Characterizing a therapeutic model of inhalational anthrax using an increase in body temperature as a trigger for treatment in New Zealand white rabbits

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Keywords: Bacillus anthracis, anthrax, monoclonal antibody, passive immunization, therapeutic, New Zealand White rabbit

Running title: Rabbit inhalation anthrax therapeutic model
The development of an appropriate animal therapeutic model is essential to assess the potential efficacy of therapeutics for use in the event of a *Bacillus anthracis* exposure. We conducted a natural history study that showed New Zealand White Rabbits exhibited a significant increase in body temperature (SIBT), changes in hematologic parameters, increases in C-reactive protein, and succumbed to disease with an average time to death of approximately 73 hours following aerosol challenge with *B. anthracis* Ames spores. The SIBT was used as a trigger to treat with a fully human monoclonal antibody directed at protective antigen (PA). Ninety percent (9/10) of the treated rabbits survived the lethal inhalational challenge of *B. anthracis*. Further characterization investigated the protective window of opportunity for anti-PA antibody administration up to 12 hours post-onset of SIBT. Eighty-three percent (5/6) of the rabbits treated at SIBT and 100% (6/6) of those treated at 6 hours after SIBT survived challenge. Only 67% (4/6) of the rabbits treated at 12 hours after SIBT survived. The increase in body temperature corresponded with both bacteremia and antigenemia (PA in the blood), indicating that SIBT is a suitable trigger to initiate treatment in a therapeutic model of inhalational anthrax.
Introduction

*Bacillus anthracis*, the etiologic agent of anthrax, is a gram-positive, spore-forming bacterium that can cause human disease via the gastrointestinal, cutaneous, or inhalation (pulmonary) routes, each exhibiting different clinical manifestations of disease, pulmonary being the most lethal (4). The virulence of *B. anthracis* is predicated on the bacterial poly-D-glutamate capsule and a tripartite toxin consisting of three polypeptides, protective antigen (PA), lethal factor (LF), and edema factor (EF), which interact to form two interlinked toxins (17). PA and LF combine to produce anthrax lethal toxin (LT), and the PA and EF combine to produce edema toxin (ET). Upon binding to the host cell, the N-terminal region of PA (PA83) is cleaved and the resulting 63 kDa protein (PA63) heptamerizes forming a ring structure with competitive binding sites for three molecules of LF and/or EF (18). Once the enzymatic moiety (EF and/or LF) binds to the oligomerized PA63, the complex enters the cell via receptor-mediated endocytosis. Conformational changes to PA63 in the acidic endosome facilitate the translocation of EF and LF into the cytosol (2). EF is a calmodulin-dependent adenylyl cyclase that forms 3',5'-adenosine monophosphate from adenosine triphosphate in many different types of cells (14). LF is a zinc metalloprotease with mitogen-activated kinase kinases 1, 2, 3, 4, 6, and 7 as the only known substrates (6, 21, 29). The enzymatic actions of these toxin components inhibit signaling cascades required for proper immune cell function and contribute to the pathology associated with disease such as edema and hemorrhage of infected tissues.

A humoral response to PA confers protection against anthrax and PA is the dominant antigen in the current licensed anthrax vaccine adsorbed (AVA). Protection of rabbits vaccinated with recombinant PA (rPA) correlates directly with anti-PA titer (15). Passive immunization...
with anti-PA antibodies has also been shown to provide protection in animal models (11, 16, 20, 22, 23).

The ability to generate high titer *B. anthracis* spores using basic microbiological techniques combined with the ability of this agent to be disseminated by aerosolization, has made anthrax a bioterrorist and military threat. It is current practice to vaccinate at-risk individuals such as military personal, first responders, and laboratory workers with AVA, but due to the rare occurrence of anthrax in the human population it may not be feasible to vaccinate the general population. However, there is the need to protect an unvaccinated population exposed to an intentional release, as evidenced in 2001 with the anthrax letter attacks. Such protection would come in the form of antibiotics, post-exposure vaccination, and passive immunization.

To develop these medical countermeasures animal models are required to assess the efficacy of vaccines and therapeutics. The three general indications of medical countermeasures against anthrax are:

1. General use prophylaxes: given prior to exposure (e.g. vaccines)
2. Post-exposure prophylaxes: given after exposure, prior to onset of symptoms (e.g. vaccines and antibiotics)
3. Therapeutics: given once the subject has presented with symptoms (e.g. antibiotics and passive immunization).

