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Pathology and Pathophysiology of Inhalational Anthrax in a Guinea Pig Model

Running Title: Inhalational Anthrax in Guinea Pig Model

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ABSTRACT:

Non-human primates (NHP) and rabbits are the most commonly used animal models for evaluating efficacy of medical countermeasures against anthrax in support of licensure under the FDA's "Animal Rule." However, a need for an alternative animal model may arise in certain cases. Development of such an alternative model requires a thorough understanding of the course and manifestation of experimental anthrax disease induced under controlled conditions in the proposed animal species. The guinea pig, which has been used extensively for anthrax pathogenesis studies and anthrax vaccine potency testing, is a good candidate for such alternative model. This study was aimed at determining the median lethal dose (LD_{50}) of the *Bacillus anthracis* Ames strain in guinea pigs and investigating natural history, pathophysiology, and pathology of inhalation anthrax in this animal model following nose-only aerosol exposure. The inhaled LD_{50} of aerosolized Ames strain spores in guinea pigs was determined to be $5.0x10^4$ spores. Aerosol challenge of guinea pigs resulted in inhalational anthrax with death occurring between 46 and 71 hours post-challenge. The first clinical signs appeared as early as 36 hours post-challenge. Cardiovascular function declined starting at 20 hours post-exposure. Hematogenous dissemination of bacteria was observed microscopically in multiple organs and tissues as early as 24 hours post-challenge. Other histopathologic findings typical of disseminated anthrax infection included suppurative (heterophilic) inflammation, edema, fibrin, necrosis, and/or hemorrhage in the spleen, lungs, and regional lymph nodes, and lymphocyte depletion and/or lymphocytolysis in the spleen and lymph nodes. The study demonstrated that the course of inhalation anthrax disease and the resulting pathology in guinea pigs are similar to those seen in rabbits and NHPs as well as in humans.

INTRODUCTION:

Bacillus anthracis, the etiologic agent of anthrax, is a spore-forming bacterium that can cause disease in humans via the gastrointestinal, cutaneous, or inhalation routes, inhalation being the most lethal. Following inhalation exposure to *B. anthracis*, the disease course in humans is typically biphasic and consists of nonspecific initial clinical signs and symptoms followed by a sudden onset of respiratory distress with dyspnea, stridor, cyanosis, and chest pain (1-5). The onset of respiratory distress is followed by shock and high mortality, even with treatment. Anthrax is considered a serious biological terrorist threat due to the stability of *B. anthracis* spores, highly lethal effects by the inhalation route, and the relative ease of dissemination.

It is neither feasible nor ethical to perform human trials to support the development of medical countermeasures against anthrax. Therefore, employment of adequate animal models of inhalational anthrax, which closely mimic human disease and accurately reflect mechanisms of host-pathogen interaction, is critical for the development and licensure of prophylactic or therapeutic countermeasures against the disease. Rabbits and nonhuman primates (NHPs) are generally considered to be the preferred animal models for studies of inhalational anthrax infection and have been widely used to study disease pathogenesis, examine bacterial characteristics such as virulence, and assess efficacy of vaccines and therapeutics (6, 7).

Rabbits (8, 9) and NHPs, including rhesus macaques, cynomolgus macaques, and African green monkeys (10-16) are generally accepted as the preferred animal

models of inhalation anthrax. They are considered to be predictive of the anthrax disease in humans because of their high susceptibility to infection with toxin-producing strains of *B. anthracis*, particularly via inhalation route. In contrast, mice appear to be equally susceptible to both toxigenic and non-toxigenic encapsulated strains, suggesting that the polyglutamate capsule of *B. anthracis* is the primary virulence factor in this model and the mechanism of disease is not toxin-mediated (17).

