Recombinant Protective Antigen Anthrax Vaccine Improves Survival when Administered as a Postexposure Prophylaxis Countermeasure with Antibiotic in the New Zealand White Rabbit Model of Inhalation Anthrax

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Inhalation anthrax is a potentially lethal form of disease resulting from exposure to aerosolized Bacillus anthracis spores. Over the last decade, incidents spanning from the deliberate mailing of B. anthracis spores to incidental exposures in users of illegal drugs have highlighted the importance of developing new medical countermeasures to protect people who have been exposed to “anthrax spores” and are at risk of developing disease. The New Zealand White rabbit (NZWR) is a well-characterized model that has a pathogenesis and clinical presentation similar to those seen in humans. This article reports how the NZWR model was adapted to evaluate postexposure prophylaxis using a recombinant protective antigen (rPA) vaccine in combination with an oral antibiotic, levofloxacin. NZWRs were exposed to multiples of the 50% lethal dose (LD50) of B. anthracis spores and then vaccinated immediately (day 0) and again on day 7 postexposure. Levofloxacin was administered daily beginning at 6 to 12 h postexposure for 7 treatments. Rabbits were evaluated for clinical signs of disease, fever, bacteremia, immune response, and survival. A robust immune response (IgG anti-rPA and toxin-neutralizing antibodies) was observed in all vaccinated groups on days 10 to 12. Levofloxacin plus either 30 or 100 μg rPA vaccine resulted in a 100% survival rate (18 of 18 per group), and a vaccine dose as low as 10 μg rPA resulted in an 89% survival rate (16 of 18) when used in combination with levofloxacin. In NZWRs that received antibiotic alone, the survival rate was 56% (10 of 18). There was no adverse effect on the development of a specific IgG response to rPA in unchallenged NZWRs that received the combination treatment of vaccine plus antibiotic. This study demonstrated that an accelerated two-dose regimen of rPA vaccine coadministered on days 0 and 7 with 7 days of levofloxacin therapy resulted in a significantly greater survival rate than with antibiotic treatment alone. Combination of vaccine administration and antibiotic treatment may be an effective strategy for treating a population exposed to aerosolized B. anthracis spores.

Bacillus anthracis is a naturally occurring, spore-forming bacterium that can cause different forms of human disease depending on the route of exposure. Inhalation anthrax is the most lethal form, with mortality rates of up to 100% in untreated people and 40 to 85% depending on the amount of exposure and when an individual seeks medical treatment (1, 9, 13). B. anthracis has been categorized as a high-bioterror threat agent due to the perceived ease with which spores can be grown and then disseminated by aerosol (15). Most spores germinate within a few days after being inhaled by a host, but germination is asynchronous and some spores may remain dormant for a prolonged time (7, 8, 20). Consequently, the Centers for Disease Control and Prevention (CDC) recommends a 60-day course of antibiotics to treat inhalation anthrax and that the treatment begin immediately “upon suspicion” that the patient has inhalation anthrax, i.e., before there is an onset of symptoms or confirmation of infection. When medical intervention is initiated before a patient has a confirmed diagnosis, the treatment is referred to as postexposure prophylaxis (PEP). After the so-called “letter attacks” in 2001, more than 10,000 people were offered a PEP course of antibiotics to be taken for at least 60 days in conjunction with the licensed anthrax vaccine (21).

Although antibiotics are highly effective prior to the onset of disease symptoms, they are active only on vegetative bacteria. Once antibiotic treatment is stopped, there is a concern that residual spores could germinate into vegetative cells and lead to disease, hence the requirement for extended antibiotic treatment. In the event of antibiotic therapy alone, insufficient exposure of the host to vegetative cells may result in inadequate immune memory and suboptimal levels of toxin-neutralizing antibody production. This would render the host unprotected if spores germinated late after exposure, after antibiotics had been discontinued, resulting in potential latent infection. In addition, people who are treated with PEP antibiotics are likely to resist complying with long courses of ciprofloxacin or doxycycline treatment due to uncomfortable side effects (2). For these reasons, there is a desire to establish efficacy of an anthrax vaccine administered in conjunction with antibiotics, particularly under a shortened antibiotic regimen. The objective is to stimulate antibody development with the vaccine while the antibiotic reduces the immediate bacteremia caused by germinating spores.