The rabbit and non-human primate have been used extensively in medical countermeasure development and are considered appropriate animal models of human inhalational anthrax (7, 27, 28, 32). Developing a true therapeutic treatment model requires that the animal demonstrate clinical signs of disease (e.g. bacteremia) prior to treatment. However, by the time bacteremia can be confirmed by culture results during the conduct of an efficacy
study, the disease may progress to a state where therapeutic intervention is no longer effective. Therefore, we explored the use of clinical and physiological changes observed following a lethal exposure to \textit{B. anthracis} as potential “triggers for treatment.” We utilized a significant increase in body temperature (SIBT) as the trigger to treat with a fully human monoclonal antibody to PA. Our hypothesis was that treatment following exhibition of SIBT would result in increased protection of animals exhibiting signs of inhalational anthrax. In the current body of work, the antibody was administered therapeutically (animals confirmed as bacteremic at the time of treatment) and showed significant protection in the New Zealand White (NZW) rabbit model of inhalational anthrax.

Materials and Methods

New Zealand White Rabbits

NZW rabbits (specific pathogen-free) were obtained from Covance Research Products (Denver, PA). The animal procedures were approved by Battelle’s Institutional Animal Care and Use Committee. All work was done in a BSL-3/ABSL-3 laboratory registered with the Centers of Disease Control and Prevention and inspected by the Department of Defense and the U.S. Department of Agriculture.

Aerosol Challenge

A modified Microbiological Research Establishment type three-jet Collison nebulizer (BGI, Waltham, MA) with a precious fluid jar was used to generate a controlled delivery of aerosolized \textit{B. anthracis} Ames spores, from a liquid suspension into a muzzle only exposure
chamber. Atmospheric samples from within the exposure chamber were collected in an impinger (Model 7541, Ace Glass Inc.) filled with approximately 20 ml of sterile water. The liquid in the impinger was diluted and enumerated by the spread plate technique to quantify viable spore counts per ml. The viable counts per ml of liquid, exposure time, and impinger sampling rate were used to determine the total number of spores per liter of atmospheric air (aerosol concentration) in the exposure chamber. During each exposure, the aerosol particle size in the exposure chamber atmosphere was determined using an APS spectrometer (APS model 3321, TSI Inc, Shoreview, MN).

Whole body plethysmography was performed real-time on each animal during agent challenge to measure important respiratory parameters. These parameters (tidal volume, total accumulated tidal volume, and minute volume) were calculated from the measured volumetric displacement of air caused by the movement of the thoracic cavity of an animal while in a sealed plethysmograph. The total accumulated tidal volume, along with the aerosol concentration was used in calculating the inhaled dose. The inhaled dose was converted to median lethal dose (LD$_{50}$) equivalents using the LD$_{50}$ cited by Zaucha et al. (1998) of $1.05 \times 10^5$ B. anthracis Ames spores (32).

Temperature Monitoring

Each rabbit was sedated with acepromazine (1-5 mg/kg) and a programmable temperature transponder (IPTT-300, BMDS, Seaford, DE) was implanted subcutaneously (at the shoulder blade level) prior to exposure to B. anthracis Ames spores. Temperature readings were taken twice daily prior to challenge to establish a normal baseline for each animal. Body temperatures were monitored hourly from 12 to 72 hours post-challenge. The threshold for an
elevated body temperature was set as the average baseline temperature plus two times the standard deviation of rabbit’s baseline temperature. The SIBT was dependent upon the inherent variation in baseline body temperature and defined as three consecutive readings that were greater than the threshold for an elevated temperature. Following the hourly temperature monitoring period, surviving rabbits were monitored twice daily.

Bacteremia

Whole blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes and streaked over blood agar plates and incubated at 37ºC for 24-48 hours. Plates containing colonies with morphology consistent with *B. anthracis* Ames were reported as positive.

