Development of protective immunity in New Zealand White rabbits challenged with 
*Bacillus anthracis* spores and treated with antibiotics and obiltoxaximab, a monoclonal 
antibody against protective antigen

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Running head: Recall immunity after inhalational anthrax treatment

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Recommended management of inhalational anthrax, a high-priority bioterrorist threat, includes antibiotics and antitoxins. Obiltoxaximab, a chimeric monoclonal antibody against anthrax Protective Antigen (PA) is licensed under the U.S. Food and Drug Administration’s (FDA’s) Animal Rule for treatment of inhalational anthrax. Because of spore latency, disease re-emergence after treatment cessation is a concern and there is a need to understand development of endogenous protective immune responses following antitoxin-containing anthrax treatment regimens. Here, acquired protective immunity was examined in NZW rabbits challenged with a targeted lethal dose of *B. anthracis* spores and treated with antibiotics, obiltoxaximab or a combination of both. Survivors of the primary challenge were re-challenged 9 month later, and monitored for survival. Survival rates after primary and re-challenge, for controls and animals treated with obiltoxaximab, levofloxacin or a combination of both were 0, 65, 100, 95% and 0, 100, 95, 89%, respectively. All surviving immune animals had circulating antibodies to PA and serum toxin neutralizing titers prior to re-challenge. Following re-challenge, systemic bacteremia and toxemia were not detected in most animals and levels of circulating anti-PA IgG titers increased starting 5 days post re-challenge. We conclude that treatment with obiltoxaximab, alone or combined with antibiotics, significantly improves survival of rabbits that received a lethal inhalation *B. anthracis* spore challenge and does not interfere with development of immunity. Survivors of primary challenge are protected against re-exposure, have rare incidents of systemic bacteremia and toxemia, and have evidence of an anamnestic response.

**Keywords:** Anthrax; Bacillus anthracis; Antitoxin; Protective Antigen; Monoclonal antibodies; Obiltoxaximab; Immune memory
INTRODUCTION

Inhalational anthrax is caused by spores of *B. anthracis*, a Gram-positive bacterium found in soils all over the world. *B. anthracis* is a top priority biowarfare Category A agent (1) and is considered a high priority public threat (2). Several antibiotics, such as ciprofloxacin, levofloxacin, and doxycycline are FDA-approved for the treatment of inhalational anthrax and must be given for 60 days to ensure complete spore clearance (3). Following the 2001 US anthrax bioterrorism attack, 11 people developed inhalational anthrax and 5 people died despite aggressive treatment with multiple antibiotics and supportive therapy (4). The high mortality rate among victims of inhalational anthrax brought forth the need to develop therapeutics against inhalational anthrax that could be adjunctive to antibiotics.

Virulence of *B. anthracis* depends critically on secretion of Lethal Toxin (LT) and Edema Toxin (ET) comprised of the enzyme moiety lethal factor (LF) or edema factor (EF), and the common binding component, protective antigen (PA). Toxins contribute to pathogenesis through mediating tissue cytotoxicity and suppressing host immune responses (5) and PA neutralization is effective in preventing the establishment and progression of inhalational anthrax in animal models (6). The level of anti-PA IgG at the time of challenge is the single most accurate correlate of protection against inhalation anthrax (7) and seroconversion has been extrapolated to predicting survival probability in humans (8, 9). Currently, CDC recommends that antitoxins against PA should be added to antimicrobial drug treatment for any patient for whom there is a high level of clinical suspicion for systemic anthrax (3). Obiltoxaximab (ETI-204) is a chimeric IgG1(κ) monoclonal antibody that binds with high affinity to PA and prevents its association with cellular receptors (10). Efficacy of obiltoxaximab has been demonstrated in animal models (11, 12) and it was recently licensed under the FDA’s Animal Rule for treatment of inhalational...
anthrax due to *B. anthracis* in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate (13).

Administration of antibiotics can lead to spore latency (14) and re-emergence of infection after discontinuation of treatment or due to non-compliance is a significant concern during inhalational anthrax. Thus, it is desirable to know whether adaptive immunity develops under differing treatment regimens and whether this memory immunity is protective against re-exposure. Here, we examined the immune status of NZW rabbits challenged with lethal dose of *B. anthracis* spores and treated with antibiotics, obiltoximab or a combination of both. The goal of the study was to evaluate the development of adaptive immune responses in spore-challenged animals given mono- or combination therapy and to compare protective immune status following differing treatment regimens. To our knowledge, this is the most comprehensive evaluation of adaptive immunity in anthrax infected and treated animals conducted to date.

**METHODS**

**Test system**

NZW rabbits (*Oryctolagus cuniculus*, specific pathogen free) weighing 2.8 to 4.2 kg with implanted vascular access ports (VAPs) were procured from Covance Research Products, Inc. (Denver, PA).

**Study Design**

The study was conducted in 2 phases, Phase 1 and Phase 2. The study was randomized, placebo-controlled, and parallel-group (50% male:50% female with exception of the control group in Phase 2 which was 100% males). In Phase 1, animals were randomized to treatment prior to
spore challenge by weight and gender. Animals were then randomized to a challenge day and challenge order such that there was approximately an equal number of animals from each group on each challenge day. In Phase 2, Phase 1 survivors and 12 additional naïve control animals were randomized to a challenge day and challenge order. In both study phases, rabbits were exposed nose only to an aerosolized dose of *B. anthracis* spores (Ames strain) targeting 200 times the median lethal dose (LD$_{50}$) (15) by real-time plethysmography. Phase 1 was open-label, but Phase 2 was blinded. Prior to the start of Phase 2, a transponder identification chip was implanted subcutaneously into the shoulder region of each rabbit that survived Phase 1 to take the place of the ear tag identification. The transponder number differed from the Phase 1 ear tag identification number which was obscured.

