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Toxin-Deficient Mutants of Bacillus anthracis Are Lethal in a Murine Model for Pulmonary Anthrax

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Bacillus anthracis, the etiologic agent of anthrax, produces at least three primary virulence factors: lethal toxin, edema toxin, and a capsule. The capsule is absolutely required for dissemination and lethality in a murine model of inhalation anthrax, yet the roles for the toxins during infection are ill-defined. We show in a murine model that when spores of specific toxin-null mutants are introduced into the lung, dissemination and lethality are comparable to those of the parent strain. Mutants lacking one or more of the structural genes for the toxin proteins, i.e., protective antigen, lethal factor, and edema factor, disseminated from the lung to the spleen at rates similar to that of the virulent parental strain. The 50% lethal dose (LD₅₀) and mean time to death (MTD) of the mutants did not differ significantly from those of the parent. The LD₅₀s or MTDs were also unaffected relative to those of the parent strain when mice were inoculated intravenously with vegetative cells. Nonetheless, histopathological examination of tissues revealed subtle but distinct differences in infections by the parent compared to some toxin mutants, suggesting that the host response is affected by toxin proteins synthesized during infection.

Prototypical virulent strains of Bacillus anthracis synthesize the anthrax toxins, lethal toxin and edema toxin, and a poly-β-glutamic acid capsule. The structural genes for the toxin proteins are located on plasmid pXO1 (182 kb; accession no. NC001496), and an operon encoding biosynthetic enzymes for capsule is found on pXO2 (96 kb; accession no. NC002146.1) (45). Evidence that massive bacteremia is not solely responsible for death during an anthrax infection led to the isolation of the three toxin proteins (43, 44) lethal factor (LF), edema factor (EF), and protective antigen (PA), encoded by lef (accession no. NC001496.1:127442-129871) (38), cya (accession no. NC001496.1:154224-156626) (27, 39, 46), and pagA (accession no. NC001496.1:133161-135455) (50, 54), respectively. These three proteins, act as A/B-type toxins (1, 28) such that PA (B or binding component) allows the translocation of either LF or EF (A or enzymatic components) into host cells to yield lethal toxin (LT) or edema toxin (ET), respectively. All three toxin genes are located noncontiguously on pXO1 but are coordinately positively regulated by AtxA, a global regulator on pXO1 (4, 9, 47).

PA is responsible for binding and toxin entry into host cells (3). Two different receptors, expressed ubiquitously on many cell types, bind the 83-kDa PA molecule (5, 42). Once PA is bound to cellular receptors, it is proteolytically cleaved to release a 20-kDa protein, allowing the residual 63-kDa protein to hepatamerize and bind three molecules of LF, EF, or both in a competitive fashion (29). The toxin complex is then endocytosed. The low pH of the endolysosome triggers PA to undergo a conformational change, form a pore, and subsequently deliver LF and EF to the cytosol (33).

Many in vitro studies have been performed to attribute enzymatic properties to LF and EF (25). These studies have demonstrated that LF is a zinc metalloproteinase that cleaves the N termini of mitogen-activated protein kinases that result in altered gene expression (7). Thus, LT can hinder the innate and adaptive immune responses, allowing for immune evasion by the bacteria. Edema factor is an adenylate cyclase that causes elevated cyclic AMP in cells and leads to skin edema observed in dying animals (34). EF also inhibits the bacterium-induced chemiluminescence response by neutrophils, demonstrating that ET hinders neutrophil oxidative metabolism and phagocytosis (30).

In vivo models used to study the role of B. anthracis toxins have predominately employed purified proteins (24, 26, 31). Moayeri et al. (24) demonstrated that LT causes hypoxic tissue injury that is fatal in a murine model. Also in a murine model, it was shown recently that ET is highly lethal at doses lower than those of LT and that ET is responsible for pathological lesions found in several tissues, including lymphocytolysis and gastrointestinal tract hemorrhage (14). The pathology suggested that administration of purified ET resulted in multiple organ failure leading to death (14). Studies utilizing purified toxins in vivo models are difficult to interpret and extrapo-
late to conditions occurring during the infection when bacilli are present, because the serum and tissue levels of toxin proteins during an infection are not known. Furthermore, the role of the toxins may be affected by other bacterial components that are missing from experiments using purified toxins.