Evaluation of the efficacy of an anthrax vaccine must be completed in well-characterized animal models, in compliance with the FDA Animal Rule (3a). These models must be relevant to
humans such that efficacy data generated can be extrapolated to predict clinical benefit in humans. The New Zealand White rabbit (NZWR) model used in this work is a widely accepted model for inhalation anthrax (10, 11, 16, 23, 25). In this PEP proof-of-concept study, the time of initiation of antibiotic treatment was chosen to be 6 to 12 h after exposure, similar to a study design used in rhesus macaques (22), mimicking a PEP situation after a suspected bioterrorist attack. Although the routes of disease pathogenesis are similar, the time course in the rabbit model is known to be much faster than that of humans or nonhuman primate models (17, 25). NZWRs become ill as early as 24 to 48 h after exposure (24; PharmAthene, unpublished data), indicating that initiation of treatment just after aerosol exposure in this model is appropriate, and the compression of disease onset phase requires earlier intervention in order to be predictive of disease management in humans. Previous model development work was performed, under the guidance of the National Institute of Allergy and Infectious Diseases, to extensively characterize the vaccine and antibiotic regimen used in this study (H. A. Lockman, unpublished data). The unpublished study confirmed that levofloxacin was well tolerated when administered by oral gavage and that it could be concurrently administered with a recombinant protective antigen (rPA) vaccine without interfering with the host immune response in unchallenged NZWRs.

The purpose of the current study was to evaluate the efficacy of an accelerated rPA vaccine schedule when used in conjunction with a shortened antibiotic treatment regimen. It required administration of the vaccine in the presence of antibiotics to mimic the standard of care prescribed for humans exposed to aerosolized B. anthracis spores. In addition, the immunogenicity of the vaccine when coadministered with levofloxacin was studied in an unchallenged cohort to determine if the expected host response was interfered with by the presence of antibiotic.

**MATERIALS AND METHODS**

**Animals.** Specific-pathogen-free New Zealand White rabbits (NZWRs) weighing between 2.5 and 3.0 kg were purchased from Covance. NZWRs were housed individually in stainless steel cages with food and water available ad libitum; environmental enrichment was provided. The facility (Battelle Memorial Institute) was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Antibiotic, vaccine, and controls.** Levofloxacin (levo) (Johnson and Johnson Pharmaceutical Research and Development) dosing solution was prepared by SRI International (Menlo Park, CA) at a target concentration of 20 mg/ml. The diluent for the antibiotic was hydroxypropyl methylcellulose (HPMC) supplied by The Dow Chemical Company. The rPA vaccine was supplied by Avecia (now PharmAthene, Inc.) as a liquid formulation at 200 μg/ml rPA adsorbed to Alhydrogel (aluminum content, 0.63 g/ml). Levo was demonstrated to be stable at 20 μg/ml, determined during method development to be an appropriate internal standard, was added to each 100-μl plasma sample (calibration standards, quality control samples, and NZWR samples). Samples were precipitated with acetonitrile and supernatants were evaporated under vacuum. After reconstitution with mobile phase, samples were analyzed by reversed-phase high-performance liquid chromatography with fluorescence detection. The lower limit of quantitation was 0.07 μg/ml. Levo was demonstrated to be stable in NZWR plasma for 120 days when stored under study conditions (−70°C).