**Treatment Administration**

Levofloxacin (Levaquin® Oral Solution; Ortho-McNeil, Raritan, NJ) was administered by gastric intubation once daily at 50 mg/kg/day for 3 days; this dose was shown to be efficacious and well-tolerated when given at the first signs of infection to NZW rabbits (12). Obiloxaximab (Elusys Therapeutics, Inc.) was administered with the first dose of levofloxacin or water as a single IV dose of 16 mg/kg. No treatment was administered in Phase 2.

**Study Conduct**

All studies were conducted at the biosafety level 3 facilities at Battelle Biomedical Research Center, Columbus, Ohio, with the approval of Battelle’s Institutional Animal Care and Use Committee.
Pharmacodynamic Measurements

Blood collection

Blood samples for pharmacodynamic measurements were taken from the medial auricular artery or the marginal ear vein. In Phase 1, blood samples were collected from all animals at 1 hour +/- 5 min post first treatment time point to confirm the appropriate dosing. Starting on day 7 post-challenge (PC), samples were taken relative to the day of challenge. In Phase 2, all PC blood samples were collected relative to the median challenge time ±1 hour. Mean challenge times were calculated from the end time of the first and last animals challenged.

Measurement of serum PA.

Serum PA63 and/or PA83 were quantified using a validated (16, 17) sandwich ELISA method as previously described (12). Briefly, obiltoxaximab (Elusys Therapeutics, Inc.) was utilized as a capture reagent followed by detection with goat anti-PA antisera and horseradish peroxidase-conjugated anti-gamma chain secondary antibody (IgG; Invitrogen; Carlsbad, CA). The assay did not detect PA20 nor PA bound to serum obiltoxaximab. The assay lower limit of quantitation (LLOQ) was 9.68 ng/mL and the upper limit of quantitation (ULQQ) was 40,000 ng/mL.

Measurement of bacterial burden (blood and tissue)

Bacterial burden was assessed by incubating cultures of blood samples for 16 to 24 hours. Fresh blood samples were diluted 1:10 serially and each dilution was plated in triplicate. In addition, at the time of gross necropsy, small tissue sections of brain, spleen, liver, and bronchial lymph node were obtained, homogenized in PBS, and streaked onto a solid agar plate. The plates were incubated at 37 ± 2°C for a minimum of 48 hours and B. anthracis colonies were enumerated to
determine bacterial tissue burden.

Measurement of serum obiltoxaximab.

Serum obiltoxaximab was detected using a validated electrochemiluminescence (ECL) method utilizing the Meso Scale Discovery (MSD) platform. Free obiltoxaximab was captured by biotinylated rPA63 (List Biological Laboratories, Inc.) bound to an MSD streptavidin coated plate and ruthenylated goat anti-human antibody was used as the detection reagent. This immunoassay method selectively detects obiltoxaximab in rabbit serum due to the usage of an anti-human IgG detection reagent. Standard curve calibrators and quality control samples were prepared in rabbit serum. The generated signal was read on an MSD platform. The ECL units were collected and regressed using a 5PL Logistic (1/Y2 weighting factor) regression.

Measurement of serum antibodies to PA and Toxin Neutralization Assay (TNA)

Serum anti-PA IgGs were quantified using a validated ECL method with MSD Platform. Biotinylated rPA63 (List Biological Laboratories, Inc.) bound to an MSD streptavidin coated plate was used as a capture reagent and ruthenylated protein A/G (Thermo Scientific, Waltham, MA) was used as the detection reagent. Standard curve calibrators and quality control samples were prepared in rabbit serum. The signal was read on an MSD platform and the ECL units were regressed using a 5PL Logistic (1/Y2 weighting factor) regression. The results were measured against obiltoxaximab as a standard. Both obiltoxaximab and endogenous rabbit anti-PA IgG can be detected in this assay. Cell based TNA was used to qualitatively assess the neutralizing antibodies to LT as described previously (18). Briefly, a serial dilution of the test samples and controls were prepared in a separate plate followed by addition of LT and incubation to allow for
LT neutralization by serum antibodies. The mixtures were transferred to the J774A.1 cells-containing plates and incubated to allow intoxication to proceed. Cell viability was determined colorimetrically using a tetrazolium salt, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) as the reporter or signal system and the optical density (OD) values were read at 570 nm using a 690 nm reference wavelength. The TNA SAS program fitted the 7-point serial dilutions of the reference serum standard and test sample serum OD values to a four-parameter logistic-log (4PL) function, which was used to calculate the reportable values, 50% neutralization factor 50 (NF$_{50}$) and 50% effective dilution (ED$_{50}$). The assay has 3 levels of acceptance criteria: 1) plate OD acceptance criteria based on inspection of the OD values and with the % Coefficient of Variation (CV) for the OD values of each dilution of the reference standard (RS) required to be $\leq$ 20%, 2) plate SAS acceptance criteria based on calculations from the SAS output, including ED$_{50}$ of RS and quality controls (QC) and the parameterization of the RS and QC curves, 3) test sample acceptance criteria ensuring that the results from the test sample were acceptable and only applied to individual results that were greater than the limit of detection (LOD) for the assay. If any of these acceptance criteria were not met for a plate then the entire plate failed and was repeated.