The course of inhalational anthrax and pathological changes observed in rabbits and NHPs are similar to those reported in humans. Findings in rabbits include necrotizing lymphadenitis, splenitis, pneumonia, and vasculitis, as well as hemorrhage, congestion, and edema in multiple tissues (8). Similar lesions are observed in NHPs, which also frequently exhibit meningitis (18), thus exhibiting a full range of lesions described in human inhalational anthrax (3).

In certain cases, however, these well-established models do not respond to a specific countermeasure (e.g., a vaccine containing a novel adjuvant) in a manner consistent with human response and, therefore, cannot be utilized for the assessment of efficacy of such countermeasure. For example, rabbits do not respond strongly to vaccine adjuvants that act via the toll-like receptor-9 (19). Such cases necessitate development of an alternative animal model that is predictive of the human response to a particular drug or vaccine, yet closely follows the standardized conditions of anthrax exposure that have been established for the rabbit and NHP models (e.g., nose-, muzzle-, or head-only exposure, use of *B. anthracis* strain virulent in humans, defined

challenge dose of the pathogen, etc.). Investigating natural history of the disease under these standardized conditions is an essential part of such model development.

The guinea pig model has been used extensively to elucidate pathogenesis of inhalational anthrax (20). Exposure of guinea pigs to *B. anthracis* by inhalation or via intratracheal instillation has been shown to result in phagocytosis of spores by alveolar macrophages followed by intracellular germination and translocation into the thoracic and mediastinal lymph nodes, with subsequent entry of vegetative bacterial cells into the circulation resulting in massive septicemia and toxemia (20). Thus, the infection mechanism in guinea pigs is very similar to that which is observed in rabbits (8, 21-23), NHPs (15, 24-26), and humans (4, 27, 28). Of note, guinea pigs have been utilized for anthrax vaccine production and potency assessment (e.g., Sterne vaccine, BioThrax® (Anthrax Vaccine Adsorbed)) as part of the batch release testing (29), as well as in proof-of-concept studies evaluating pre- and post-exposure efficacy of anthrax countermeasures (23, 30, 31).

Despite extensive use of guinea pigs in anthrax research, there are no published lethal dose estimates using a nose-only aerosol exposure system. The commonly used whole-body aerosol exposure system is not ideally suited for modeling a disease where the respiratory system is the primary portal of entry for the causative agent (32). The nose-only exposure limits the entry of the challenge materials to the respiratory tract, avoiding skin contamination, and minimizes oral exposure (33). There are limited data available on the aerosol exposure of guinea pigs to the Ames strain of *B. anthracis,* which has been utilized in the rabbit and NHP aerosol challenge models (8, 13, 15, 34,

35). Additionally, the Ames strain is highly virulent in humans (36) and is most likely to be the basis for any weaponized strain (37, 38).

This paper describes determination of the inhaled median lethal dose (LD_{50}) of *B. anthracis* (Ames) spores in guinea pigs using a nose-only aerosol exposure system and the natural history of inhalation anthrax in guinea pigs, as well as discusses the comparability of the guinea pig model to other established animal models and its fidelity to the pathophysiology of the disease in humans.

MATERIALS AND METHODS:

Animals

Animal studies were conducted in compliance with the Animal Welfare Act and followed the principles of the Guide for the Care and Use of Laboratory Animals from the National Research Council. The animal procedures were approved by Battelle's Institutional Animal Care and Use Committee (IACUC). All work was performed in a BSL-3/ABSL-3 laboratory registered with the Centers for Disease Control and Prevention (CDC) and inspected by the Department of Defense and the Department of Agriculture.

Hartley strain guinea pigs, weighing between 635 and 874 g (equal numbers of male and female animals), were purchased from Charles River Laboratories (Wilmington, MA). All animals were in good health, free of malformations, and free of clinical signs of disease prior to placement on the study. Animals were individually housed in suspended polycarbonate bedding cages on a stainless steel rack equipped with an automatic watering system. The light/dark cycle was 12 hours each per day,

regulated using fluorescent lighting. Manufacturer tested and certified Guinea Pig Chow pellets (PMI Nutrition International, St. Louis, MO) and water were available *ad libitum*.