Circulating Protective Antigen Enzyme-Linked Immunosorbent Assay

Serum was isolated from whole blood collected in serum separator tubes (SST). Serum samples were then evaluated for quantitative circulating PA levels by the enzyme-linked immunosorbent assay (ELISA). A double affinity-purified polyclonal, monospecific rabbit anti-PA IgG “capture antibody” (produced by Battelle, Columbus, OH; purified from rPA-vaccinated rabbit serum and first passed over a protein A column, and then over a PA column) was used to coat the wells of a 96-well plate at a concentration of 2.0 µg/ml. The plates were blocked with 5% skim milk then incubated with rabbit serum samples containing native PA (Lot No. NR-164; BEI Resources), reference standard, or quality control samples consisting of rPA spiked differentially into naïve rabbit serum. The PA was detected by first incubating with diluted goat anti-PA serum, followed by incubation with a bovine anti-goat secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biologicals, Santa Cruz, CA), developed with a 2,2'-azinobis
[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt substrate for 30 minutes, and reaction terminated with the supplied stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The plates were read at 405 nm and the data were analyzed using a four parameter logistic-log (4PL) model to fit the eight point calibration curve. The concentrations of the PA in unknown samples were determined by computer interpolation from the plot of the reference standard curve data (Softmax Pro, Molecular Devices). The limit of detection (LOD) for this assay was 2.0 ng/ml.

**Complete Blood Cell Counts and C-Reactive Protein Levels**

Complete blood cell counts (CBCs) were performed on blood samples collected into EDTA blood collection tubes with the Advia® 120 Hematology Analyzer (Siemens, Deerfield, IL). Plasma was then harvested from the whole blood by centrifugation and C-reactive protein (CRP) levels determined using the Advia® 1200 Chemistry Analyzer (Siemens). The LOD for CRP was 0.5 mg/dL.

**Characterization of Inhalational Anthrax in New Zealand White Rabbits**

Twelve NZW rabbits weighing between 2.5 - 3.5 kg (50% male and 50% female) were aerosol challenged with a mean dose of 225 ± 35 LD50 of *B. anthracis* Ames spores; two rabbits (one male and one female) were utilized as unchallenged controls. The unchallenged animals were handled in the same manner as the challenged group with the exception of not being placed the challenge chamber or subjected to plethysmography. Blood samples were taken every 6 hours between 24 and 48 hours post-challenge and at 60 and 72 hours post-challenge. Blood samples were assayed for bacteremia, hematology parameters, CRP, and PA.
Efficacy of Passive immunization

Nineteen vascular access port (VAP)-implanted, NZW rabbits weighing between 2.5-3.5 kg (Covance) were challenged with a mean dose of 325 ± 61 LD₅₀. Ten rabbits (5 male, 5 female) received 8 mg/kg (intravenously via the VAP) of a fully human monoclonal anti-PA antibody (23, 25, 30) on an individual basis immediately following exhibition of SIBT. Nine rabbits (6 male, 3 female) were included in an untreated control group.

Efficacy of Delayed Passive immunization

Twenty-four VAP-implanted NZW rabbits (50% male, 50% female) weighing approximately 2.5 -3.5 kg were challenged with 325 ±71 LD₅₀. The rabbits were treated on an individual basis with 10 mg/kg of fully human monoclonal antibody (six rabbits per group) immediately following a SIBT, 6 hours following a SIBT, or 12 hours following a SIBT. Six rabbits served as untreated controls.

Statistical analysis

The time to death was calculated from the end of the aerosol exposure to the time the animal was found dead or euthanized due to morbundity. One-sided Fisher’s exact tests were utilized to compare the survival rates between groups. Kaplan-Meier curves were plotted and the log-rank test was computed to determine if differences were statistically significant. If the log-rank test was significant, pairwise log-rank tests were computed to determine group
differences. The Bonferroni-Holm adjustment was used to maintain an overall 0.05 level of significance. For the purposes of determining the exact time from challenge to positive bacteremia, circulating PA, CRP, or an abnormal hematology result, the time from the end of aerosol exposure until the scheduled blood draw were used. A result from the PA-ELISA or measured CRP was considered positive if the value was greater than the LOD for each assay. For each respective hematology parameter, the threshold for an abnormal parameter was defined as each individual animal’s baseline parameter value plus or minus two times the root mean squared error from an analysis of variance (ANOVA) model. Significant differences in group geometric mean PA and CRP levels and cell counts were determined using ANOVA models with any group effect fitted separately for each time point. If there was a significant group effect in the data, Tukey’s multiple comparisons were performed to determine which pairs of groups were significantly different. Data were analyzed using SAS software, Version 8 (SAS Institute Inc. Cary, NC).