The TNA has two reportable values, ED$_{50}$ and NF$_{50}$. The ED$_{50}$ is the reciprocal of the dilution of a serum sample that results in 50% neutralization of the LT. The neutralization capacity of each test serum in relation to that of the reference serum (50% neutralization factor, NF$_{50}$) is the quotient of the ED$_{50}$ of the test sample (ED$_{50}$TS) and the ED$_{50}$ of the reference serum standard (ED$_{50}$RS). NF$_{50}$ is calculated as follows: NF$_{50} = \frac{\text{ED}_{50}^{\text{TS}}}{\text{ED}_{50}^{\text{RS}}}$. The NF$_{50}$ serves as a relative measure of toxin neutralization.
Statistical Considerations

The primary endpoint was the survival rate defined as the percentage of animals alive at the scheduled Phase 2 termination. The survival rate in each of the groups was compared to the placebo (all challenged animals) using one-sided Boschloo's exact test with a Berger-Boos correction of gamma=0.001 (19). Animals did not receive treatment in Phase 2 and their group assignments were pre-defined based on the primary challenge. Re-challenge was considered successful if the mortality rate in the re-challenge control population exceeded 90%. Survival rates were summarized for Phase 1 based on the treatment administered and including only animals surviving to receive treatment. Analyses were conducted with R using the package "exact". Exact 95% confidence intervals for differences in survival rates are based on the score statistic (Proc Freq of SAS® version 9). Baseline characteristics (gender, weight, and anti-PA IgG levels) were summarized at Phase 2 pre-challenge using descriptive statistics. Descriptive statistics for serum PA, quantitative bacteremia, anti-PA IgG and TNA levels were generated for all Phase I and Phase 2 time points. Anti-PA IgGs and TNA levels were analyzed using analysis of variance (ANOVA) models fitted to each time point and Tukey’s multiple comparisons were used to test whether levels significantly differed between the groups.

RESULTS

Establishment of memory immunity in NZW rabbits.

The overview of the study design is shown in Table 1. In Phase 1, NZW rabbits were spore challenged and treated 30 hours later with either placebo, a single 16 mg/kg dose of obiltoximab, first of 3 daily doses of levofloxacin, or obiltoximab in combination with levofloxacin. Published data from patients with cutaneous anthrax (20) suggest that
seroconversion to detectable anti-PA IgG responses occurs only in the patients with evidence of systemic infection. In *B. anthracis* spore-challenged NZW rabbits, mean time to first appearance to PA in blood was approximately 30 hours (21). Thus, 30 hours was selected as treatment time to ensure that majority of treated animals had evidence of systemic disease. Pre-treatment disease parameters are summarized in Table 2. The mean challenge spore doses were comparable in all groups. Systemic infection, as measured by presence of blood bacteremia, was detected in 85%-90% of rabbits receiving active treatment and the geometric means for bacteremia were similar among groups that received levofloxacin, obiltoxaximab or a combination of both. For all four groups, PA levels were below level of quantitation in most animals.

All eight Phase 1 control animals succumbed to *B. anthracis* infection, while 65% (13/20), 100% (20/20), and 95% (19/20) of the animals treated with obiltoxaximab, levofloxacin, or obiltoxaximab and levofloxacin, respectively, survived to re-challenge (Figure 1A). The proportion of animals surviving the primary challenge in Groups 1 through 3 was significantly greater than that of the Phase I control group (Table 3). Complete resolution of bacteremia occurred by day 7 following primary challenge for all rabbits that survived to that time point and was maintained through the Phase 1 observation period.

**Development of anti-PA antibody responses following challenge and treatment.**

Levels of circulating anti-PA IgG titers were measured using a Protein A/G method that is species non-specific and detects both endogenous rabbit antibodies to PA and obiltoxaximab. To understand the relative contributions of endogenously secreted anti-PA antibodies and obiltoxaximab to the overall anti-PA IgG profile, obiltoxaximab was additionally measured by a human IgG-specific assay that detects obiltoxaximab but not rabbit antibodies. In infected
rabbits, intravenously administered obiltoxaximab has a serum half-life of approximately 1 day and is cleared or significantly diminished in circulation by day 28 post-challenge (Figure 1B and data not shown). In Phase 1 of the study, anti-PA antibody levels were not detected prior to challenge but were elevated by day 7 post-challenge in all groups (Figure 1C). On days 7 and 14 following challenge, the mean anti-PA IgG levels in the group treated with levofloxacin only (Group 2) were lower than those in the group treated with obiltoxaximab or the group treated with obiltoxaximab and levofloxacin (Figure 1C). These differences were attributed to detection of only endogenous anti-PA IgG in Group 2 (levofloxacin monotherapy group). In contrast, both endogenous antibodies and obiltoxaximab were detected in Group 1 and 3 animals during the first 4 weeks following challenge due to the non-specific format of the assay. By 28 days following challenge, obiltoxaximab levels were either below detection or markedly reduced compared to previous time points (Figure 1B). On day 56 post-challenge, all rabbits had circulating anti-PA IgGs except for one animal in Group 2 that did not have detectable anti-PA IgG at any Phase 1 time point. Comparable levels of anti-PA IgG were observed in all groups and those levels were maintained for the duration of the observation period with only 2 rabbits in Group 3 having no detectable anti-PA IgGs at days 112, 140 and 168 (Figure 1C).