Aerosol Exposure

B. anthracis Ames strain spores were prepared and characterized as described previously (39). Spores were stored at 4ºC to 8ºC in sterile water (Thermo Scientific HyClone, Logan, UT) with 1.0% phenol (Sigma-Aldrich, St. Louis, MO). Prior to use, the spores were washed with endotoxin-free water four times and were diluted to the appropriate concentration in endotoxin-free sterile water and 0.01% Tween-20. The spore suspensions contained less than 5% vegetative cells and debris as confirmed by phase contrast microscopy (Leica Microsystems, Wetzlar, Germany). Prior to aerosolization, the spores were enumerated and diluted to the proper concentration required to yield the targeted dose.

Aqueous suspensions of *B. anthracis* spores were aerosolized by a six-jet Collison nebulizer and delivered to the guinea pigs using the nose-only aerosol exposure system (CH Technologies Tower, Westwood, NJ). Airflow was regulated using mass flow meters (MFM), and mass flow controllers (MFC) to monitor the aerosol flow. The aerosol was sampled for viable concentration dose determination of *B. anthracis* using an impinger (Model 7541, Ace Glass, Inc., Vineland, NJ). The liquid in the nebulizer and impinger was diluted and enumerated by the spread plate technique to quantify the number of viable bacteria (colony-forming units, cfu) per mL. The inhaled dose was determined based on the calculated bacterial concentration and the animal respiration parameters estimated based on animal weights using Guyton's formula (40). Prior to exposure, guinea pigs were acclimated to the challenge restraint tubes to

reduce animal distress and its potential effect on respiratory parameters. Animals were not anesthetized or sedated during the acclimation or challenge procedure.

LD_{50} Determination

The LD_{50} was determined using an iterative approach. Three iterations were performed with a total of 160 animals (equal number of males and females). Groups of animals were exposed to targeted inhaled doses ranging between 1×10^3 and 1×10^6 spores/animal, with challenge dose levels in the second and third iteration based on the survival results from the previous iteration(s). In each iteration, guinea pigs were monitored for mortality twice daily for up to 21 days.

The LD_{50} of inhaled aerosolized anthrax spores was estimated using a probit regression model of the probability of survival at each (log) dose level. All probit models were fitted in SAS® (ver. 9.1) using PROC PROBIT (SAS Institute, Cary NC). Model diagnostics were examined to confirm the appropriateness of the model assumptions. Estimated parameters of the probit model were used to compute the LD_{50} and LD_{90}, and Fieller's method (41, 42) was used to compute a 95% confidence interval for each estimate.

Natural History Study

Animals and Treatments

Forty-one guinea pigs (20 males and 21 females) were randomly assigned to nine treatment groups as outlined in Table 1. Of these, 37 animals (18 males and 19 females) were exposed via the aerosol route to a target dose of 200 LD_{50} of *B. anthracis* spores. Nine of the challenged animals (4 males and 5 females) were surgically implanted with a telemetry transmitter (TL10M2-C50-PT, Data Sciences International,

St. Paul, MN) and were monitored for clinical signs of disease. One group of four animals (2 males and 2 females) was not subjected to aerosol inhalation; these animals were euthanized on Day 0 to serve as unchallenged controls.

One group of four guinea pigs (2 males and 2 females per group) was scheduled to be euthanized at each of the following time points: 24, 30, 36, 48, 60, 72, and 96 hours after challenge (Table 1). At the specified time point, all surviving animals in the group were exsanguinated and humanely euthanized.

Prior to blood and cerebrospinal fluid collection as well as prior to euthanasia, animals were anesthetized with 80 mg/kg of ketamine and 10 mg/kg of xylazine administered via intraperitoneal (IP) and subcutaneous (SC) injection, respectively.