Results

Characterization of Disease Progression

To characterize the disease progression in the NZW rabbit model of inhalational anthrax, twelve rabbits were challenged with *B. anthracis* Ames strain and monitored for bacteremia, circulating PA, changes in body temperature, hematology parameters, and CRP levels. All twelve challenged rabbits died with a mean time to death of 73.37 ± 24.07 hours (Figure 1). A febrile response (defined by SIBT) was observed for challenged rabbits, while the body
temperature for naïve rabbits remained relatively unchanged at the pre- and post-challenge phases (Figure 2). The mean time from challenge to SIBT was 27.1 ± 7.2 hours for challenged animals (Table 1) in which the average time to death from SIBT was 46.3 ± 26.2 hours. There was no correlation between the onset of fever and the post-challenge survival time.

Bacteremia, circulating PA levels, hematology parameters, and CRP were examined in addition to monitoring body temperature. The times from challenge to bacteremia, detection of circulating PA, changes in hematology parameters, or CRP levels for each animal are listed in Table 1. The mean time to the first positive blood culture was 25.5 ± 3.6 hours; all terminal blood samples from challenged rabbits were positive for *B. anthracis* (data not shown). In addition, PA detection in the serum corresponded with positive blood cultures for 68% of the rabbits. The mean time to the detection of circulating PA was 28.0 ± 4.3 hours, with the mean levels peaking at 60 hours post-challenge. While the times from challenge to detectable levels of PA were relatively consistent, a wide range (e.g., 15.6 to 4365.5 ng/mL) in the levels of circulating PA was observed (Figure 3).

White blood cells (WBCs), in particularly lymphocytes, decreased in all challenged rabbits from 30 to 42 hours post-challenge and increased toward baseline levels at subsequent collection time points (Figures 4a and b). There was a significant decrease in lymphocyte counts from challenged animals at 36 hours post-challenge compared to controls (P=0.0008). Heterophil levels increased 6 hours after challenge and remained elevated until 30 hours post-challenge (Figure 4c). While empirical changes to total WBC and heterophil counts occurred after challenge, there was no statistical difference between challenged and control levels. Changes from baseline occurred at 31.5 ± 3.7, 28.51 ± 8.0, and 21.8 ± 20.1 hours post-challenge for WBCs, lymphocytes and heterophils, respectively (Table 1). The challenged rabbits exhibited
an increase in CRP levels at a mean time of 23.0 ± 14.9 hours post-challenge (Table 1). The CRP levels were significantly higher in the challenged animals compared to control from 36 to 72 hours post-challenge (P<0.05; Figure 4d).

Efficacy of Passive Immunization at SIBT.

Consistent with above, a febrile response was observed following challenge with the mean time from challenge to SIBT of 26.11 ± 2.96 hours in the treatment group (Figure 5; Table 2). The threshold for an elevated body temperature was set as the average baseline temperature plus two times the standard deviation of rabbit’s baseline temperature. Treatment was initiated after three consecutive readings above threshold; therefore the rabbits exhibited the first febrile responses three hours prior to passive immunization, approximately 23 hours post-challenge. The mean body temperature continued to increase after treatment and the febrile response did not resolve until 84 hours post-challenge. The observed resolution of fever was not surprising as the monoclonal targets PA, neutralizes the toxins and allows the host immune response to clear the infection.

Treatment with the monoclonal antibody (8 mg/kg) to PA at SIBT protected 90% (9/10) of the rabbits (Figure 6). The lone treated rabbit that succumbed to disease died two days following challenge and approximately 16 hours after treatment administration. All untreated, challenged controls died with an average time to death of 77.46 ± 35.44 hours following challenge (Figure 6). A significant increase in survival was observed in the treated animals when compared to the untreated controls (P < 0.001, Fisher’s Exact test). The passive immunization dose was based the previously published results using this antibody in the B. anthracis challenged Dutch-belted rabbits (23). In that report, a dose of 10 mg/kg was used in the delayed
treatment experiment. In order to conserve the antibody for additional studies we decided to use a dose slightly lower (8mg/kg) when treating at SIBT.

