor is the antiphagocytic poly-N-glutamic acid capsule encoded on a separate 90-kb plasmid known as pXO2 (13, 48).

PA binds to a cell surface receptor, where it is proteolytically activated (26, 41), creating a site for EF or LF binding. Once assembled, the toxin complex is internalized by receptor-mediated endocytosis (11, 12). PA serves as a carrier to facilitate entry of LF and EF into the host cell cytoplasm (10, 30, 41). Consistent with the central role of PA in anthrax toxin action, vaccination with PA alone can induce protective immunity to anthrax (23).

The anthrax vaccine currently licensed for human use in the United States is composed of a sterile culture supernatant of the Sterne veterinary vaccine is used in humans in the former Soviet Union (39) although reactogenicity may be a problem (42). Other efforts have focused on the creation of live, recombinant anthrax vaccines by using B. subtilis, vaccinia virus, or Salmonella typhimurium as a vector to express the cloned PA gene in the vaccinated host (7, 17, 18, 20, 22, 49). However, no attempts have been made to create a recombinant, live anthrax vaccine by using small, high-copy-number plasmids in B. anthracis to obtain enhanced expression of the PA gene.

We report here the construction of three new gram-negative/gram-positive shuttle vectors that express the B. anthracis PA gene alone in two nontoxicogenic, nonencapsulated anthrax strains. Our objective was to assess these recombinant strains for the ability to serve as live anthrax vaccines and to test the most promising strains as one-shot vaccines in guinea pigs. A derivative of one of these strains may fulfill our goal of replacing the current human anthrax vaccine with a safer, efficacious, and more easily administered vaccine effective in a single dose.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1.

Enzymes. Restriction endonuclease BstYI was obtained from New England Biolabs Inc. (Beverly, Mass.). All other nucleases, proteases, phosphatases, and ligases were from Gibco BRL (Grand Island, N.Y.) and were used as recommended by the suppliers.

Experimental animals. Female Hartley guinea pigs weighing 400 to 450 g at the start of vaccination were obtained from Charles River Laboratories (Wilmington, Mass.). In conducting the research described in this report, we adhered to the Guide for the Care and Use of Laboratory Animals (6) as promulgated by the Institute of Laboratory Animal Resources, National Research Council. Our facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

DNA extraction and purification. Plasmid DNA was extracted from Escherichia coli by the boiling method (31); resuspended in 10 mM Tris-1 mM EDTA (pH 8.0) (all chemicals were purchased from Sigma Chemical Co. [St. Louis, Mo.] unless otherwise noted), mixed with 8 volumes of saturated LiCl, and purified with a CirclePrep kit (Bio 101, Inc., La Jolla, Calif.). B. subtilis plasmid DNA was extracted in identical fashion, except that cells were preincubated for 30 min at 37°C with fivefold more lysozyme. B. anthracis plasmid DNA was prepared as previously described (24), except that E buffer and lysis buffer contained 15% (wt/vol) sucrose, samples were rapidly heated to 60°C in a boiling water bath and lysed for 1 h, and lysis was terminated by adding 1/4 volume of 2 M Tris (pH 7) containing 3-mg/ml protease K followed by 30 min of incubation at 37°C.

Construction of shuttle vectors expressing anthrax PA. Plasmids pBLKSPPA in E. coli and pC194 and pUB110 in B. subtilis were prepared as described above.
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source (reference)(l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. anthracis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>∆ANR</td>
<td>Non-toxicogenic (pX01⁻) non-capsulated (pX02⁻)</td>
<td>USAMRIID*</td>
</tr>
<tr>
<td>ΔSterne-1</td>
<td>Non-toxicogenic, non-capsulated</td>
<td></td>
</tr>
<tr>
<td>Ames</td>
<td>PA⁺, LF⁺, EF⁺ (pX01⁺) Cap⁺ (pX02⁺)</td>
<td>USD*A</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>F⁻ mcrB mrr hsdS20 recA13^* dam-13; Tn5 dcm-6 hrdA2 mcrA mcrB1</td>
<td>Gibco BRL*</td>
</tr>
<tr>
<td>GM2163</td>
<td>S. Leppa, NIH*</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pC194</td>
<td>Cm' repH</td>
<td>USAMRIID (14)</td>
</tr>
<tr>
<td>pUB110</td>
<td>Kan' Pml' repI</td>
<td>USAMRIID (30, 31)</td>
</tr>
<tr>
<td>pPA102</td>
<td>pUB110 with PA gene inserted</td>
<td>USAMRIID (19)</td>
</tr>
<tr>
<td>pBLKSPPA</td>
<td>pPA gene inserted into pBluescript (28)</td>
<td>This study</td>
</tr>
<tr>
<td>pJB1</td>
<td>pBLKSPPA fused with pC194</td>
<td>This study</td>
</tr>
<tr>
<td>pJB2</td>
<td>pBLKSPPA fused with pUB110</td>
<td>This study</td>
</tr>
<tr>
<td>pJB3</td>
<td>pBLKSPPA fused with pUB110 in opposite orientation to pJB2</td>
<td>This study</td>
</tr>
</tbody>
</table>

*USAMRIID, U.S. Army Medical Research Institute of Infectious Diseases.