Post-Challenge Observations and Body Weight Measurements

Animals were observed every 6 hours post-challenge until scheduled termination, or until the animal was found dead or was euthanized due to moribund condition. Signs of illness (i.e., dyspnea, forced abdominal respirations, unresponsive to touch or external stimuli) were monitored and recorded. Body weights were measured daily beginning on the day prior to challenge (baseline) until the scheduled terminal endpoint, or until the animal was found dead or euthanized due to moribundity. To determine if the mean weight at each post-challenge study day was significantly different from that at baseline, paired t-test analysis was performed.

Blood Collections

Blood was collected by cardiac puncture or from the cranial vena cava. Blood from each animal was collected into serum separator tubes (SST), sodium polyanethol sulfonate (SPS) and ethylenediaminetetraacetic acid (EDTA) tubes.

193

194 **Bacterial Burden Assessment**

195 For the qualitative bacteremia assessment, 30-40 µL of whole blood was
196 inoculated onto blood agar plates and the plates were incubated at 37°C for a minimum
197 of 48 hours. A plate containing at least one colony with morphology consistent with *B.*
198 *anthracis* was considered positive for *B. anthracis*. For the quantitative assessment, 100
199 µL of whole blood was plated in triplicate on tryptic soy agar (TSA). In addition, 10-fold
200 serial dilutions were performed by transferring 100 µL of whole blood or previous
201 dilution into 900 µL of phosphate buffered saline (PBS). For each dilution prepared, 100
202 µL was plated in triplicate on TSA. Plates were incubated at 37°C for 16-24 hours,
203 bacterial colonies enumerated, and the corresponding concentration (cfu/mL)
204 calculated.

205 For qualitative bacteremia assessment, estimates with 95% confidence intervals
206 for the proportions of bacteremic animals were calculated within each group. For
207 quantitative assessment, geometric means and 95% confidence intervals were
208 calculated within each group. The limit of detection (LOD) for quantitative bacteremia
209 was 100 cfu/mL. The concentrations reported to be below the LOD were replaced with
210 50 cfu/mL for the purposes of statistical analysis.

211 Quantitative polymerase chain reaction (qPCR) analysis for the presence of
212 bacterial DNA was performed by amplification of a small fragment within the coding
213 region of the *rpoB* gene on the *B. anthracis* chromosome.

Serum PA ELISA

For detection of anthrax protective antigen (PA) in sera of infected animals, anti-PA IgG "capture antibody" (Battelle Memorial Institute, Columbus, OH) purified from recombinant PA (rPA)-vaccinated rabbit serum using a protein A column followed by a PA column, was used to coat the wells of a 96-well plate at a concentration of 2 µg/mL. The plates were blocked at 37°C for 30- 90 minutes with skim milk, then incubated at 37°C for 60 minutes with rabbit serum samples containing native PA [Lot No NR-164; Biodefense and Emerging Infections Research Resources Repository (BEI Resources)], or a reference standard and quality control samples consisting of rPA spiked differentially into naïve guinea pig serum. The PA was detected by incubating the plates at 37°C for 60 minutes with diluted goat PA anti-serum, followed by incubation at 37°C for 60 minutes with a bovine anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biologicals, Santa Cruz, CA), then a 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt substrate and a stop solution (both from Kirkegaard and Perry Laboratories, Gaithersburg, MD).

The plates were read using a BioTek ELx800, SPECTRAmax PLUS^{384} at a wavelength of 405 nm with a reference wavelength of 490 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). All PA-ELISA observations reported to be below the limit of detection (LOD) of 1.30 ng/mL were replaced with 0.65 ng/mL for the statistical analysis.

Hematology and Clinical Chemistry

Hematology assessment was performed on whole blood samples using an Advia® 120 Hematology Analyzer (Siemens, Deerfield, IL) and included the following parameters: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV) and total and differential white blood cell parameters.

Serum samples were analyzed using an Advia® 1200 Chemistry Analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) for serum proteins, liver function enzymes, kidney function parameters, electrolytes, and C-reactive protein (CRP).