Other animal studies have employed toxigenic, nonencapsulated Sterne strains (pXO1⁻ pXO2⁻), which are significantly attenuated in most murine models (53). Pezard et al. (34) utilized live Sterne strain toxin mutants to assess the roles of individual toxin components in vivo, using a subcutaneous murine model. The 50% lethal dose (LD₅₀) for the acapsular parent strain was 10⁶ spores per mouse, whereas the LF- and PA− mutants were not lethal with a dose of 10⁸ spores per mouse. The EF− mutant caused lethal infections in half of the mice at a dose of 10⁴ spores. Since these strains do not harbor pXO2, the results may not be germane for the role for toxin proteins during an infection with a fully virulent B. anthracis strain. We reported previously that a strain carrying both pXO1 and pXO2 but deleted specifically for the capsule bio- synthetic operon capBCADE on pXO2 was completely attenuated when spores were delivered intratracheally (i.t.) in a murine model of inhalation anthrax (12). Our data indicated that capsule was required in the murine model, but the contribution of toxins was not addressed.

Death resulting from B. anthracis infection is thought to result from a combination of massive bacteremia as well as the associated severe toxemia (18, 37). Anthrax acquired via the pulmonary route consists of two distinct stages during which lethal toxin and edema toxin might play major roles (25). The initial stage occurs in the lung and lung-associated draining lymph nodes, where the toxins may contribute to germination, bacterial survival, and/or dissemination from the lung. The second stage of infection begins with dissemination into the bloodstream, where the toxins might contribute to the ability of the bacilli to replicate and cause damage to vital organs. In this study, we employed a virulent (pXO1⁺ pXO2⁺) parent B. anthracis strain (12) and isogenic toxin gene mutants to assess the importance of the secreted toxins during the pulmonary and systemic stages (mimicked by an intravenous route of inoculation) of anthrax infection. We also examined the effects of the toxins on pathology during the systemic stage. We found that toxin production by B. anthracis is not required for death in the pulmonary or systemic murine model of anthrax, although the presence of toxins causes subtle and reproducible, histopathologically distinct features that likely add to the pathophysiology of infection. Virulence factors can have synergistic and antagonistic effects. Therefore, it was imperative to examine the role of the individual toxin components in the presence of capsule in this model.

MATERIALS AND METHODS

Animals. Female, 6-week-old BALB/c mice were purchased and maintained in a specific-pathogen-free facility at the University of New Mexico. The mice were housed in individually HEPA-filtered cages (Tecniplast, Phoeniexville, PA) with autoclaved Tek-Fresh bedding (Harlan Teklad, Madison, WI). Food and water were supplied to the mice ad libitum. Animals were allowed to acclimate to their surroundings for 1 week prior to use. The University of New Mexico Institutional Animal Care and Use Committee approved all protocols.

Strain construction. In mutants UT539, UT540, NM1, and UT541, the coding sequences for lef, cya, pagAR, and lef/cya, respectively, were replaced with antibiotic resistance cassettes by using a protocol described previously (41). For construction of the cyu-null mutant, the cyu coding sequence (accession no. NC001496:1:154224-156626) (62 bp upstream from the translational start site to 104 bp downstream from the translational stop site) was replaced with an omega-kanamycin cassette. The lef mutation was constructed by replacement of the lef coding sequence (accession no. NC001496:1:127442-129871) (167 bp downstream from the translational start site to 59 bp upstream from the translational stop site) with the omega-kanamycin resistance cassette. For construction of UT541 (cya lef), the cya gene was replaced with an omega-spectinomycin cassette (41) as described above for the cya-null kanamycin cassette. This mutation was transduced into UT539 with selection for spectinomycin resistance to generate UT541. For pagA4 mutant construction, a central portion of the pagA coding sequence (accession no. NC001496:1:133161-135545) (87 bp downstream from the deleted gene of interest in combination with oligonucleotide primers to the omega-kanamycin or omega-spectinomycin cassette. Reverse transcription-PCR confirmed that the pagA4 mutation had a polar effect on the expression of pagB (accession no. NC001496:1:139399-132328), pagC (104 bp downstream of pagA), and pagD (16, 17) and does not appear to affect virulence in a murine model, since the pagA4 mutant exhibited an LD₅₀ similar to that of the parent strain (see Results).