**Phlebotomy.** Blood was collected into sodium EDTA tubes on days −7, 0, 1, 6, and 7 to determine plasma levo concentrations. Blood was collected into serum separator tubes on days −7, 7, 7, 10, 12, 14, 21, and 30 for analysis by IgG anti-PA enzyme-linked immunosorbent assay (ELISA) and/or toxin neutralization assay (TNA). NZWRs in the aerosol challenge groups had blood collected in EDTA tubes on days −7, 4, 7, 10, 14, 21, and 30 and at time of death when possible, for qualitative bacteremia analysis by plate culture and hematology assessment, which included white blood cell count, differential leukocyte (absolute) count, hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelet count, and mean platelet volume.

**Clinical observations.** NZWRs were observed twice daily (for health in unchallenged groups or for clinical signs of illness due to anthrax infection in other groups; moribundity, respiratory distress, appetite, activity, and clinical observations). Rabbits determined to be moribund were anesthetized and administered an overdose of a euthanasia agent containing pentobarbital.

**BT.** NZWRs were implanted with subcutaneous temperature transponder chips (IPTT-300 from BMDS, Seaford, DE) in both the rump and shoulder. Temperatures were recorded in the morning and afternoon for 5 time points before the challenge to establish a baseline reading for each animal. After aerosol exposure, temperatures were recorded twice daily. An elevated body temperature (BT) was defined as an increase from the baseline (recorded prior to aerosol challenge) temperature of more than 2.5°F, or a temperature of more than 103°F (measured from the rump transponder) or 104°F (measured from the shoulder transponder).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>Drug (50 mg/kg levo or control [HPMC])</th>
<th>Vaccine dose (μg rPA/dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>Levo 100</td>
<td>HPMC Not administered</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>Levo 30</td>
<td>HPMC Not administered</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>Levo 10</td>
<td>HPMC Not administered</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>Levo Diluent Only</td>
<td>HPMC Not administered</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>HPMC</td>
<td>Not administered</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Levo 30</td>
<td>HPMC 30</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>Levo 30</td>
<td>HPMC 30</td>
</tr>
</tbody>
</table>

**TABLE 1** Postexposure prophylaxis study design

The study for evaluation of bacteremia, immunogenicity assays, and assessment of antibiotic levels.

**Aerosol exposure.** On study day 0, rabbits in groups 1 to 5 were placed in a plethysmography chamber in a biological safety class III cabinet system and aerosol challenged via a nose-only system. The target aerosol exposure of B. anthracis spores was 200 LD50s (lethal dose in 50% of the population) (25) generated with a 3-jet Collison nebulizer. Aerosol samples were collected in an all-glass impinger for analysis of B. anthracis concentration via plate count on tryptic soy agar plates. Plethysmography was performed in real time during the aerosol exposure.

**Plasma antibiotic concentrations.** Samples were collected for baseline, peak, and trough readings. Levo analysis was completed at SRI International. Ciprofloxacin (100 μl, 1.4 μg/ml), determined during method development to be an appropriate internal standard, was added to each 100-μl plasma sample (calibration standards, quality control samples, and NZWR samples). Samples were precipitated with acetonitrile, and supernatants were evaporated under vacuum. After reconstitution with mobile phase, samples were analyzed by reversed-phase high-performance liquid chromatography with fluorescence detection. The lower limit of quantitation was 0.07 μg/ml. Levo was demonstrated to be stable in NZWR plasma for 120 days when stored under study conditions (−70°C).
TABLE 2 Summary of reportable clinical observations, survival, and bacteremia in NZWRs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of NZWRs with 2 or more of the following observations:</th>
<th>% survival (no. that survived/total)</th>
<th>% NZWRs with bacteremia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Respiratory</td>
<td>Activity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Challenged cohort</td>
<td>100 μg rPA + levo (group 1)</td>
<td>10/18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 μg rPA + levo (group 2)</td>
<td>4/18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10 μg rPA + levo (group 3)</td>
<td>12/18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0 μg rPA + levo (group 4)</td>
<td>15/18</td>
<td>0</td>
</tr>
<tr>
<td>Untreated controls (group 5)</td>
<td>9/10</td>
<td>2/10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2/10</td>
</tr>
<tr>
<td>Unchallenged cohort</td>
<td>30 μg rPA + levo (group 6)</td>
<td>2/6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 μg rPA only (group 7)</td>
<td>1/6</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Gastrointestinal (GI) includes eating, no stool, and/or soft stool.
<sup>b</sup> Includes recumbency or moribundity.
<sup>c</sup> One observation time point because NZWRs were found dead at the next observation period.
<sup>d</sup> One NZWR in this group was bacteremic on days 14 and 17.