**USD, U.S. Department of Agriculture, Ames, Iowa.

†Grand Island, N.Y.

‡NIH, National Institutes of Health.

pC194 was digested with BsrYI and pUB110 and pBLKSPPA were digested with BamHI. Plasmid pBLKSPPA was then treated with phosphatase. pBLKSPPA was mixed with pC194 or pUB110, and the vectors were fused by ligation with T4 DNA ligase before transformation into E. coli HB101.

Transformation and selection of E. coli and B. anthracis. E. coli HB101 and GM2163 were transformed by a 45-s heat shock in calcium-containing medium previously described (31). E. coli transformants were initially selected on L agar plates containing 5 μg of chloramphenicol (CM)/ml for pC194-based vectors or 12.5 μg of kanamycin (KAN)/ml for pUB110-based vectors with subsequent selection including 100 μg of ampicillin/ml for shuttle vectors. For successful transformation into B. anthracis, plasmids had to be demethylated by passage through GM2163 (38). B. anthracis strains were grown in BYGT medium (1.9% brain heart infusion extract, 0.5% yeast extract, 0.2% glucose, 0.4% glycerol, 0.1 M Tris [pH 8.0]) (all dehydrated culture media were purchased from Difco Laboratories, Detroit, Mich.) to an optical density at 590 nm of 0.2 to 0.4 absorbance units and transformed by electroporation in 0.4-cm cuvettes at 10 kV/cm as previously described (8) with 0.1 μg of KAN/ml added during recovery to induce drug resistance gene expression from pC194-based vectors. Anthrax transformants were selected on L agar plates containing 10 μg of CM/ml for pC194-based vectors or 25 μg of KAN/ml for pUB110-based vectors. After each transformation, vector integrity was verified by analytical restriction digestion with Clal, HindIII, BglII, or EcoRI.

Preparation of B. anthracis culture supernatants. Five-milliliter aliquots of FA medium (3.5% tryptone, 2% yeast extract (diluted overnight against water), 2.0% s-histidine, 0.8% NaHPO₄, 0.4% KH₂PO₄, 0.74% NaCl) were inoculated into a medium (3.3% tryptone, 2% yeast extract [dialyzed overnight against water], 0.5% yeast extract, 0.2% glucose, 0.4% glycerol, 0.1 M Tris [pH 8.0]) (all dehydrated culture media were purchased from Difco Laboratories, Piscataway, N.J.) in water, as previously described (22), and washed once more with water. They were then sedimented to a pellet at 10,000 x g for 20 min at 6°C.

Vaccination and challenge of guinea pigs. Hartley guinea pigs in groups of 17 to 20 each received one 0.5-ml dose i.m. of a live vaccine strain containing 10⁹ spores in PBS with 0.1% gelatin or one 0.5-ml dose of PBS-gelatin alone as a control. Six weeks after vaccination, guinea pigs were challenged i.m. in the thigh with 200,000 spores of the virulent B. anthracis Ames strain (10⁹ spores = 1.0% lethal dose [LD₅₀] which had been prepared and stored as previously described (22). Deaths of animals were recorded for 3 weeks after challenge.

Statistical analysis. Product limit survival estimates involving time to death after challenge were used to compare five vaccines to a PBS control and to each other. The product limit survival estimates were calculated by using the Lifetest procedure of the SAS statistical software package (SAS Institute Inc., Cary, N.C.). The association between protective efficacy and anti-PA antibody response was also determined by the Lifetest procedure.

RESULTS AND DISCUSSION

Construction and stability of shuttle vectors containing the PA of B. anthracis. Three new gram-negative/gram-positive shuttle vectors containing the anthrax protective antigen gene were constructed in E. coli and transformed into the ∆ANR and ΔSterne strains of B. anthracis (Table 1), both of which lacked pX01 and pX02, as described in Materials and Methods and Fig. 1. In addition to their ability to replicate in E. coli and Bacillus species, all three of these vectors contained six unique cloning sites in the multiple cloning site derived from pBluescript.