Spore preparation. Spore stocks were streaked onto NYB-NaCO₃ (11) plates and incubated overnight at 37°C. A few single colonies were then added to 50 ml of phage assay medium (15) in a 500-ml flask. The culture was shaken at 250 rpm and then incubated for 5 to 7 days at 30°C. Samples were examined for growth. The initial optical density (OD₆₀₀) of the culture was measured, and 10 ml of 5× growth medium was added with shaking to maintain the OD₆₀₀ at 0.4. At this density, all cultures contained 10⁷ spores/mL. The plates were incubated in an atmosphere of 5% CO₂ at 39°C for approximately 24 h to ensure maintenance of pXO2 (capsule-positive phenotype). A few colonies from each plate were used to inoculate 15 ml of Luria broth (2) containing 0.5% glycerol and antibiotics when appropriate. Cultures were then heated at 68°C for 40 min to kill any remaining vegetative cells. Spores were collected by centrifugation for 30 min at 4,000 rpm and washed three times prior to being resuspended in sterile phosphate-buffered saline (PBS). The suspension was aliquotted and frozen at −80°C. The titers of individual aliquots were determined by serial dilution and plating using an Autoplate 4000 (Spiral Biotech, Bethesda, MD).

Preparation of vegetative cells for intravenous infections. B. anthracis spores were streaked onto nutrient broth yeast agar plates (15) containing 0.8% bicarbonate and antibiotics when appropriate (kanamycin [50 μg/ml] and/or spectinomycin [100 μg/ml]). The plates were incubated in an atmosphere of 5% CO₂ at 37°C for approximately 24 h to ensure maintenance of pXO2 (capsule-positive phenotype). A few colonies from each plate were used to inoculate 1 ml of Luria broth (2) containing 0.5% glycerol and antibiotics when appropriate. Cultures were shaken at 200 rpm for approximately 12 h at 37°C in air. Cells were then subcultured in Luria broth containing 0.5% glycerol (no antibiotics; initial optical density at 600 nm of 0.1) at 37°C to an optical density at 600 nm of approximately 0.4. At this density, all cultures contained ~1×10⁹ CFU/ml. Cultures were then centrifuged for 3 min at 16,000 × g, the medium was removed, and the cells were washed with Dulbecco’s PBS (pH 7.2) (catalog no. 20012-050; Invitrogen, Carlsbad, CA) using a volume equivalent to the initial culture volume. The cells were washed two more times before resuspension in a volume equivalent to the original culture volume. Prior to infection, an aliquot of washed cells was diluted and plated on blood agar plates to obtain the final CFU/ml employed in the infection (inoculating dose).

Inoculations. All infections were carried out in an ABSL 3 containment area. Intratracheal infections were performed as described previously (21). Briefly, mice were anesthetized and restrained on a small surgical board. A small incision was made in the skin over the trachea, and the salivary gland was separated to expose trachea. A bent 30-gauge needle attached to sterile polypropylene tubing at one end and to a 1-ml syringe at the other was inserted into and parallel with the trachea, where a 50-μl spore inoculum was delivered. After inoculation, the lungs from two or three random mice were immediately harvested to determine the actual B. anthracis deposition. Intravenous infections were performed by restraining the mice and delivering 100 to 200 μl of inocula by tail vein injection using a 30-gauge needle attached to a 1-ml syringe.

LD₅₀ calculation. LD₅₀s were calculated using the method of Reed and Muench (36).
Germination and dissemination. For germination and dissemination studies, an inoculum of approximately 50,000 spores (five times the LD50 of UT500) was given to all animals. At 3, 24, 48, and 72 h, the lungs and spleens were isolated and placed in a tube containing 1 ml PBS and 250 μl of 2.5-μm-diameter silica-zirconia beads (Biospec Products, Bartlesville, OK). The samples were homogenized in a bead beater (Biospec Products). To assess germination, one half of the sample was plated immediately onto tryptic soy agar with 5% sheep blood (Remmel, Lenexa, KS) using Autoplate 4000 (Spiral Biotech, Bethesda, MD). The other half of the sample was heated at 68°C for 40 min before plating. Colony counts were enumerated using Qcount (Spiral Biotech).