Analysis of variance (ANOVA) models with an effect for group were fitted separately to each hematology and clinical chemistry parameter. These models were used to determine if the levels in each challenge group were significantly different from those in the control group. Hematology and clinical chemistry data from each challenged group were compared to those in the control group. An analysis of variance (ANOVA) model was fitted separately to each hematology parameter with an effect for group in order to check the model assumption of normality and to identify potential outliers. The models were fitted to base-10 log-transformed data.

Gross Pathology and Histopathology

Complete necropsies were performed on all guinea pigs found dead, euthanized due to moribund condition, or euthanized at scheduled termination time points. Brains,

hearts, large intestines (cecum, colon, and rectum), kidneys, livers, lungs, lymph nodes (bronchial, mediastinal, mandibular, and mesenteric), mammary glands, pancreases, small intestines (duodenum, jejunum, and ileum), spleens, and stomach as well as any other abnormal tissues and gross lesions were collected. Tissue samples were preserved in 10% neutral buffered formalin, paraffin-embedded, processed to slides, stained with hematoxylin and eosin. The microscopic findings were graded semi-quantitatively. A numerical score (Grade 1 through 4) was used to describe average severity grade for each lesion as well as the extent of bacterial invasion of the tissue. Gross and microscopic diagnoses were entered into the PATH/TOX SYSTEM (Xybion Medical Systems Corporation, Cedar Knolls, New Jersey) for data tabulation and analysis.

Telemetry

One group of 9 guinea pigs (4 males and 5 females) was implanted with telemetry transmitters (TL10M2-C50-PT, Data Sciences International. St. Paul, MN) to collect data on the following parameters: body temperature, systolic and diastolic blood pressure (BP), mean arterial pressure (MAP), pulse pressure, heart rate, and activity. The data were recorded for a period 30 seconds every 15 minutes, beginning five days prior to challenge (baseline levels) and continued until the animal was euthanized or succumbed to infection.

The mean value for each parameter measured by telemetry was computed for every 15 minutes for 24 hours (00:00, 00:15,…, 23:45) prior to challenge (baseline). Data collected post-challenge was then baseline adjusted according to the associated clock time. One-hour averages were computed for the baseline adjusted values such

that, for example, the 1 a.m. average for a given study day included measurements recorded at 1:00, 1:15, 1:30, and 1:45. The standard deviation of all one-hour averages at baseline was calculated for each animal and used to form the upper and lower limits for indications of abnormality. The upper limit was defined to be three standard deviations above zero, while the lower limit was defined to be three standard deviations below zero. A parameter was defined as abnormal if three consecutive baseline adjusted one-hour averages were outside the upper or lower limits following challenge. The time of onset for abnormality was defined as the time associated with the third abnormal value during the first occurrence of three consecutive abnormal values following challenge.

Proportions of abnormal animals and exact 95% confidence intervals were calculated for each telemetry parameter. Among those animals that became abnormal, mean time to abnormality and 95% confidence intervals were also calculated for each telemetry parameter.

RESULTS:

LD_{50} Determination

Using a nose-only aerosol exposure system, guinea pigs were challenged with inhaled doses of *B. anthracis* Ames spores ranging from 6.4×10^3 to 7.69×10^5 spores per animal (Table 2). Analysis of the mortality data showed that there was a statistically significant dose-response relationship ($p < 0.0001$) with higher dosages of the agent resulting in a higher probability of death (Figure 1). The nose-only anthrax spore LD_{50} for guinea pigs was found to be approximately 5.0×10^4 spores per animal (Table 3,

Figure 1). There was no impact on the LD_{50} ($p = 0.11$) when the gender of the animals was considered, nor was there an interaction between gender and inhaled dose ($p = 0.97$).