Although the replication of these shuttle vectors in B. anthracis was under the control of pC194 or pUB110, both of which were originally isolated from Staphylococcus aureus (15, 32, 33), none of the vectors showed any sign of deletion or rearrangement in B. anthracis (data not presented). Some of the vectors were also well maintained in the absence of antibiotic selection pressure in vitro (Table 2), a feature which is essential to their use in a live vaccine since they must be maintained in the host, where no selection pressure is present.

Also shown in Table 2 is the stability of plasmid pPA102, pUB110 derivative that contains the PA gene but can only replicate in gram-positive species (Table 1). This vector was also well maintained in the absence of antibiotic selection pressure.

Characterization of PA production by the recombinant anthrax strains. All of the recombinant anthrax strains produced...
FIG. 1. Construction of shuttle vectors for expression of PA in B. anthracis. Plasmid pBLKSPPA is a pBluescript vector which contains the anthrax PA gene and its endogenous promoter inserted into a multiple cloning site on a ColI-BamHI fragment. This vector was digested with BamHI, treated with phosphatase, and ligated to gram-positive vectors pUB110 and pC194, which had been digested with BamHI and BuYI, respectively, to create PA-producing shuttle vectors pJB1, pJB2, and pJB3.

and secreted mature PA of approximately 83 kDa, as determined by reactivity with anti-PA antibodies and comigration with purified PA (Fig. 2B, lane 5). In all strains, at least 80% of the PA was present as the intact 83-kDa species. The additional bands in some samples (Fig. 2A, lanes 2 and 5) were the degradation products of PA as determined by reactivity with anti-PA antibodies.

The amounts of PA produced by the various strains were determined by a macrophage lysis assay, which measures biological activity of PA by comparison with known amounts of purified PA (Table 3). Levels of PA production were significantly greater from the pUB110-based plasmids than from the pC194-based plasmids (Table 3). The differences in PA production did not appear to be due to altered growth characteristics of the strains because all strains grew to approximately the same final optical density at 590 nm (data not presented). In some cases, variations in the amount of PA produced may have been due to transcriptional interference. For instance, the accessory repH and PA genes on pJB1 were transcribed in opposite directions, and pJB2 and pJB3 were identical, except for the orientation of the pUB110 insert (Fig. 1). Transcriptional interference may also explain the reduced stability of pJB2 relative to pJB3 in ΔSterne (Table 2).

The data revealed that the host background had no demonstrable effect on the level of PA production by these plasmids. Plasmids pPA102, pJB1, and pJB3 produced equivalent levels of PA, whether in ΔANR or in ΔSterne (Table 3).

The B. anthracis strains containing pPA102 produced 49 to 55 µg of PA/ml in vitro (Table 3). This was more than the 15 to 20 µg of PA/ml produced with added bicarbonate by the Sterne strain containing native plasmid pX01 (20, 28) but was equivalent to the reported 42 µg/ml produced by B. subtilis containing pPA102 (20). With the native plasmid, toxin production is regulated by a trans activator encoded on the pX01 toxin plasmid and is increased in the presence of bicarbonate.

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Parenta</th>
<th>Amt (µg/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔANR(pPA102)</td>
<td>pUB110</td>
<td>49.2 ± 21.7</td>
</tr>
<tr>
<td>ΔSterne(pPA102)</td>
<td>pUB110</td>
<td>54.9 ± 16.7</td>
</tr>
<tr>
<td>ΔANR(pJB1)</td>
<td>pC194</td>
<td>2.74 ± 0.52</td>
</tr>
<tr>
<td>ΔSterne(pJB1)</td>
<td>pC194</td>
<td>2.34 ± 0.65</td>
</tr>
<tr>
<td>ΔSterne(pJB2)</td>
<td>pUB110</td>
<td>21.4 ± 3.1</td>
</tr>
<tr>
<td>ΔANR(pJB3)</td>
<td>pUB110</td>
<td>6.35 ± 0.82</td>
</tr>
<tr>
<td>ΔSterne(pJB3)</td>
<td>pUB110</td>
<td>7.06 ± 1.17</td>
</tr>
</tbody>
</table>

a Parent plasmid of the shuttle vector that directs plasmid replication and drug resistance in the B. anthracis host.
b Results are the mean ± the standard error of the mean of three or four experiments measuring PA release into the culture supernatant during a 16-h incubation.
(2, 27, 47). However, pX01 was not present in the recombinant anthrax strains (Table 1), and the high levels of PA production reported in Table 3 were measured in the absence of bicineptate.