**Bacteremia.** Using the spread plate method, 100 μl of blood was applied to tryptic soy agar plates. Plates were incubated at 37°C for at least 24 h, and colonies were manually counted.

**ELISA.** The ELISA to detect antibody to protective antigen (PA) was adapted from methods published by Quinn et al. (19). Briefly, PA was passively adsorbed to a 96-well microtiter plate overnight at approximately 4°C. Samples and reference sera were diluted in PBS (pH 7.4)–5% skim milk–0.5% Tween 20. Plates were washed with PBS–0.1% Tween 20; samples, reference sera, and controls were added, and plates were incubated for 60 min at 37°C. Plates were then washed, and horseradish peroxidase-conjugated goat anti-rabbit IgG diluted in PBS–5% skim milk–0.5% Tween 20 was added to the plates. After incubation at 37°C for 60 min, plates were washed, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate was added, and plates were incubated at 37°C for 30 min. Plates were read at a 405-nm wavelength (with a 490-nm reference). Samples were quantitated against the reference material calibrated in μg/ml using a four-parameter logistic regression (4PL). Sample concentrations below the limit of detection (1.0 μg/ml) were assigned a value of 1.0.

**TNA.** The TNA was adapted from methods published by Quinn et al. (18). Briefly, diluted serum samples were preincubated with lethal toxin (LT) containing 50 ng/ml PA and 40 ng/ml lethal factor (LF) in a 96-well plate. Preincubated serum-LT mixtures were transferred to a 96-well plate that had been seeded with J774A.1 cells. Following incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the plates for detection of viable cells. Solubilization buffer was then added. After 16 to 20 h, optical density (OD) values were read to determine cell viability (14). All incubations were carried out at 37°C in approximately 5% CO₂. Neutralization of LT was manifested as a suppression of cytotoxicity and, hence, the preservation of cell viability. A 4PL model was used to analyze the OD versus the serum dilution. The result was reported as the reciprocal of the effective dilution at 50% inhibition (ED<sub>50</sub>) and also as the NF<sub>50</sub> (the quotient of a reference sample ED<sub>0.5</sub> and the test sample ED<sub>50</sub>).

**Pathology.** Complete gross necropsies were conducted on all rabbits. Protocol-specified tissues (brain, kidneys, spleen, lungs, liver, mediastinal lymph nodes, and gross lesions) were fixed in 10% neutral buffered formalin, processed for routine hematoxylin and eosin staining, and examined by a board-certified veterinary pathologist.

**Statistics.** To establish the efficacy of vaccine and antibiotic combination therapy, one-sided Fisher’s exact tests were used to compare survival rates between each antibiotic-vaccine treatment group and the corresponding control groups. Log rank tests, with Bonferroni-Holms adjustment as required, were used to calculate significant differences in time-to-death (TTD) data. For blood bacteremia analyses, two-sided Fisher’s exact tests were performed for each group in order to determine whether bacteremia and group were independent. If bacteremia and group were found to be dependent, then all possible pairwise Fisher’s exact tests were performed. Independence between death and whether the animal was ever bacteremic was tested using a two-sided Fisher’s exact test. The challenge control animals (group 5) were not included in the analysis. Analysis-of-variance (ANOVA) models were used to determine if changes in temperature, hematology parameters following challenge, and immunogenicity data (ELISA and TNA results) were statistically different among treatment groups.