Histopathology. Mice were inoculated via the intravenous route with vegetative cells of the UT500, UT539, UT540, or NM1 strain of *B. anthracis* (three mice per bacterial strain per experiment, two separate experiments), or with PBS as a control, followed by sacrifice at 18 h postinoculation. Necropsies were performed on all mice. Lungs, spleen, and liver were collected from each mouse for microscopic examination. Lungs were removed from the thorax en bloc and inflated with 10% neutral buffered formalin via a tracheal cannula. Lungs and other tissues were fixed for 24 to 72 h and subsequently trimmed for paraffin embedding. Lungs were trimmed along the edges of the left main stem bronchus and the right cranial, middle, and caudal lobar bronchi. Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin for blinded histological analysis by a board-certified veterinary pathologist. Lesions were graded on a semiquantitative scale based upon the severity and the distribution of lesions (minimal, 1; mild, 2; moderate, 3; and marked, 4). Lesion scores for the two experiments were pooled for statistical analysis. Slides from these two experiments were also Gram stained using the method of Brown and Brenn (20), and bacilli in the lung were enumerated by counting five separate fields (magnification, ×60) per mouse. Bacterial counts for the two experiments were pooled and analyzed statistically.

Statistics. A one-way analysis of variance was performed to compare the LD50 of all strains and the enumeration of the bacilli in the lungs. Contingency tables utilizing Fisher’s exact test were employed to analyze the lesion scores for the two experiments. Slides from embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin for blinded histological analysis by a board-certified veterinary pathologist. Lesions were graded on a semiquantitative scale based upon the severity and the distribution of lesions (minimal, 1; mild, 2; moderate, 3; and marked, 4). Lesion scores for the two experiments were pooled for statistical analysis. Slides from these two experiments were also Gram stained using the method of Brown and Brenn (20), and bacilli in the lung were enumerated by counting five separate fields (magnification, ×60) per mouse. Bacterial counts for the two experiments were pooled and analyzed statistically.

RESULTS

Euthanasia by CO2 affects germination of *B. anthracis* in the lung environment. We previously reported a high rate of germination initiation after spores were deposited in the lungs as determined by measuring heat-sensitive and heat-resistant CFU (12). Our data indicated significant germination in the lung but no growth within the alveolar spaces (12, 21). The high percentage of heat-sensitive cells that we identified was in contrast to results from other labs (8, 35). In the studies reported here, we systematically addressed each step of the infection and lung harvest procedures to account for the observed differences. We found that euthanasia by regulated CO2 (the IACUC-recommended method at the University of New Mexico) resulted in high rates of spores becoming heat sensitive. In contrast, pharmacologic euthanasia with avertin resulted in low numbers of heat-sensitive cells, indicating minimal levels of germination (Fig. 1).

We hypothesized that the CO2 method of euthanization altered the fluid environment of the lung by causing microvascular leakage and therefore entry of germinants. To test this hypothesis, we harvested alveolar fluid from CO2-euthanized and avertin-euthanized mice and tested each for the ability to induce spore germination in the UT500 strain. The alveolar fluid from CO2-euthanized mice was very effective at inducing spore germination (i.e., high numbers of heat-sensitive cells were found), while the alveolar fluid from avertin-euthanized mice was not (data not shown). We speculate that germination is induced by microvascular leakage induced by the CO2 euthanization procedure and accumulation of serum components in the air spaces that are capable of initiating germination. Therefore, in the studies reported here, we employed avertin euthanasia when examining germination of spores in the lung environment.

Dissemination from the lung and subsequent lethality do not require toxin synthesis. To assess whether the toxin components are required for *B. anthracis* to escape from the lung, we inoculated mice i.t. with our parent strain and toxin-deficient mutants (Table 1) and compared the doses of parent and toxin-deficient spores that killed half of the mice (LD50) (Table 2). We also calculated the mean time to death (MTD) for each strain (Table 2). The mean LD50 of the parent strain UT500 was 1.5 × 104 spores, with a MTD of approximately 2 days (5 × 104 spores/mouse). The mean LD50 of the *lef* mutant UT539, the *cya* mutant UT540, the *lef cya* mutant UT541, and the *pagAR* mutant NM1 were not significantly different from each other or from that of the parent strain (Table 2). The MTD of mice receiving the mutant strains averaged 2 days, similar to

![FIG. 1. Heat sensitivity of UT500 in the lungs of mice at 3 h postinfection when euthanized using CO2 or Avertin. The data shown are from one experiment (n = 5). Error bars indicate standard deviations.](http://iai.asm.org/)
that observed for the parent strain. The MTD shown in Table 2 for each deletion mutant is from a representative experiment. Notably, the MTD varied between 2 and 3 days for all strains in subsequent experiments, with no pattern to distinguish differences among strains (data not shown).