These high PA production level may have been due to a copy number effect. While plasmid copy number was not measured, pUB110 is known to be a multicopy plasmid in B. subtilis (5), whereas pX01 is present in only one or two copies per cell of B. anthracis (25). In addition, pPA102 has undergone a spontaneous deletion which may have removed a negative regulatory region upstream of the PA gene (20). Alternatively, the deletion may have allowed readthrough from the adjacent phleomycin resistance (pm\textsuperscript{r}) gene, derived from pUB110, into the PA gene to increase PA transcription (Fig. 1) (20). Further studies are needed to address these possibilities. This deletion has not occurred in pJB2 or pJB3, and the pm\textsuperscript{r} gene is far removed from the PA gene (Fig. 1).

Persistence of the recombinant B. anthracis strains and plasmid stability in vivo. The recombinant PA-producing strains are highly attenuated. They lack the antiphagocytic poly-n-glutamic acid capsule, the ability to produce functional toxins, and other possible virulence factors encoded on pX01 and pX02 (Table 1). Neither death nor obvious illness occurred when animals received a dose of 10\textsuperscript{8} ΔANR or ΔSterne spores containing the recombinant plasmids (data not presented).

To ascertain if these strains were still capable of prolonged survival in the host, doses of 10\textsuperscript{9} spores of three of the strains were injected i.m. into guinea pigs. The isolated, inoculated muscles were homogenized and cultured 1, 3 and 7 days later. Bacteria from the animals were also tested for retention of plasmid stability in vivo.

Differences in survival of the strains in vivo, suggesting that neither host background nor level of PA production affected persistence (Fig. 3). These results are consistent with observations of Pezar et al., who showed that persistence of Sterne strain-derived mutants in the mouse footpad is unrelated to the production of PA (35).

Protective efficacy of the live, recombinant anthrax vaccines. With plasmid stability (Table 2) and PA production (Table 3) as criteria, the five most promising recombinant anthrax strains were selected for use in a live-vaccine trial with the guinea pig, the animal model most frequently used in recent studies to evaluate anthrax vaccines (21). Each guinea pig was vaccinated once with 10\textsuperscript{9} spores of a selected strain and challenged i.m. 6 weeks later with 2,000 LD\textsubscript{50} of Ames, a fully virulent anthrax strain.

While only three of the live vaccines partially protected animals from death after this rigorous challenge (Table 5), detailed analysis of survival estimates based on time to death after challenge (Fig. 4) demonstrated that all of the live vaccines provided statistically significant protection relative to the PBS control (Table 5). This analysis also revealed that the best protection was induced by ΔSterne(pPA102) and ΔANR (pPA102) and that there was no statistically significant difference between these two strains. A live anthrax vaccine containing a modified pX01 plasmid that produces only the PA component of anthrax toxin provides mice with similar levels of protection as a live vaccine containing a modified pX01 plasmid that produces PA.

TABLE 4. Plasmid stability in vivo\textsuperscript{a}

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔANR(pPA102)</td>
<td>108 ± 8\textsuperscript{b}</td>
<td>113 ± 38</td>
<td>70 ± 30</td>
</tr>
<tr>
<td>ΔSterne(pPA102)</td>
<td>96 ± 8</td>
<td>98 ± 4</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>ΔSterne(pJB2)</td>
<td>98 ± 14</td>
<td>95 ± 21</td>
<td>127 ± 12</td>
</tr>
</tbody>
</table>

\textsuperscript{a} B. anthracis strains were inoculated on day 0 and isolated from the leg muscles of three guinea pigs at each time point and plated in the presence and absence of KAN to determine plasmid retention.

\textsuperscript{b} Values represent the mean ± the standard deviation from three animals.

TABLE 5. Protective efficacy of a single dose of recombinant B. anthracis against anthrax infection in guinea pigs

<table>
<thead>
<tr>
<th>Vaccination\textsuperscript{a}</th>
<th>No. survived/no. challenged (%)\textsuperscript{b}</th>
<th>Time to death (days)\textsuperscript{c}</th>
<th>P value vs PBS\textsuperscript{d}</th>
<th>Serological response\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔSterne(pPA102)</td>
<td>7/17 (41)</td>
<td>9.8 ± 1.3</td>
<td>0.0001</td>
<td>0.1534</td>
</tr>
<tr>
<td>ΔANR(pPA102)</td>
<td>4/17 (24)</td>
<td>6.0 ± 0.7</td>
<td>0.0001</td>
<td>0.3145</td>
</tr>
<tr>
<td>ΔSterne(pJB2)</td>
<td>2/19 (11)</td>
<td>5.1 ± 0.6</td>
<td>0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td>ΔSterne(pJB3)</td>
<td>0/19 (0)</td>
<td>2.8 ± 0.2</td>
<td>0.0002</td>
<td>0.6673</td>
</tr>
<tr>
<td>ΔANR(pJB3)</td>
<td>0/19 (0)</td>
<td>2.8 ± 0.2</td>
<td>0.0005</td>
<td>0.0005</td>
</tr>
<tr>
<td>PBS</td>
<td>0/20 (0)</td>
<td>2.0 ± 0.0</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Guinea pigs were vaccinated i.m. with a single dose of 10\textsuperscript{9} spores or PBS.