Toxin-neutralizing antibody levels were measured via the TNA in serum of surviving rabbits on days 28 and 56 post-challenge in Phase 1. The primary TNA endpoints were the effective dilution-50 (ED$_{50}$) and the neutralization factor-50 (NF$_{50}$). As expected, TNA ED$_{50}$ (Figure 2A) and NF$_{50}$ (Figure 2B) were below the limits of detection for all animals in Groups 1 through 3 prior to primary challenge. By day 28 following primary challenge, all surviving animals had developed a TNA ED$_{50}$ and NF$_{50}$ titer which continued to be detected at 2 months following challenge (Figure 2), except for one animal in Group 2 that also did not have a
measurable anti-PA IgG response in Phase 1 (discussed above). However, this rabbit had a quantifiable anti-PA IgG titer immediately prior to re-challenge albeit in the absence of detectable TNA activity.

**Survival following re-challenge.**

Prior to re-challenge, the mean body weights, ages, anti-PA IgG levels, and TNA ED$_{50}$ and NF$_{50}$ titers were comparable across all Phase 1 treatment groups (Table 4). All primary challenge survivors (Groups 1 through 3) had measurable anti-PA IgG levels and TNA activity prior to the re-challenge (day -7 or 9 month post primary challenge) with exception of two rabbits in Group 3 and one rabbit in Group 2 (discussed earlier). In two Group 3 rabbits, anti-PA IgGs were detectable until 4 and 5 months (days 140 and 168) post-primary challenge and declined thereafter.

All Phase 1 survivors and 12 naïve approximately age- and weight-matched control rabbits received re-challenge with a targeted dose of 200 LD$_{50}$ of *B. anthracis* spores. The administered spore doses were comparable across all groups and no treatment was administered during Phase 2. All twelve Phase 2 naïve control animals succumbed to *B. anthracis* infection, while 100% (13/13), 95% (19/20), and 89% (17/19) of the animals treated with obiltoxaximab, levofloxacin, or obiltoxaximab and levofloxacin during primary challenge, respectively, survived to the end of the Phase 2 observation period (Figure 3A). The proportion of surviving animals in each Phase 1 obiltoxaximab treatment group was significantly greater than the Phase 2 control group at the 0.025 level (p < 0.0001 for each group as compared to the control). There were no significant differences in the proportion of animals that survived re-challenge among Groups 1, 2 and 3. The time from re-challenge to death was also not significantly different among the groups.
Development of systemic disease following re-challenge.

Bacteremia and toxemia development following re-challenge were examined. In the control group, 75% (8/12) of rabbits were bacteremic 24 hours following challenge and bacteremia levels rose exponentially in all rabbits (Figure 3B) with mean terminal bacteremia levels of $7.9 \times 10^6$ cfu/ml. All control animals were bacteremic at least at one time point following challenge. In contrast, *B. anthracis* was not detected in blood cultures at nearly all time points for animals in Groups 1 through 3 that had been challenged nine months prior to a second challenge. In addition, positive *B. anthracis* cultures in survivors were transient and only present in low numbers (1/13 Group 1; 1/19 Group 2; 2/17 Group 3) (Figure 3B). The animals in Groups 2 and 3 that succumbed to disease during the re-challenge all had positive blood terminal cultures ($1.7 \times 10^2$ cfu/ml, Group 2 non-survivor; $4.5 \times 10^2$ cfu/ml and $3.1 \times 10^6$ cfu/ml, Group 3 non-survivors).

In addition, bacterial dissemination to the peripheral tissues (bronchial lymph node, brain, liver and spleen) was examined in all survivors at the end of the study and in all Phase II non-survivors. *B. anthracis* was detected in at least three of the tissues evaluated in all animals that succumbed during Phase II. In contrast, all animals that survived re-challenge did not have a positive culture from any tissue assessed.

Presence of circulating PA was examined in each group prior to and following Phase 2 challenge (Figure 3C). The Phase 2 naïve control animals had detectable PA levels as early as 24 hours after challenge and all control animals had elevated PA levels prior to succumbing to infection. In contrast, the majority of re-challenged animals did not have detectable PA throughout Phase 2 period (Figure 3C). Prior to re-challenge (day -7), all animals in Groups 1
through 3 were negative for PA with exception of one animal in Group 1 (obiltoxaximab Phase 1
treatment). PA levels for this animal were above the limit of quantitation and at a constant low
level until the scheduled sacrifice on day 21. Persistent PA detection in this animal may have
been related to cross-reactivity with circulating anti-obiltoxaximab antibodies due to the assay
format. *B. anthracis* was not observed in blood cultures at any Phase 2 time points for this
animal (including day -7) and levels of PA detected in this animal were low and did not change
significantly throughout Phase 2. In addition, transient PA increase following re-challenge was
observed in several Group 2 and 3 animals. Two animals in Group 2 were positive for PA on day
5 post-secondary challenge, and both animals were also positive for bacteremia at this time point.
One of these animals survived to the scheduled sacrifice, while the second PA positive animal
succumbed shortly after day 5 time point. In addition, two Group 3 animals had quantifiable PA
levels at the day 3 time point and both animals succumbed to infection.