We also assessed the ability of the parent strain and toxin mutants to disseminate from the lung by measuring CFU in the spleens of infected mice at 3, 24, 48, and 72 h. At each time point, groups of five mice were harvested, except when the mice had already died. Figure 2 depicts combined data from two separate experiments. All mice had similar CFU in the lungs at 3 h, with no extrapulmonary colonization (data not shown). The number of heat-sensitive CFU in the lung at 3 h postinfection was low and did not significantly differ for mice infected with the parent or mutant \textit{B. anthracis} strains (data not shown). All \textit{B. anthracis} strains colonized the spleen by 24 h. In all groups, mice began to succumb to anthrax infection by 48 h, whereby \textit{B. anthracis} was recovered from the spleens at death. In some mice, dissemination at 48 and 72 h was accompanied by a sharp increase in the number of organisms in the lung that were heat sensitive, consistent with hematogenous seeding of the lung, as previously described (21).

**Toxin synthesis does not affect the \( \text{LD}_{50} \) and MTD during the systemic phase of infection.** Spores escape from the lungs of individual mice at different time points after i.t. inoculation, making comparisons among individual mice within identical experimental groups difficult. To synchronize the systemic infection so that similar numbers of organisms would colonize the organs of all mice at approximately the same rate, we infected mice intravenously with vegetative cells of \textit{B. anthracis}. As seen in the pulmonary phase of infection, the \( \text{LD}_{50} \) of all strains were not significantly different (Table 3). Similarly, the numbers of organisms in the spleens of infected mice at 18 h, when significant morbidity was evident, were not significantly different among the mice infected with the parent and mutant strains (data not shown). Finally, mice given \( 1 \times 10^5 \) organisms (\( \text{LD}_{100} \) of the parent strain) had MTDs for all five strains that were not different among groups; across groups, mice succumbed at between 20 and 23 h postinfection.

**Toxin-deficient mutants elicit subtle histopathological differences during the systemic phase of infection.** The microscopic pathology of mice infected with UT500 and the mutant strains was examined 18 hours after intravenous infection, when mice were moribund. Vegetative \textit{B. anthracis} cells were detected microscopically in pulmonary alveolar septal capillaries, splenic sinuses, and hepatic sinusoids of all mice. There

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>( \text{LD}_{50} ) (CFU)</th>
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<tbody>
<tr>
<td>UT500</td>
<td>+ + +</td>
<td>8–64</td>
</tr>
<tr>
<td>UT539</td>
<td>+ - +</td>
<td>9–110</td>
</tr>
<tr>
<td>UT540</td>
<td>- + +</td>
<td>14–37</td>
</tr>
<tr>
<td>UT541</td>
<td>- - +</td>
<td>16–94</td>
</tr>
<tr>
<td>NM1</td>
<td>+ - -</td>
<td>34–64</td>
</tr>
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\( \text{LD}_{50} \) generated from three different sets of experiments. Each experiment had three or four different groups (\( n = 6 \)) receiving different doses.
was no evidence of inflammation or other host response in the lungs of any mice. Microscopic lesions in the livers consisted of occasional foci of neutrophilic inflammation (cya, cya lef, and pagAR mutants only) and necrotic/apoptotic cellular debris within sinusoids. In the spleens, lesions consisted of variable red pulp neutrophilic infiltrates and of necrosis/apoptosis and depletion of red and white pulp compartments, often together with large number of vegetative cells of the organism (Fig. 3). Depletion of the red and white pulp of the spleen was presumed to be secondary to previous cell death.