\textsuperscript{b} Animals were challenged with 2,000 LD\textsubscript{50} of Ames spores i.m. 6 weeks after vaccination.

\textsuperscript{c} Harmonic mean time to death ± the standard error of the mean.

\textsuperscript{d} P value versus PBS control of the survival estimate calculated using the Log Rank test from the Lifetest procedure of the SAS Statistical Software package.

\textsuperscript{e} P value versus the subsequent entry in this table [i.e., entry 2, ΔANR(pPA102), versus entry 3, ΔSterne(pJB2)] of the survival estimate calculated using the Log Rank test from the Lifetest procedure of the SAS Statistical Software package.

\textsuperscript{f} Reciprocal geometric mean anti-PA titer of prechallenge sera (determined by enzyme-linked immunosorbent assay).
protection against the attenuated Sterne strain (36). However, fully virulent strains were not tested in the latter study. In two additional experiments, ΔSterne(pPA102) gave 64% (7 survivors; 11 challenged) and 50% (6 survivors; 12 challenged) survival against a challenge dose of 100 LD₅₀ (data not shown).

The protection induced by ΔSterne(pPA102) was superior to that induced by ΔSterne(pJB2) (P = 0.0114). ΔSterne(pJB2) provided significantly better protection than ΔSterne(pJB3) (P = 0.0004) (Table 5, P value versus next entry). Likewise, ΔANR(pPA102) protected significantly better than ΔANR(pJB3) (P < 0.0001). Statistical analysis of all of the groups revealed a strong correlation between the anti-PA response induced by the strain (Table 5) and protection measured by either survival (P = 0.0001) or time to death (P = 0.0001). These differences in protective efficacy (Table 5) also correlated strongly with PA production in vitro (Table 3). Thus, the pPA102 strains that produced 49 to 55 μg of PA/ml gave the best protection, the pJB2 strain producing 21 μg of PA/ml gave moderate protection, and the pJB3 strains producing 6 to 7 μg of PA/ml gave minimal protection. Statistical analysis supported the correlation between PA production (Table 3) and both survival (P = 0.0056) and time to death (P = 0.0285). The strains with the higher PA production in vitro (Table 3) also generated the higher anti-PA titers (Table 5) (P = 0.0146). This suggests that PA production in vitro is proportional to production in vivo.

The importance of in vivo production of PA was further supported by an experiment comparing the efficacy of live versus irradiated nonviable spores. In this experiment, all guinea pigs given 10⁹ ΔSterne(pPA102) spores produced an anti-PA antibody response. Six of 12 animals challenged with 100 LD₅₀ of a virulent strain survived. In contrast, none of 12 animals inoculated with irradiated spores survived the lethal challenge and none developed an immune response to PA (data not presented).

The correlation of survival with the anti-PA antibody response, as well as with the production of PA, is most clearly seen when comparing the ΔSterne strains containing the three different plasmids. Thus, survival with ΔSterne(pPA102) was significantly greater than that with ΔSterne(pJB2), which was significantly greater than that with ΔSterne(pJB3) (Table 5). PA production in vitro was 55, 21, and 7 μg/ml, respectively, for the three strains (Table 3), while the reciprocal geometric mean anti-PA antibody titers were 2,111, 192, and 33, respectively (Table 5).

In the course of this study, there were no statistically significant differences between the ΔANR and ΔSterne strains with regard to plasmid stability in vivo (Table 4), PA production (Table 3), persistence in the host at the site of inoculation (Fig. 3), and protective efficacy (Fig. 4 and Table 5).

A single dose of either the ΔANR(pPA102) or the ΔSterne (pPA102) live vaccine was as effective (Table 5) in the guinea pig model as a single dose of the currently licensed nonliving human anthrax vaccine (23). As the primary factors that correlated with live-vaccine protective efficacy were the immune response to PA and PA production, future efforts to enhance the level of PA production may generate a live vaccine which is superior to current vaccines, even when administered at doses lower than those used here.

ACKNOWLEDGMENTS

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