**Boost in memory immunity after re-challenge.**

Figure 4 summarizes results for anti-PA IgG and TNA levels by time point from day -7 through
day 21 post-re-challenge. There were no significant differences among the re-challenged groups
at any of these time points. With the exception of two Group 3 rabbits, all treated animals that
survived the primary challenge had measurable anti-PA IgG levels prior to the re-challenge and
those levels remained unchanged for first three days after challenge. The anti-PA IgG levels
increased for all treatment groups by 5 days post-re-challenge and remained elevated through the
end of the in-life period.

On day 21 post re-challenge, TNA ED$_{50}$ and NF$_{50}$ titers increased from the pre-challenge
baseline in all surviving animals (Figure 4B, C and Table 5). To determine if the TNA levels
were significantly different among the groups, an ANOVA model was fitted to the base-10 log-
transformed TNA ED$_{50}$ by Phase 2 time point. There were no significant differences among the
groups for TNA ED$_{50}$ and NF$_{50}$ titers either before or after re-challenge (Table 5).

**DISCUSSION**

We examined development of protective immunity in rabbits challenged with a lethal
dose of *B. anthracis* spores and treated with obiltoxaximab administered intravenously alone or
in combination with antibiotics. This study models a post-exposure scenario of treatment with an
antibiotic and/or anthrax antitoxin following inhalational exposure to spores and development of
systemic disease. Currently, several monoclonal and polyclonal antitoxins are stockpiled by the
US government and antitoxins are recommended for use in conjunction with antimicrobial
therapy in patients suspected of having systemic anthrax (23).

Early during infection, anthrax toxins suppress host immunity through impairment of
innate immune cells and stifling efficient priming and effector functions of adaptive immunity
(5, 24, 25). Therefore, it is plausible that toxin inhibition by obiltoxaximab administered alone or
in combination with antibiotics may improve immune function following infection and promote
establishment of long term immunological memory. Alternatively, it could be envisioned that
PA-binding antitoxins may sequester free PA from antigen presenting cells and interfere with
priming of anti-PA responses. Thus, the objectives of this study were to establish that treatment
with obiltoxaximab alone or in combination with antibiotics following primary anthrax challenge
results in the development of protective immune and to compare the magnitude of recall immune
responses in animals administered differing treatment regimens.

To assess protective immunity following challenge and treatment, we examined survival
and development of bacteremia and toxemia after immune animals were re-exposed to lethal
dose of spores. In addition, kinetics of endogenously produced anti-PA IgGs were characterized
throughout the study. The results of this study are consistent with the hypothesis that
obiltoxaximab administration does not interfere with priming of adaptive immunity and animals
treated with obiltoxaximab after exposure to *B. anthracis* develop an immune response
protective against re-challenge. In addition, we demonstrate that humoral immune responses (as
measured by circulating rabbit anti-PA IgGs) develop comparably in animals given antibiotics,
antitoxin, or a combination of both. Cumulatively, our study outcomes project that toxin
neutralization via either administration of antitoxin that directly removes toxin from circulation
or via administration of antibiotic that limits toxin production by bacterial cells enables host
survival and progression toward development of adaptive immune responses. These results are
in alignment with the published analyses of immune responses in human victims of anthrax
outbreak following antibiotic treatment (26).

In Phase 1 of the study, rabbits were treated with obiltoxaximab, levofloxacin, or a
combination of obiltoxaximab and levofloxacin 30 hours following spore challenge. The
treatment time was selected based on a previously published study where mean time to PA
appearance in blood was 30 hours post-challenge (21). While the majority of animals in our
study were negative for blood PA at the time of treatment administration, we attribute this
discrepancy to the differences in the sensitivity of assays for PA measurement employed in our
study and in the study by Migone et al (limits of detection of 9.68 ng/mL and 0.6 ng/mL,
respectively). Notably, 85-90% of the animals in treatment groups were positive for blood
bacteremia in our study and it can be projected that more animals would have had positive PA
measurements if a more sensitive assay were employed. The observed Phase 1 survival rates
were consistent with previously published results (12). All treated animals had evidence of endogenous immune responses to PA and there was no evidence of reemergence of disease due to spore latency in any of the treatment groups. Following re-challenge, survivors of primary challenge were highly protected against re-exposure irrespective of the treatment administered. Toxemia and bacteremia were detected in less than 10% of re-challenge survivors, and were transient and only present in low concentrations, suggesting that memory immunity was effective in curbing development of systemic disease upon re-exposure. In addition, none of the animals that survived re-challenge had evidence of tissue bacterial burden.

Development of anti-PA antibody responses and the functional ability of serum to neutralize lethal toxin was characterized in survivors of the primary challenge. Based on observations in survivors of the 2001 US anthrax bioterrorism attack (26), it was anticipated that the anti-PA IgG levels would steadily decrease over the six-month period following primary challenge. However, the anti-PA IgG titers for nearly all animals remained stable between 2 and 9 months post primary challenge. Immediately prior to re-challenge, all but two rabbits had an anti-PA IgG titer, and levels of circulating anti-PA antibodies or toxin neutralizing activity were comparable between rabbits in the obiltoxaximab treatment arms and levofloxacin alone arm.