FIG. 3. Splenic lesions at 18 h after intravenous inoculation with *B. anthracis*. (A) Parent. (B) LF− mutant. (C) EF− mutant. Note the presence of bacilli in tissues from mice infected with each strain (arrows in panels A and B). Tissues from mice infected with the LF− mutant (B) display more red and white pulp depletion than tissues from mice infected with the parent (A) and EF− (C) strains. In panel C abundant neutrophils are present in red pulp (arrowhead).
inhalation anthrax. In the present study we utilized pXO1 absolutely required for dissemination in a murine model for sole biosynthetic genes and demonstrated that capsule is ab-

The qualitative nature of the microscopic lesions was similar for all mice. However, there were subtle differences in the severity of the lesions, most notably in the spleen, for mice inoculated with different mutants (Fig. 3). Hepatic and splenic lesion severity was scored semiquantitatively for six mice inoculated in two separate blind experiments (Table 4). The spleens of mice infected with the parent strain and the lef mutant displayed significantly more red and white pulp necrosis/apoptosis and depletion than those of mice infected with the cya, cya lef, and pagAR mutants. Surprisingly, mice infected with the lef mutant displayed more white and red pulp depletion than mice infected with the parent strain. In contrast, mice infected with the parent strain and the lef mutant had fewer splenic neutrophilic infiltrates than mice infected with the other three strains.

The bacilli were Gram stained and counted in the lungs, where evaluation could be made without interference by necrotic cell debris and the host inflammatory response. Interestingly, mice infected with the lef mutant showed a statistically significant increase in the number of bacilli in the lungs compared to those inoculated with the parent strain, while the lungs of mice inoculated with the cya, cya lef, and pagAR mutants had significantly fewer bacilli than those of mice inoculated with the parent strain (Fig. 4). Although not counted, a similar trend was observed in the relative number of splenic bacteria.


discussion

The toxin proteins and capsule produced by B. anthracis are considered to be critical virulence factors contributing to morbidity and mortality of anthrax infections. It is important to determine the role of these virulence factors in infection in order to develop therapeutics during an anthrax infection. The majority of previous studies examined the virulence of strains lacking entire plasmids or tested the effects of purified toxin components in animal models. In a previous study, we employed pXO1+pXO2+ strains of B. anthracis deleted for capsule biosynthetic genes and demonstrated that capsule is absolutely required for dissemination in a murine model for inhalation anthrax. In the present study we utilized pXO1+pXO2- strains of B. anthracis deleted for the specific anthrax toxin genes to investigate the roles of EF, LF, and PA in the murine model. We determined that the toxin genes were not required for lethality in this particular animal model but that the addition of toxins alters the pathophysiology in a manner that could contribute to the final outcome. Mutants deficient in EF, LF, both EF and LF, or PA exhibited LD50s and MTDs that were similar to those of the parent strain. Furthermore, delivery of vegetative cells of the parent and toxin mutant strains using intravenous inoculation, a route which bypasses the requirements for escape from the lungs and spore germination, also killed mice with similar MTDs and LD50s.

A common belief about B. anthracis infections is that the alveolar macrophage-endospore interaction is critical for traffic-ficking of the pathogen to the lymph nodes and for development of vegetative cells from spores. Histopathological studies by Ross initially identified endospores within macrophages in draining lymph nodes after i.t. inoculation into guinea pig lungs (40). Subsequent in vitro studies suggested that toxin production was required for efficient spore germination within macrophages (10). Our data indicate that neither edema toxin nor lethal toxin is required for escape from the lung. We found that the LD50s were identical for the parent strain and toxin-deficient mutants following intratracheal delivery. In addition, the germination and the kinetics of dissemination from the lung to the spleen were identical for all strains.