**RESULTS**

**Challenge doses.** Aerosol challenges were completed over 3 days. The delivered average doses ± standard deviations were 2.91 × 10⁷ ± 0.48 × 10⁷, 3.00 × 10⁷ ± 0.41 × 10⁷, and 2.16 × 10⁷ ± 0.31 × 10⁷ CFU per animal. The average challenge dose, overall, was 2.7 ± 0.5 × 10⁷ CFU, which is equivalent to 256 ± 52 LD<sub>50</sub>.

**Levo concentration in plasma.** After levo administration (groups 1 to 4 and 6), individual peak concentrations in plasma were always greater than trough concentrations. Mean peak concentrations in plasma ranged from 2.86 to 4.68 μg/ml. The mean trough concentrations in plasma ranged from 0.12 to 0.22 μg/ml. The in vitro MIC<sub>90</sub> is 0.12 μg/ml, so NZWRs had trough concentrations near the MIC<sub>90</sub>. All control NZWR samples (groups 5 and 7) contained no measurable levo, and predose samples from day −7 were also negative for levo (data not shown). There were no gender differences in concentrations in plasma.

**Clinical observations.** Observations related to anthrax disease are summarized in Table 2. No statistical analysis was performed due to the small sample numbers.

**BT.** BT data for all groups are shown in Fig. 1. There were no statistically significant differences in BT between vaccinated and unvaccinated groups. However, the incidence of individual NZWRs with an elevated temperature decreased with increasing vaccine dose. The most obvious changes in body temperature were observed in untreated challenged controls (group 5), and all had a fever prior to death (Fig. 1). In the low-dose vaccination group 3 (10 μg rPA plus levo), 6/18 NZWRs developed a fever after antibiotic treatment was complete and 2 of those died. In the unvaccinated levo-treated NZWRs (group 4), only 7/18 rabbits...
developed a fever, and all 7 died from anthrax (data not shown). In the two groups of unchallenged NZWRs, body temperatures did not change from baseline throughout the course of the study.

Survival. NZWRs that were challenged with *B. anthracis* spores were observed for 30 days postexposure. Survival data are shown in Fig. 2. Among the untreated controls, all NZWRs died by day 6 postexposure, with an average TTD of 4 days (range, 2.8 to 5.8 days). Survival was seen in both combination therapy and levo-alone treatment groups, although the mortality rates were significantly lower in the groups treated with 100 μg, 30 μg, and 10 μg rPA plus levo (*P* = 0.0014, 0.0014, and 0.0300, respectively) than in those treated with levo alone. When NZWRs received the antibiotic regimen only (0 μg rPA), there was a 56% survival rate, but when vaccine was combined with antibiotic treatment, the survival rate increased to 100%, even with an rPA dose as low as 30 μg. The groups receiving the 30- and 100-μg rPA vaccine doses had significantly (*P* ≤ 0.05) increased protection as defined by TTD compared to controls, with 100% survival in those vaccinated groups. In the group receiving the lowest vaccine dose (10 μg rPA) in combination with levo, there was also significantly increased TTD (12.8 days) compared to the control group (TTD = 4.1 days). Gross pathology findings consistent with anthrax were observed in a dose-dependent manner in all NZWRs that died or that were euthanized in a moribund state.

Bacteremia. Blood samples were drawn in treated groups, beginning on day 4, to evaluate bacteremia; time points were approximately every 3 days for 21 days postchallenge and then day 30. All rabbits that died were bacteremic on at least one sampling. Survival data are shown in Table 2. No NZWRs were detectably bacteremic on days 4 to 7, while on antibiotic treatment. The total number of treated animals that became bacteremic was 12/72 (17%), and only 1 of these was positive at two consecutive time points.

**FIG 1** There were no statistical differences in body temperature changes between any treated NZWRs. However, the untreated, challenged group developed and maintained a fever up to the point of death on approximately day 5.