Notably, within 5 days following re-challenge, anti-PA IgG levels for nearly all animals increased compared to the re-challenge baseline, suggesting that these animals had developed a memory B-cell response following primary challenge. Levels of TNA NF₅₀ in all treatment groups were lower at re-challenge as compared to predicted level of 0.56 corresponding to a 70% probability of survival in rabbits vaccinated with Anthrax Vaccine Absorbed (18). It is plausible that antibodies elicited by active infection may be more effective as compared to a vaccine or infection may activate other immune components such as antibodies to non-PA antigens or
While nearly all survivors of re-challenge had anti-PA IgG titers and the functional ability to neutralize toxin, two of the survivors had notable results. An anti-PA IgG titer declined in one combination-treated rabbit by 4 months post-primary challenge but this animal had a quantifiable anti-PA IgG titer starting 7 days post-re-challenge and did not have bacteremia at any point throughout the re-challenge period. In contrast, one levofloxacin-treated animal did not have a detectable anti-PA IgG or TNA activity throughout Phase 1 period. This animal developed an anti-PA IgG titer approximately 9 months post-primary challenge (in the absence of quantifiable TNA) and continued to have a quantifiable anti-PA IgG titer throughout the re-challenge period. While this animal survived, there was a detectable bacteremia for this animal at days 3 and 5 post-re-challenge.

The anti-PA IgG and TNA results for 2 non-survivors of the re-challenge were examined closely. One non-survivor was treated with obiltoxaximab and levofloxacin in Phase 1 and had an anti-PA IgG titer that decreased after 2 months and was undetectable by 5 months post-challenge. The anti-PA IgG titer in this animal remained below limit of quantitation until 3 days post-re-challenge when the animal succumbed to infection. In contrast, one re-challenge non-survivor from the levofloxacin treatment group had a detectable anti-PA IgG titer and TNA activity prior to re-challenge. This animal died despite having an anti-PA IgG titer through 5 days post-re-challenge. These results suggest that the presence of circulating-PA IgG titers and toxin neutralization titers after priming of adaptive immunity may not be always sufficient to protect against repeat exposure to spores.

To our knowledge, this study provides the most comprehensive longitudinal investigation of anti-PA antibody responses in rabbits following B. anthracis infection and treatment. Previous
study found that rabbits administered a human monoclonal antibody to PA at the time of spore challenge were protected against re-challenge 5 weeks later (27). However, definitive conclusions regarding role of endogenous immunity could not be drawn from this study because injected antibody was still found in circulation at the time of re-challenge. It should be noted that our study has some limitations. All challenges were conducted with uniformly high spore dose, and no conclusions can be drawn regarding relationship between spore dose and anti-PA IgG levels. In addition, only responses to PA were investigated and anti-PA IgG and TNA were sole correlates of protection. Following infection and treatment, adaptive responses against other bacterial and toxin epitopes may develop and contribute to protection (28, 29, 30, 31). Analysis of cross-species survival data across multiple anthrax vaccination studies suggested that TNA is highly predictive of survival in animal models, and the cross-species results support that extrapolation from animal to human data may be informative (8, 9). Finally, results of animal studies should not be over-interpreted in the absence of further confirmation through analyses of human data gathered during inhalational anthrax outbreaks. However, we believe that results obtained in rabbit model of inhalational anthrax reasonably predict what would occur in humans under similar circumstances. NZW rabbits have been a valuable model for testing and development of vaccines and therapeutics against anthrax and are an accepted model for approval of anthrax therapeutics under the FDA’s Animal Rule (32). The course of inhalational anthrax in the rabbit model is reasonably comparable to human disease and rabbits are expected to react with a response predictive for humans. Protective Antigen is thought to have immunomodulatory activity toward human neutrophils and cells of mononuclear origin function and has been proposed to directly suppress human innate immune responses (24). Results of the studies with toxin-deficient strains of *B. anthracis* in NZW rabbits established that protective
antigen plays a major role in the anthrax pathogenesis in rabbits through modulating innate host effector mechanisms with possible immunomodulating effect on heterophils, the rabbit equivalent of human neutrophils (33). While additional future studies can address the specific mechanisms of PA-induced immunosuppression in cells of human and rabbit origin, the interference of toxin with innate immune system has been plausibly established in both species and analyses of immune responses in treated rabbits have been warranted. Therefore, we believe that our findings have relevance to understanding the immunological outcomes that follow anthrax infection in humans.

Notes

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Figure 1. Kinetics of anti-PA antibodies in challenged and treated animals.

NZW rabbits were aerosol challenged with targeted 200 LD_{50} of *B. anthracis* spores and treated with obiltoxaximab (circles), levofloxacin (triangles) or combination of both (squares) at 30 hrs PC. (A) Kaplan-Meier curves representing time to death from challenge and survival data for each group are shown. (B, C) Serum samples were collected at indicated times relative to treatment (post-treatment, PT) or challenge for specific assessment of obiltoxaximab (B) or non-specific assessment of all circulating anti-PA IgGs (C). (A) Each symbol represents individual animal values. Vertical lines indicate means and standard error for each group at each indicated time point (B). (C) Vertical lines indicate means and standard deviations for each group at each indicated time point. Numbers of animals with measurable total anti-PA IgG levels (n) and the total numbers of animals surviving to each time point (N) are shown on the bottom. Total anti-PA pool is comprised of endogenous rabbit antibodies to PA as well as circulating obiltoxaximab. Dotted lines indicate lower limit of quantitation for each assay. For statistical computations, levels below limit of detection were replaced with ½ LLOQ. LLOQ was 50 ng/ml in each assay.

Figure 2. Toxin neutralizing activity in serum of challenged and treated animals (Phase 1).