In the current study, the mean time from challenge to a positive blood culture was 26.15 ± 8.61 hours in the treated animals and 90% of the rabbits were bacteremic prior to treatment (Table 2). The one rabbit not bacteremic prior to treatment was culture positive approximately three hours following treatment. These data suggest that using SIBT as a non-invasive surrogate for bacteremia, enables the treatment of a majority of the animals in a therapeutic manner.

The mean time from challenge to PA detection was 26.41 ± 2.96 and 37.65 hours in the treated and untreated rabbits, respectively. Most of the untreated animals became toxemic within the same timeframe as the treated rabbits. However, PA was not detected in two untreated animals until the 60 and 72 hour blood collection time points resulting in a greater mean time to toxemia. Thirty percent of rabbits were positive for circulating PA prior to treatment (Table 2).

In the untreated rabbits, the mean PA level was 592.5 ± 583.0 ng/ml at the last blood sample collected prior to death. The one treated animal that succumbed to disease had a PA level of 53.9 ng/ml prior to treatment at 24 hours post-challenge. The PA levels reached 3433.3 ng/ml at the last blood draw time point prior to death. After treatment, the PA levels in all surviving animals began to decline and were below the limit of detection by 96 hours post-challenge. Conversely, PA levels in untreated rabbits continued to increase until death (Figure 7).

**Efficacy of Delayed Passive Immunization following SIBT**

To determine the window of therapeutic opportunity, treatment with the monoclonal antibody (10 mg/kg) was administered individually to rabbits either immediately following SIBT, or at 6 or 12 hours after SIBT. The antibody dose was increased from 8 mg/kg to 10
mg/kg to maximize the effect in the delayed treatment groups. All groups exhibited a febrile
response with the mean time to SIBT of 28.20 ± 6.22 hours across groups. Table 3 shows the
mean time from challenge to SIBT and treatment for each group. Eighty-three percent (5/6) of
the rabbits treated immediately following SIBT survived challenge with the one death occurring
at 2.6 days after challenge. All (6/6) of the rabbits treated 6 hours following exhibition of SIBT
survived, while only 67% (4/6) of those treated 12 hours following exhibition of SIBT survived
(Figure 8). It is important to note one of the animals in the 12 hour group died before receiving
treatment. All of the untreated control rabbits (6/6) succumbed to infection following challenge
with a mean time from challenge to death of 90.5 ± 22.1 hours. A Fisher’s exact test
demonstrated that all treatment groups had a significantly greater survival rate than untreated
control group (P<0.05).

Sixteen of the 17 treated rabbits were bacteremic prior to passive immunization. One
rabbit treated 12 hours following a SIBT did not exhibit bacteremia and survived to the end of
the study. Only 33% (2/6) of the rabbits treated immediately following SIBT were positive for
PA and 33% (2/6) of the rabbits did not exhibit detectable PA levels in circulation. The only
rabbit to succumb to infection in the group treated immediately following a SIBT had 255 ng/mL
of circulating PA at the time of terminal blood draw. All animals treated at 6 or 12 hours after
SIBT had detectable levels of PA in circulation prior to passive immunization (Table 3). Figure 9
shows the PA levels over course of the study and mean time to treatment in each group. After
treatment, the PA levels reached a plateau but continued to increase in the untreated controls
(Figure 9).
Prior to 2001, the last case of inhalational anthrax in the U.S. was reported in 1978 (26). In the 2001 anthrax attack, the median incubation time from exposure to symptom onset was 4 days. Patients sought care a median of 3.5 days (range 1 to 7 days) after this onset of symptoms. The most common symptoms were fever, fatigue, malaise, lethargy, cough, nausea or vomiting and dyspnea. Upon examination by a health care professional, physical findings were fever, tachycardia, elevated WBCs, neutrophilia, abnormal chest X-ray findings (10). Blood cultures from 7 patients prior to treatment showed all were positive for *B. anthracis*. With multiple antibiotic treatment regimens and supportive care, the mortality rate was 45% (9, 10). This intentional release of *B. anthracis* spores in 2001 highlights the need for animal models to test the safety and efficacy of medical countermeasures against intentional release of biological agents.