**FIG 2** NZWRs were observed for 30 days after exposure. *, the mortality rate was significantly lower in the vaccinated groups than in the group that received levo only (group 4). The average time to death (TTD) in group 3 was 12.8 days, and in group 4 it was 15.1 days; the control group died in an average of 4.1 days. Groups 1 to 4 had 18 NZWRs per group, and group 5 had 10 NZWRs.
points. Four of the NZWRs that survived had at least one bacteremic time point that occurred after antibiotic treatment was complete. There appeared to be a vaccine dose-response effect, with fewer rabbits becoming bacteremic as the vaccine dose increased. There was a significant difference between levo-treated unvaccinated rabbits (group 4) and the levo-treated group administered 100 µg rPA (P < 0.001), where no vaccinated rabbits became bacteremic. In the vaccinated groups, only 4 rabbits (7%) became bacteremic, and 3 of those survived. In the unvaccinated group receiving levo, 8 of 18 NZWRs became bacteremic, and 7 of those died from anthrax. In the untreated control group, all 10 rabbits became bacteremic by day 4 and died.

**IgG anti-PA antibodies—ELISA.** Antibody responses over time are shown in Fig. 3A and B. On day 7 postchallenge, the IgG concentrations were below the limit of detection for all treatment groups. There was a significant difference between levo-treated unvaccinated rabbits (group 4) and the levo-treated group administered 100 µg rPA (P < 0.001), where no vaccinated rabbits became bacteremic. In the vaccinated groups, only 4 rabbits (7%) became bacteremic, and 3 of those survived. In the unvaccinated group receiving levo, 8 of 18 NZWRs became bacteremic, and 7 of those died from anthrax. In the untreated control group, all 10 rabbits became bacteremic by day 4 and died.

**Functional activity of PA antibodies—TNA.** Both ED50 and NF50 values were determined in the TNA. Since the relative values and conclusions were similar, only ED50 is shown in Fig. 3. The challenged cohort is shown in Fig. 3C: on day 7 postchallenge, ED50 values began to rise in a dose-response fashion and leveled by day 14. The group treated with only levo did develop some response in the TNA by day 30, as a means of a natural host immune response. (D) Results of the TNA, reported as geometric mean titers for the cohort of NZWRs that were not challenged. The presence of levo had no effect on ED50 titers induced by vaccination with rPA vaccine.

FIG 3 (A) IgG anti-rPA antibodies in the challenged cohort were tested on study days shown. There were significant differences (P < 0.05) between two of the vaccinated groups on both days 10 and 12: 100 µg rPA plus levo compared to 30 µg rPA plus levo and 30 µg rPA plus levo compared to 10 µg rPA plus levo. (B) IgG anti-rPA antibodies in the unchallenged cohort were tested on the study days shown. There were no significant differences between the two treatment groups, indicating that levo had no adverse effect on the immune response induced by vaccination. (C) Results of the TNA, reported as geometric mean titers for the cohort of NZWRs that were challenged. Vaccination occurred on days 0 and 7, daily levofloxacin treatment on days 0 to 7. On day 10 postexposure, TNA titers began to rise in a dose-response fashion and leveled by day 14. The group treated with only levo did develop some response in the TNA by day 30, as a means of a natural host immune response. (D) Results of the TNA, reported as geometric mean titers for the cohort of NZWRs that were not challenged. The presence of levo had no effect on ED50 titers induced by vaccination with rPA vaccine.
the TNA responses reached a plateau. The ED\textsubscript{50} and NF\textsubscript{50} data were supported by the ELISA data in that there was no significant difference between the unchallenged groups, shown in Fig. 3D, at any point (30 \( \mu \)g rPA with and without levo). There was no correlation between TNA results and survival, as there was a 100% survival rate in two dose groups.