Serum samples were collected immediately prior to primary challenge or at indicated times post-challenge for the assessment of TNA ED_{50} (A) and NF_{50} (B). Vertical lines indicate means and standard error. For statistical computations, TNA ED_{50} and NF_{50} levels below limit of detection were replaced with 11.5 and 0.027 (½ of each respective LOD).
Figure 3. Survival and systemic disease development in immune animals following re-challenge.

Nine month after primary challenge, Phase 1 survivors and 12 additional naïve controls were challenged with targeted 200 LD$_{50}$ of B. anthracis spores and monitored for 21 days. (A) Kaplan-Meier curves representing time to death from challenge and survival data for each group are shown. (B, C) Whole blood samples were collected prior to treatment (D -7) or at indicated times post re-challenge (PC) for the assessment of quantitative bacteremia (B) and circulating free PA (C). Shown are means and SEM for bacteremia and free PA at each indicated time point. Dotted lines represent limit of detection (LOD) for bacteremia or limit of quantitation (LOQ) for PA.

Numbers of animals with measurable levels for each parameter (n) and the total numbers of animals surviving to each time point (N) are indicated at the bottom. Shaded areas indicate that no animals survived to the time point. For statistical computations, bacteremia levels below LOD were replaced with 2 cfu/mL (½ LOD) and PA levels below limit of quantitation (LOQ) were replaced with 4.84 ng/ml (½ LOQ).

Figure 4. Assessment of anti-PA IgG and TNA responses following re-challenge.

Whole blood samples were collected prior to re-challenge (D -7) or at indicated times post-re-challenge (PC) for the assessment of circulating anti-PA IgG levels (A), TNA ED$_{50}$ (B) and TNA NF$_{50}$ (C). Shown are means and SD (A) and SEM (B, C) at each indicated time point. Dotted lines represent LLOQ (anti-PA IgG) and LOD (TNA ED$_{50}$ and NF$_{50}$).
### TABLE 1 Study design

| Group | n   | Targeted LD_{50} Challenge Dose (mg/kg, IV) | Obiltoxaximab (mg/kg, IV) | Levofloxacin (mg/kg/day, PO) x 3 days | Treatment Time (hrs, PMC) |
|-------|-----|------------------------------------------|------------------------|-------------------------------------|--------------------------|
|       |     | **Phase 1 (Primary challenge)**          |                        |                                     |                          |
| 1     | 20  | 200                                      | 16                     | 0 (vehicle)                         | 30                      |
| 2     | 20  | 200                                      | 0 (saline)             | 50                                  | 30                      |
| 3     | 20  | 200                                      | 16                     | 50                                  | 30                      |
| 4     | 8   | 200                                      | 0 (saline)             | 0 (vehicle)                         | 30                      |
|       |     | **Phase 2 (Re-challenge)**               |                        |                                     |                          |
| 1     |     | Survivors Group 1 (Phase 1)              |                        |                                     |                          |
| 2     |     | Survivors Group 2 (Phase 1)              |                        |                                     |                          |
| 3     |     | Survivors Group 3 (Phase 1)              |                        |                                     |                          |
| 5     | 12  | 200                                      | None                   | None                                | NA                      |

IV, intravenous; PO, oral; PMC, post mean challenge; NA, not applicable
| Group # | Dose and Route (Obiltoxaximab + Levofloxacin) | Mean LD₅₀ Challenge Spore Dose (SD) | Bacterial Burden at PTT (cfu/mL) | PA at PTT (ng/mL) |
|--------|---------------------------------|-------------------------------|-------------------------------|----------------|
| 1      | Obiltoxaximab (16 mg/kg IV) + Water (0 mg/kg/day PO x 3 days) | 238.1 (58.6) | 17/20 (85) | 5.9E+02 (1.24+02, 2.8+03) | 4/19 (21) | 6.8 (4.8, 9.6) |
| 2      | Saline (0 mg/kg IV) + Levo (50 mg/kg/day PO x 3 days) | 209.2 (41.0) | 18/20 (90) | 3.4E+02 (9.7+01, 1.2+03) | 1/20 (5) | 5.5 (4.3, 7.0) |
| 3      | Obiltoxaximab (16 mg/kg IV) + Levo (50 mg/kg/day PO x 3 days) | 207.1 (37.4) | 17/20 (85) | 5.9E+02 (1.14+02, 3.1+03) | 4/19 (21) | 6.5 (4.9, 8.7) |
| 4      | Saline (0 mg/kg IV) + Water (0 mg/kg/day PO x 3 days) | 221.9 (47.0) | 4/8 (50) | 2.4E+01 (1.9+00, 3.1+02) | 0/8 (0) | NA |

PTT, Prior to Treatment; CI, confidence interval; cfu, colony forming units; NA, not applicable
## TABLE 3 Phase 1 Survival Rates

| Group # | Dose and Route (Obiltoxaximab + Levofloxacin) | Time of administration (hrs post-challenge) | Survival % (# Survived/#Treated) | P-value | 95% CI$^1$ |
|---------|---------------------------------------------|--------------------------------------------|----------------------------------|---------|------------|
| 1       | Obiltoxaximab (16 mg/kg IV) + Water (0 mg/kg/day PO x 3 days) | 30 ± 4 | 65% (13/20) | 0.0018 | (0.156, 0.846) |
| 2       | Saline (0 mg/kg IV) + Levo (50 mg/kg PO x 3 days) | 30 ± 4 | 100% (20/20) | 0.0010 | (0.631, 1.000) |
| 3       | Obiltoxaximab (16 mg/kg IV) + Levo (50 mg/kg/day PO x 3 days) | 30 ± 4 | 95% (19/20) | 0.0010 | (0.604, 0.999) |
| 4       | Saline (0 mg/kg IV) + Water (0 mg/kg/day PO x 3 days) | 30 ± 4 | 0% (0/8) | NA | NA |