Rabbits are considered an acceptable animal model to evaluate potential anthrax vaccine candidates because the pathophysiological response to disease closely resembles human inhalational anthrax (1, 32). The work presented here suggests that the NZW rabbit follows a similar clinical course to humans, although disease progression is more rapid.

These results confirm the findings of Yee et al., which demonstrated that bacteremia was closely followed by detection of PA in circulation by electrochemiluminescence (ECL) with fever occurring shortly following the detection of the toxin component (31). The correlation between detection of PA and bacteremia was also demonstrated by Koliber et al. (2006) using the ECL assay (12). In the current study, PA levels were variable over time and may be due to differences in the disease progression of each individual animal. A similar variation was observed in guinea pigs after intranasal inoculation of 20-50 LD$_{50}$ of Vollum stain spores (12).
The confounding factors of asynchronous germination of the *B. anthracis* spores and animal-to-animal variability make it difficult to compare PA levels at set time points post-challenge. More work is needed to correlate PA levels at the time of onset of bacteremia and just prior to death as a reliable maker, or surrogate at these stages of disease.

The febrile response after being challenged with *B. anthracis* spores is not limited to the NZW rabbit model. Through constant telemetric monitoring, Lawrence *et al* (2009) showed that Dutch-belted dwarf rabbits exhibit increases in body temperature just prior to death (13). While the results of the series of studies presented here show that NZW rabbits consistently produce fevers following challenge, results from primate studies are more variable. Studies with African Green Monkeys determined that a febrile response was inconsistent after challenge with *B. anthracis* spores and did not always correlate with bacteria and toxemia (24). This finding suggests that the use of body temperature as a trigger for treatment may be limited to rabbit models of inhalation anthrax.

To determine the efficacy of a compound in a truly therapeutic manner the test subject must be treated after clinical symptoms. In animal models, a positive bacteremic culture, prior to treatment, has been considered a requirement for signaling administration of therapeutics (1). In this study, the correlation of SIBT with bacteremia and PA levels in blood following inhalational exposure to *B. anthracis* Ames spores was used as a trigger for treatment with an anti-PA monoclonal antibody. Ninety percent of these monoclonal antibody-treated animals survived challenge, which was similar to rabbits treated with raxibacumab (human antibody to PA) after SIBT or PA detection where a 40 mg/kg dose protected 44% of the rabbits from a lethal inhalational anthrax exposure (11).
In addition to treatment efficacy studies, there are published studies using human monoclonal antibodies to PA that were designed to determine a window of opportunity for antibody treatment at a defined time post-challenge. The antibody ETI-204 (20 mg/rabbit) administered at 24 or 36 hours post-aerosol challenge with *B. anthracis* Ames spore showed significant protection; however, no difference in survival was observed when the antibody was administered 48 hours post-challenge (19). Similarly, the human monoclonal antibody, AVP-21D9, demonstrated significant protection when given 6, 12, or 24 hours post-nasal instillation challenge with *B. anthracis* spores in the Dutch Belted rabbit model (23). While not statistically significant, 60% of rabbits were protected when treatment was delayed until 48 hours post challenge. Using SIBT as the primary indicator for delayed treatment, the present study showed significant protection above controls when treatment was delayed up to 12 hours following SIBT. This protection afforded by the monoclonal was consistent with observation in previous studies (14, 18).

While passive immunization against anthrax is promising as a prophylactic/therapeutic, it is likely that future recommendations for the individuals at risk of exposure will include active immunization and antibiotic treatment, as well as neutralizing monoclonal anti-PA antibodies. In 2006, a 44-year-old Pennsylvania man was diagnosed with naturally-acquired inhalational anthrax and was treated with antibiotics, supportive care, and human anthrax immunoglobulin; the patient survived the infection (23). However, the definitive effect of passive immunization is difficult to determine from this single instance and more studies, on the passive immune therapy during anthrax infections are needed.

The work presented here indicates that using SIBT as a trigger for treatment may be a valuable tool in evaluating therapeutics in the rabbit model of inhalational anthrax. There have
been recent advances in sensitive methods to detect circulating anthrax toxins and capsular material (3, 5, 8) which may also be a suitable trigger for initiation of treatment in a therapeutic model. However, laboratories that do not have access to these assays may find the febrile response in the rabbit model as a reliable, cost effective trigger for treatment when testing therapeutics against anthrax. The model may also be acceptable for determining if passive immunization adds benefit over antibiotic treatment alone. An aggressive antibiotic regimen along with supportive care is the current standard of care for anthrax patients. Any adjunct therapy will most likely have to show added benefit above the current standard. The logical next phase in the characterization of the NZW rabbit as a therapeutic model will be to determine if it can be used in these added benefit studies.