Given the absence of a germination or dissemination phe-

notype for the toxin mutants in our pulmonary model, we determined whether toxin synthesis affected the host response during the systemic phase of anthrax. Although the presence or absence of the toxin genes did not affect the MTDs for mice in a synchronous intravenous model of infection, our histopathology studies revealed subtle, but distinct, differences in mice infected with the parent strain compared to nontoxigenic B. anthracis strains. Mice infected with EF-, EF- and LF-, and PA- mutants, all lacking edema toxin activity, had elevated neutrophilic infiltration, decreased levels of necrosis/apoptosis, and postnecrotic depletion in their spleens compared to those infected with the LF- mutant and the parent strain. We speculate that loss of edema toxin activity results in enhanced host neutrophilic response with subsequent reductions in bacterial burden and decreased necrosis/apoptosis in the spleen. Also, infection with the LF-deficient mutant, possessing edema toxin activity, resulted in greater numbers of bacilli in lung tissue and increased splenic cell death compared to the parental strain. This indicates that the toxins may have an antagonistic effect and that EF clearly plays an as-yet-undefined role in dampening the host's ability to inhibit bacillus proliferation. These findings, combined with those in our previous report (12),

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<th>Strain (relevant genotype)</th>
<th>Liver</th>
<th>Spleen</th>
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<tr>
<td></td>
<td>Score (mean ± SD)*</td>
<td>Score (mean ± SD)*</td>
</tr>
<tr>
<td></td>
<td>Necrosis/apoptosis in sinusoids</td>
<td>Polymorphonuclear leucocytes</td>
</tr>
<tr>
<td>UT500 (parent)</td>
<td>1.7 ± 0.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>UT539 (lef)</td>
<td>2.3 ± 0.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>UT540 (cya)</td>
<td>1.2 ± 1.0</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>UT541 (lef cya)</td>
<td>0.5 ± 0.5*</td>
<td>0.7 ± 0.5*</td>
</tr>
<tr>
<td>NM1 (pagAR)</td>
<td>0.8 ± 0.8</td>
<td>1.5 ± 0.5*</td>
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* For explanation of scoring, see Materials and Methods. Data were pooled from two separate experiments (n = 6) for each B. anthracis strain. *, Significantly different from score for strain UT500 (P < 0.05).
reveal that individual toxin components contribute to the pathogenesis of a *B. anthracis* infection in a murine model but that when capsule is present, it is the prevailing virulence factor. Overall, the histopathologic results suggest that edema toxin might play a larger role in producing pathological changes than lethal toxin, but clearly, further studies must be done.

In previous studies using a subcutaneous inoculation murine model, Pezard and coworkers (34) demonstrated that toxin-negative mutants of *B. anthracis* displayed decreased virulence compared to a parental strain. The parent and mutant strains used in their study were devoid of the 96-kb plasmid pXO2 (22, 23), which harbors the capsule biosynthetic operon (6). In addition, Welkos demonstrated that toxin-negative strains (pXO1−) maintained greater virulence than capsule-negative strains (pXO2−) in a murine injection model (52). It is important to note that these studies used attenuated strains missing entire virulence plasmids and used routes of infection that make it difficult to extrapolate to pulmonary infection models. Previous reports demonstrating that antibodies generated against capsule are protective (19) and that a pXO1− pXO2− *B. anthracis* mutant deleted for the capsule biosynthetic operon is avirulent (12), combined with our results presented here, indicate that capsule plays a dominant role in a murine model of inhalation anthrax.

The concept that toxins are not required for lethality in mice raises the question as to why the current vaccine for human infection (AVA) is protective, given that PA is the major immunogen in the vaccine. In studies performed by Welkos and coworkers (51), protective immune serum from AVA-vaccinated animals had antispore properties and therefore could likely inhibit the very early infection process prior to germination. Thus, the protective nature of the vaccine might be due to the timing and location of expressed PA on spores and/or vegetative cells during the early phases of the infection, rather than to inhibition of the enzymatic function of the toxin.

Although the data support the notion that the *B. anthracis* capsule plays a dominant role in virulence in the murine pulmonary model, our results should not be construed to mean that the lethal and edema toxins do not play roles in the pathogenesis of human disease or even in the pathogenesis of the murine infection. It is likely that all these virulence factors act in concert to ensure survival of the bacterium during infection, but their level of contribution may depend on a variety of factors yet to be determined. Perhaps in the absence of one or both of the toxins the effects of other virulence factors are unmasked. The pXO1− pXO2− toxin-deficient mutants will permit us to address the influence of specific toxins on molecular responses by specific host cells and to examine other aspects of the host response by using more sophisticated assays. Future investigations will address whether the toxins are required for virulence in other animal models, such as the rabbit and nonhuman primate models, and to possibly rethink optimal targets for development of effective and preventative therapeutic interventions against inhalation anthrax.

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