NA, not applicable

$^1$ Boschio Test (with Berger-Boos modification of gamma=0.001) compared to control

$^2$ Exact 95% confidence interval of difference in survival rate
### TABLE 4 Phase 2 Demographics

| Group #       | n   | Mean Age (SD) | Mean Weight\(^1\), kg (SD) | Geometric Mean Anti-PA IgG, ng/mL (%CV)\(^3\) | Geometric Mean TNA ED\(_{50}^{50}/\text{NF}^{50}\)\(^3\) | Mean LD\(_{50}\) Challenge Spore Dose (SD) |
|---------------|-----|---------------|-----------------------------|-----------------------------------------------|-------------------------------------------------|------------------------------------------|
| 1 (Survivors Group 1 Phase 1) | 13  | 17.0 (0.00)   | 3.8 (0.43)                  | 2700 (77)                                    | 256/0.321                                      | 238.1 (58.6)                             |
| 2 (Survivors Group 2 Phase 1) | 20  | 17.0 (0.00)   | 3.9 (0.41)                  | 3140 (212)                                   | 383/0.558                                      | 209.2 (41.0)                             |
| 3 (Survivors Group 3 Phase 1) | 19  | 17.0 (0.00)   | 3.9 (0.39)                  | 1520 (343)                                   | 163/0.287                                      | 207.1 (37.4)                             |
| 5 (Naïve controls)           | 12  | 11.3 (0.98)   | 3.9 (0.12)                  | <LOQ (0)                                     | NA                                             | 221.9 (47.0)                             |

\(^1\)Measured on day of challenge  
\(^3\)Measured only in Phase 1 survivors  

LOQ Limit of quantitation=50.0 ng/mL for anti-PA IgG ELISA.
### TABLE 5 Phase 2 ANOVA Results for TNA by Time Point

| Day Post Re-challenge | Geometric Mean | Group Effect | P Value |
|-----------------------|----------------|--------------|---------|
|                       | Group 1 | Group 2 | Group 3 |         |
| TNA ED$_{50}$         |       |         |         |         |
| -7                    | 256    | 383     | 163     | 0.1031  |
| 21                    | 5020   | 6000    | 3670    | 0.6469  |
| TNA NF$_{50}$         |       |         |         |         |
| -7                    | 0.321  | 0.558   | 0.287   | 0.0938  |
| 21                    | 8.36   | 14.1    | 12.7    | 0.0633  |

1Phase 2 re-challenge occurred 9 months following primary challenge.
Proportion of animals with measurable total anti-PA responses in Phase 1 (n/N)

|          | Group 1 | Group 2 | Group 3 |
|----------|---------|---------|---------|
|          | Obiltoxaximab | Levofloxacin | Combination |
| Day 0  | 13/13  | 13/13  | 13/13  |
| Day 1  | 13/13  | 13/13  | 13/13  |
| Day 2  | 13/13  | 13/13  | 13/13  |
| Day 3  | 13/13  | 13/13  | 13/13  |
| Day 4  | 13/13  | 13/13  | 13/13  |
| Day 5  | 13/13  | 13/13  | 13/13  |
| Day 6  | 13/13  | 13/13  | 13/13  |
| Day 7  | 13/13  | 13/13  | 13/13  |
**Proportion Survival**

| Time to death (days) | Control Phase 2 (Gr 5) | Obiltoxaximab (Gr 1) | Levofloxacin (Gr 2) | Combination (Gr 3) |
|----------------------|------------------------|----------------------|---------------------|--------------------|
| 0                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 1                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 2                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 3                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 4                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 5                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 6                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 7                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |
| 8                    | 2.0                    | 3.0                  | 4.0                 | 5.0                |

**LOD**

| Control Phase 2 (Gr 5) | Obiltoxaximab (Gr 1) | Levofloxacin (Gr 2) | Combination (Gr 3) |
|------------------------|----------------------|---------------------|--------------------|
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |
| 10                    | 10                   | 10                  | 10                 |

**B. anthracis (cfu/mL blood)**

| Group 1 | Group 2 | Group 3 | Group 4 |
|---------|---------|---------|---------|
| 0/12    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |

**PA ng/ml blood**

| Group 1 | Group 2 | Group 3 | Group 4 |
|---------|---------|---------|---------|
| 0/12    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |

**Proportion of animals with measurable bacteremia in Phase 2 (n/N)**

| Group 1 | Group 2 | Group 3 | Group 4 |
|---------|---------|---------|---------|
| 0/12    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |

**Proportion of animals with measurable PA in Phase 2 (n/N)**

| Group 1 | Group 2 | Group 3 | Group 4 |
|---------|---------|---------|---------|
| 0/12    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |
| 1/13    | 1/20    | 0/19    | 0/19    |
| 0/13    | 0/20    | 0/19    | 0/19    |

**Time to death (days)**

A. Proportion Survival

B. B. anthracis (cfu/mL blood)

C. PA ng/ml blood
A

Anti-PA IgG (ng/ml)

Day post challenge (Phase 2)

B

Mean TNA ED50

Day post challenge (Phase 2)

C

Mean TNA NF50

Day post challenge (Phase 2)