**DISCUSSION**

The primary objective of this study was to determine if a combination of short-term levo treatment in conjunction with an accelerated vaccination schedule would increase survival rates in the NZWR anthrax model. The second objective was to determine if rPA vaccination would lengthen survival times (increase time to death [TTD]), allowing the adaptive immune response induced in the host to protect individuals after antibiotics were discontinued. NZWRs were challenged with an average of 256 LD\textsubscript{50}s of aerosolized *B. anthracis* spores. There was a 100% survival rate for NZWRs that received levo with the two highest doses of rPA vaccine (30 or 100 \( \mu \)g), and even with the lowest dose of vaccine (10 \( \mu \)g), the survival rate was 89\%, which was significantly greater than that of the group that received levo alone (56\%). All of the groups that received combination treatment had significantly lower mortality rates than the group that received levo alone. By reducing the vaccine dose to 10 \( \mu \)g and observing breakthrough in protection, the requirement for both vaccination and antibiotic therapy to achieve protection in a PEP scenario was demonstrated in this model.

The analysis of TTD data provided strong evidence that adding this rPA vaccine to a standard-of-care antibiotic treatment provided an increased benefit in a PEP scenario. When the groups receiving 0 and 10 \( \mu \)g rPA were compared using a pairwise log rank test, the TTD was found to be significantly different (\( P \approx 0.0406 \)), suggesting that even a dose of 10 \( \mu \)g rPA affords more protection than if antibiotic were administered alone.

A third objective of this study was to examine parameters that would substantiate the model and enhance its value and acceptance for use in the FDA Animal Rule. When establishing an animal model, documentation of clinical observations is important for cross-laboratory comparisons; therefore, we have documented our findings for clinical signs, body temperature, hematology, bacteremia, and host immune response. A comprehensive profile of clinical observations was presented, and while these are perhaps subjective observational data, one sign that seemed related to the disease was reduced appetite, seen at a higher incidence in infected NZWRs. Respiratory distress occurred in 20\% of the untreated controls (but not in treated rabbits) just before death. Another clinical sign that was predictably more often associated with infected NZWRs was elevated body temperature, or fever. While the most drastic changes were observed in untreated animals, which all had fevers before death, it should be noted that some degree of elevated temperature was also seen in the treated groups but that the temperature then returned to normal and the rabbits survived. There were 11 routine hematology endpoints evaluated, and all these parameters showed a large degree of individual variability; mean hematology parameters were not useful for differentiating between treatment groups in either the challenged or unchallenged cohorts. Similar hematological tests were also performed in the clinical cases reporting to the hospital in the 2001 attack, without remarkable abnormal findings on initial assessment (16).

When increase in body temperature and incidence of reduced appetite were analyzed, individual variability made comparisons difficult, and therefore no statistically significant group effects could be identified. Hematology testing and careful observation of respiratory status did not identify group trends or early indicators of disease status. While it is disappointing that these parameters will not be useful surrogate markers of efficacy of an anthrax vaccine, it is promising that they follow a trend which is predictive of the human population. In an exposed group of people, there will also be a vast range of individual variability. As seen in the illnesses reported after the letter attacks in 2001, all of the 10 patients did have a fever at some point, but the degree and onset were variable (9). It should also be noted that respiratory distress was documented in advanced stages of the clinical cases, so overall, it is encouraging that the NZWR model has a clinical presentation that authentically represents disease in a human population.

As expected, no animal was positive for bacteremia while receiving levo. The first study day that animals began testing positive for bacteremia was day 10, but this was also the first time blood was drawn after the levo regimen was completed on day 7. A conclusion cannot be made on how quickly spores began germinating, based on bacteremia data, because of this 3-day lag period. In general, caution should be taken when making inferences as to the lack of consecutive bacteremic time points. Samples could not be taken at very close intervals for ethical reasons; therefore, a comprehensive illustration of the time course is not possible for this study. Of importance is the fact that there was a lower incidence of bacteremia in all groups treated with vaccine plus levo than in groups treated with levo alone. When NZWRs received combination treatment, animals that became bacteremic remained bacteremic for only one time point. In 4 of these rabbits, the bacteremia was cleared by the time the sequential sample was taken 3 to 4 days later, but in one case, the animal died first. This confirms that the animals were infected and suggests that while the antibiotics were controlling early bacteremia, the vaccine was inducing adaptive immunity in the host, as some animals survived. When levo was discontinued, if spores continued to germinate, the NZWR had mounted an immune response that cleared the infection. Elevated levels of functional antibody (TNA data in Fig. 3C) were being produced by day 10, just 3 days after antibiotic treatment was discontinued.