Natural History

Aerosol Challenge

The total inhaled dose was calculated from the impinger sample concentrations, sampling parameters, and exposure duration. The mean of the inhaled dose was 406 ±13 of LD_{50} equivalents (based on the LD_{50} determined in the experiment described above). The spray factor determined for the spore lot used for these studies was different than the spray factors observed during the animal challenges (the spray factor was better during the animal challenges). This accounted for the difference observed in actual challenge dose verses the target dose. The mass median aerodynamic diameter (MMAD) was determined to be 1.21 µm (with a geometric standard deviation (GSD) of 1.28 µm), indicating that the aerosol particle size was around the optimal size (~1 µm) for delivery of the spores to alveoli (43).

Mortality, Clinical Signs, and Body Weights

Of the 37 anthrax-infected animals, sixteen survived to scheduled termination time points, including all animals scheduled to be sacrificed at 24, 30, and 36 hours post-challenge; 3 of 4 animals scheduled to be sacrificed at 48 hours post-challenge, and 1 of 4 animals scheduled to be sacrificed at 72 hours post-challenge (Table 4). The remaining 21 anthrax-infected animals were found dead or euthanized due to moribund

condition (Table 4). The time to death for these animals ranged between 46 and 71 hours post-challenge, with a mean of 56 hours.

Animals did not show any clinical signs of disease during the first 36 hours after challenge. Within a few hours after the appearance of symptoms, animals either died or reached moribund condition and met the criteria for euthanasia. The clinical manifestations were consistent with inhalational anthrax and similar to those observed in rabbit and NHP models, including labored breathing, cough, lacrimation, weakness, cyanosis, and lethargy. There were no significant changes from the baseline weights for any animal following challenge.

Telemetry Data

Of nine animals implanted with telemetry transmitters, seven showed abnormal results for each of the telemetry parameters except activity (Figure 2). The mean time to abnormal readings ranged from approximately 44 hours post-challenge (increased heart rate) through approximately 54 hours post-challenge (decreased systolic BP) (Table 6). In comparison to baseline, temperatures were significantly greater than baseline at 1, 23, 26, 36, and 37 hours post-challenge. Heart rates were significantly elevated from baseline during the first 3 hours post-challenge and for 5 consecutive hours beginning at 49 hours post-challenge (Figure 2). MAP, pulse pressure, diastolic and systolic BP decreased significantly compared to baseline levels between 20 and 51 hours post-challenge and generally remained significantly lower than baseline until death (Figure 2). Activity was measured by adding up the number of movements over the telemetry sampling period as detected by the telemetry receiver.

Bacterial Burden and Toxemia

Inhalational anthrax in mammalian species is characterized by the dissemination of bacilli via the circulatory system, resulting in septicemia and toxemia that lead to secondary invasion of internal organs and tissues, including central nervous system (CNS), culminating in fatal outcome (44). Accordingly, bacterial burden in blood was assessed in this study. The first positive blood cultures were detected as early as 24 hours post-challenge (one out of four animals was positive in the qualitative bacteremia assessment), and the proportion of bacteremic animals as well as bacterial counts increased as disease progressed (Figure 3, A). The results of the qPCR analysis were consistent with the quantitative bacteremia data (data not shown).

PA is one of the key virulence factors in a *B. anthracis* infection, as it combines with edema factor (EF) to form edema toxin (ET) and with lethal factor (LF) to form lethal toxin (LT). Thus, measurement of PA is considered an appropriate marker of anthrax infection and has been utilized in the NHP and rabbit models as a biomarker of disease (34). In this study, PA was first detected in the serum at 30 hours post-challenge and its levels continued to increase with time (Figure 3, B).