Acknowledgements

We would like to thank Drs. James Rogers, Judy Hewett, Tracy Macgill, and Raymond Slay for critically reading the manuscript and providing helpful comments. This work was funding by NIAID contract number N01-AI-30061. Anthrax Protective Antigen (PA), NR–164, was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH.
References


FIG. 1. Time to death after aerosol exposure to *B. anthracis* Ames strain spores. Twelve New Zealand White rabbits were exposed to 225 ± 35 LD$_{50}$ of *B. anthracis* (Ames strain) spores via aerosol challenge and monitored for 28 days post-challenge. Closed circles (●) represent the percent survival of challenged rabbits. Open circles (○) represent the unchallenged controls. The mean time to death 73.4 ± 24.1 hours for challenged animals.

FIG. 2. Body temperatures following challenge with *B. anthracis* spores. Temperature readings were taken twice daily prior to and hourly from 24 hours to 72 hours post-challenge using a programmable temperature transponder (IPTT-300, BMDS, Seaford, DE) implanted subcutaneously (at the shoulder blade level). Closed circles (●) represent mean body temperatures in degrees Celsius of 12 rabbits challenged with 225 ± 35 LD$_{50}$ of *B. anthracis* (Ames strain) spores. Open circles (○) represent the mean body temperature of two naive rabbits. The mean time from challenge to a significant increase in body temperature (2 standard deviations above baseline) was 27.1 ± 7.2 hours for challenged animals.

FIG. 3. Circulating protective antigen levels following challenge. Twelve New Zealand White rabbits were exposed to 225 ± 35 LD$_{50}$ of *B. anthracis* (Ames strain) spores via aerosol challenge. Circulating levels of PA were measured through 72 hours post-challenge using an ELISA based method. Open circles (○) represent individual rabbit levels at each time point while the closed circles (●) represent the mean of all samples assayed at that time point.
FIG. 4. Changes in hematology parameters and C-reactive protein levels. Complete blood cell
counts (CBCs) were performed on blood samples collected through 72 hours post-challenge with
the Advia® 120 Hematology Analyzer (Siemens, Deerfield, IL). Plasma was then harvested from
the whole blood by centrifugation and C-reactive protein (CRP) levels determined using the
Advia® 1200 Chemistry Analyzer (Siemens). Closed circles (●) represent mean counts or levels in
of 12 rabbits challenged with 225 ± 35 LD₅₀ of B. anthracis (Ames strain) spores. Open circles
(○) represent the mean counts or levels of two naïve rabbits. Changes from baseline (an increase
or decrease of 2 standard deviations or greater) occurred at 31.5 ± 3.7, 28.5 ± 8.0, and 21.8 ±
20.1 h post-challenge for WBCs, lymphocytes and heterophils, respectively. The challenged
rabbits exhibited a significant increase in CRP levels (over lower limit of detection of 0.5 mg/dL)
at an average of 23.0 ± 14.9 hours post-challenge.

FIG. 5. Body Temperature used as a trigger to treat after challenge with 325 ± 71 LD₅₀ of B.
anthracis Ames strain spores. Temperature readings were taken twice daily prior to challenge to
establish a normal baseline for each animal. Body temperatures were monitored hourly from 12
hours to 72 hours post-challenge. A significant increase in body temperature (SIBT) was
defined as three consecutive readings that were greater than two times the standard deviation of
that rabbit’s baseline average temperature. Following the hourly temperature monitoring period,
surviving rabbits were monitored twice daily. Closed circles (●) represent mean body
temperatures in degrees Celsius of rabbits treated intravenously with 8 mg/kg of the monoclonal
antibody to PA at SIBT. Open circles (○) represent the mean body temperatures of the
untreated control rabbits. The vertical dashed line denotes the mean time of antibody
administration for the treatment group. A SIBT response was observed in all animals following challenge with a mean time from challenge to SIBT of $28.2 \pm 10.4$ hours.