Two assays were used to evaluate the host immune response: ELISA and TNA. The functional activity measured by TNA clearly correlated with ELISA IgG concentrations (\( R^2 = 86\% \)). The magnitude of a rapid antibody response on days 10 to 12 was dependent on the vaccine dose and was followed by a plateau that was independent of dose. Antibody concentrations for all three vaccinated groups measured after the aerosol challenge were significantly greater than for the levo (challenged and unvaccinated) group, suggesting that levo did not adversely affect the immune response to the vaccine. Too few animals died in the groups that received vaccine to be able to draw any correlation between IgG anti-PA antibody or TNA levels and survival (only 2 animals died in the groups treated with 10, 30, and 100 \( \mu \)g rPA combined). This illustrates the difficulty of extrapolating correlates of protection in a PEP model and emphasizes the importance of discussion at a Vaccines and Related Biological Products Advisory Committee meeting convened by the Food and Drug Administration in November 2010 (4, 5). Although a position was not formalized, there was a strong agreement that correlates of protection should be determined only using a more straightforward model where ani-
mals are vaccinated preexposure and the immunological profile compared to the endpoint of survival.

The last objective of the study was to demonstrate that concomitant use of antibiotics would not suppress the vaccine-induced immune response. Therefore, two cohorts were not challenged with *B. anthracis*. The peak plasma levo concentrations were similar to levels achieved in humans receiving 500 mg daily (3, 6, 12). Trough levels remained near the *in vitro* MIC₉₀, suggesting that the NZWRs were not excessively dosed with antibiotic. Concentrations in plasma were not affected by vaccine dose or by anthrax infection. Levo treatment did not result in a negative impact on the host immune response in the healthy cohort receiving rPA vaccine, as demonstrated by ELISA and TNA data. In addition, the levo-only group developed an immune response to the B. *anthracis* challenge, although as expected, with a much slower time course than in rabbits that received the vaccine as well, illustrating the benefit of combining vaccination with levo in the PEP scenario.

Survival in the levo-only group was similar to that seen in humans during the 2001 anthrax attacks (9). In the NZWR model, the combination of this rPA vaccine and levo treatment significantly increased the survival rate (P values were 0.0014, 0.0014, and 0.0300 in groups 1 to 3, respectively), compared to that of animals receiving antibiotic treatment alone. Pharmacokinetic analysis demonstrated that peak antibiotic levels in the rabbit were similar to concentrations seen in humans, so it is reasonable to conclude that this was a valid model in which to demonstrate our primary objective that combination PEP therapy would increase survival in the NZWR. Administration of the rPA vaccine while a host is undergoing antibiotic therapy should allow a robust immune response to develop so that when antibiotics are discontinued, there would be adequate protection against residual late-germinating spores.

It has been demonstrated in an accelerated two-dose regimen of this rPA vaccine with a dose as small as 10 μg, administered in conjunction with 7 days of levo, that there was a significantly greater survival rate than with antibiotic treatment alone. This vaccination regimen also produced measurable immune responses that provided potential long-term protection in NZWRs exposed to multiples of the lethal dose of *B. anthracis* spores. The coadministration of levo and rPA vaccine did not impair the ability of the vaccine to induce a fast and robust specific IgG response to rPA in the rabbit model of inhalation anthrax. Future studies are planned to optimize the dose and schedule of rPA vaccine that can be administered in the human population during a PEP emergency.

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