Hematology and Clinical Chemistry

Hematologic parameters can potentially serve as important markers of local and systemic inflammation induced by an infection, vascular barrier disruption (5), and immune suppression (45) caused by the *B. anthracis* lethal toxin. However, humans rarely exhibit drastic hematological changes during early stages of anthrax infection (46). Similarly, during the first 48 hours post-challenge, only minor fluctuations in the red blood cell parameters, judged to be not biologically relevant, were observed in guinea pigs (Table 6). One animal that survived until scheduled termination at 72 hours post-

challenge, as well as four animals from which blood was collected prior to euthanasia due to moribund condition, showed significant decreases in RBC, HGB, and HCT levels, increases in MCH, MCHC, and RDW levels and elevated PLT counts. Heterophil leukocytosis, lymphocytosis or lymphopenia, increased heterophil/lymphocyte ratio, as well as occasional monocytosis, eosinophilia, and basophilia, were also noted in these five animals.

No clinically significant changes in clinical chemistry parameters were observed (data not shown).

Pathology

Necropsy examination revealed no gross lesions in animals that died or were euthanized prior to 48 hours post-challenge. In contrast, animals that died or were euthanized at later time points exhibited changes consistent with anthrax infection, including enlarged and darkened bronchial, mediastinal, and/or mesenteric lymph nodes. These lesions corresponded microscopically to mild to moderate hemorrhage.

Microscopic findings in animals sacrificed at 24 hours post-challenge included minimal hemorrhage and interstitial nonsuppurative inflammation in the lungs; minimal to mild mandibular, mediastinal, and mesenteric lymph node and splenic lymphocyte depletion/lymphocytolysis, and/or hemorrhage and hepatocellular necrosis. Minimal presence of bacteria was observed in the liver in this group of animals. The nonsuppurative inflammation was likely preexisting. Animals sacrificed at 30 hours post-challenge exhibited minimal nonsuppurative inflammation in the lungs and minimal lymphocyte depletion/lymphocytolysis in regional and mesenteric lymph nodes and spleen. Minimal to mild hemorrhage was observed in the mediastinal (Figure 4A) and

mandibular lymph nodes. Submucosal edema was present in the stomach of one animal. In livers, hepatocellular necrosis with minimal bacteria was also noted. By 36 hours post-challenge, bacteria (minimal and, primarily intravascular) were observed in the meninges of the brain. Figure 4B shows meningeal blood vessels that contain bacilli. Minimal to mild lymphoid destruction (lymphocyte depletion/lymphocytolysis) occurred in the mediastinal and mesenteric lymph nodes, and in the spleen. No other organs including the lungs were affected in animals sacrificed at this time point. Similar findings but with increased severity of the lesions, were noted in animals sacrificed at later time points.

Microscopic lesions found in the 21 guinea pigs that did not survive to their scheduled termination time points were typical of the disseminated anthrax infection and included the presence of bacilli, suppurative heterophilic inflammation, edema, fibrin, necrosis, and/or hemorrhage in the spleen, lungs, and lymph nodes (bronchial, mediastinal, mandibular, and mesenteric). The lungs exhibited alveolar edema and hemorrhage with interstitial inflammation (Figure 4C). Bacteria were present in pulmonary interstitial blood vessels, alveoli (rarely), and in hepatic sinusoids. Minimal hepatocellular necrosis was present in the liver of one animal. Lymphocyte depletion and/or lymphocytolysis were present in most lymph nodes and were prevailing in the bronchial and mediastinal lymph nodes and the spleen. Lymphocytolysis was characterized by increased apoptotic lymphocytes within and outside of germinal centers. Bacilli were abundant in renal vessels (Figure 4D) and the splenic red pulp and lymph node sinuses (Figure 4E). Bacilli were also present in vasculature of the

pancreas, mammary gland, heart (Figure 4F) and gastrointestinal tissues, and occasionally in meningeal vessels.

DISCUSSION:

Animal models of diseases that afflict humans are critical tools for developing medical countermeasures against life-threatening conditions, such as inhalational anthrax, for which clinical evaluation of efficacy is not feasible. The use of an animal model for the assessment of efficacy of a medical countermeasure is contingent upon thorough characterization of the course and manifestation of the disease of interest in that animal model. This is particularly important in cases where an established and universally accepted model is not suitable for the evaluation of a particular drug or vaccine and an alternative model needs to be developed. In such cases, assessment of the natural history of the disease under the appropriate and controlled conditions similar to those utilized for the established models is essential.