FIG. 6. Survival rate after treatment at a significant increase in body temperature. Ten NZW rabbits were challenged with $325 \pm 71$ LD$_{50}$s of *B. anthracis* Ames strain spores and treated intravenously with a human monoclonal antibody to PA (10 mg/ml). Nine animals served as untreated controls. Treating with the monoclonal antibody to PA at SIBT protected 90% (9/10) of the rabbits, whereas all of the controls died following challenge. Closed circles (●) represent the percent survival in treated rabbits while open circles (○) represent percent survival in untreated controls. There was significant increase in survival in the treated animals when compared to the untreated controls ($P < 0.001$, Fisher’s Exact test).

FIG. 7. Circulating protective antigen levels in treated and untreated rabbits following challenge. Nineteen New Zealand White rabbits were exposed to $325 \pm 71$ LD$_{50}$s of *B. anthracis*. Circulating levels of PA were measured through 96 hours post-challenge using an ELISA based method. Ten rabbits were treated with a monoclonal antibody to PA and 9 rabbits served as untreated controls. Open circles (○) represent the mean PA levels of treated animals at each time point. Closed circles (●) represent the mean PA levels of untreated animals at each time point. Bars denote standard error.
FIG. 8. Survival rates after delayed treatment based on a significant increase in body temperature. Groups of 6 rabbits were with 325 ±71 LD₅₀s then treated with monoclonal antibody (10 mg/kg) at SIBT (●), 6 hours following exhibition of SIBT (○), or 12 hours following exhibition of SIBT (▼). A fourth group of 6 rabbits served as untreated controls (▲). All treatment groups showed significantly greater survival than the untreated control group (P<0.05, Fisher’s exact test).

FIG. 9. Circulating protective antigen levels in rabbits in delayed treatment groups. Groups of 6 rabbits were with 325 ±71 LD₅₀ then untreated (a) or treated with monoclonal antibody (10 mg/kg) at SIBT (b), 6 hours following exhibition of SIBT (c), or 12 hours following exhibition of SIBT (d). Circulating levels of PA were measured through 72 hours post-challenge using an ELISA based method. Closed circles (●) represent the mean of all samples assayed at that time point and the bars denote standard error. The vertical dashed lines indicate the mean treatment time for each group.
Table 1. Time from Challenge to Event in New Zealand White Rabbits.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Challenge Dose (LD50s)</th>
<th>SIBT</th>
<th>Bacteremia</th>
<th>PA</th>
<th>WBC Count</th>
<th>Heterophil Count</th>
<th>Lymphocyte Count</th>
<th>H:L Increase</th>
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</table>

NA= Not applicable, animal did not have a significant change from baseline
SIBT= Significant increase in body temperature
WBC= White blood cell
H:L= Heterophil:lymphocyte ratio
CRP= C-reactive protein
Table 2. Mean Time from Challenge to Event in Animals Treated at a Significant Increase in Body Temperature.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Time from Challenge to Event (h)</th>
<th>% with detectable PA prior to Treatment</th>
<th>% Bacteremic Prior to Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIBT</td>
<td>Treatment</td>
<td>Bacteremia</td>
</tr>
<tr>
<td>Untreated</td>
<td>30.46 ± 14.93</td>
<td>NA</td>
<td>34.40 ± 19.35</td>
</tr>
<tr>
<td>Treated</td>
<td>26.11 ± 2.96</td>
<td>26.40 ± 9.94</td>
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</tr>
</tbody>
</table>

NA= Not applicable
SIBT= Significant increase in body temperature
PA=Protective antigen
Table 3. Mean Time from Challenge to Event in Delayed Treatment Groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Time from Challenge to Event (h)</th>
<th>% with detectable PA prior to TX</th>
<th>% Bacteremic Prior to Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIBT</td>
<td>Treatment</td>
<td>Bacteremia</td>
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<tr>
<td>Control</td>
<td>26.16 ± 5.91</td>
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<td>24.14 ± 8.08</td>
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<td>25.05 ± 4.14</td>
<td>25.27 ± 5.92</td>
<td>23.55 ± 5.61</td>
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<td>39.97 ± 4.97</td>
<td>24.14 ± 8.08</td>
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</tbody>
</table>

NA = Not applicable
SIBT = Significant increase in body temperature
PA = Protective antigen