In the case of experimental inhalation anthrax, the standard conditions employed in anthrax challenge studies in rabbits and NHPs aimed at evaluation of anthrax vaccines and therapeutics include the use of the highly virulent Ames strain of *B. anthracis* which is known to be highly virulent in humans (36); standardized and validated spore preparation and characterization methods that ensures consistency of the challenge material (39, 45, 46); an appropriate target challenge dose (200 LD_{50}) that ensures universal lethality in untreated animals; and an appropriate aerosol exposure system that ensures delivery of the aerosolized spores to the lung while minimizing non-pulmonary exposure. Therefore, this study was focused on the natural history of

inhalational anthrax in guinea pigs under these standardized conditions as well as on comparing clinical signs, disease progression, and pathology observed in guinea pigs with those previously described for human disease and other animal models.

As summarized in Table 7, the inhaled LD_{50} of the Ames strain of *B. anthracis* in guinea pigs in a nose-only exposure system was determined to be somewhat different from the previously reported LD_{50} values obtained using the whole body exposure system (47) or intranasal instillation (48). However, it is comparable to the estimated human LD_{50} (49-52) as well as to the published muzzle-only LD_{50} for NZW rabbits (8) and head-only LD_{50} for rhesus and cynomolgus macaques (13-15, 35), which indicates that the guinea pig sensitivity to the *B. anthracis* infection following inhalation exposure is similar to that in the established animal models.

It is believed that cardiovascular dysfunction including decreased cardiac output, hypotension, and altered heart rate caused by synergistic effect of lethal and edema toxins (LT and ET) results in toxic (hypovolemic) shock in humans (53, 54). Telemetry data clearly demonstrate a decline of cardiovascular functions in guinea pigs, reflecting development of septic shock and death within several hours, which is not uncommon in humans (3) and correlates with previous findings in rabbits (9, 55) and African green monkeys (56).

Depending on the exposure dose, severity and duration of the disease, there is wide variation in lesions associated with inhalational anthrax in humans (57). Postmortem findings reported in human cases include peripheral transudate surrounded fibrin-rich edema; necrosis of arteries and veins leading to hemorrhages; displacement of infiltrating tissue resulting in necrosis of mediastinal lymph nodes; mediastinitis;

464 pneumonia; pleural effusions; mesenteric lymphadenitis; multiple gastrointestinal
465 submucosal lesions and apoptosis of lymphocytes (57-59).

466 Various rodent and non-rodent species have been used as animal models for
467 inhalational anthrax. The mouse model has been used to study pathogenesis of *B.*
468 *anthracis* infection and host-pathogen interactions, as well as to generate proof-of-
469 concept data with anthrax countermeasures. However, the mouse is not very predictive
470 of the pulmonary anthrax infection in humans (60). This is primarily due to the fact that
471 immunocompetent mice are resistant to infection with non-encapsulated toxinogenic
472 strains such as the Sterne strain (61). Complement-deficient mouse models, such as
473 A/J and DBA/2 are susceptible to the effects of LT (62); however, they do not exhibit
474 typical pathological changes observed in humans, guinea pigs, rabbits, and NHPs (7,
475 18). Although a rat *in vivo* lethal toxin neutralization model has been developed and
476 used to evaluate antitoxin therapeutics (63), rats are highly resistant to *B. anthracis*
477 spore exposure (estimated LD_{50} of 1×10^6) and are, therefore, not considered an
478 appropriate model for studying inhalation anthrax (17).

479 In contrast, guinea pigs, rabbits, and several species of NHPs, have been shown
480 to be predictive of inhalation anthrax in humans (18). Both rabbits and guinea pigs
481 rapidly develop fulminant systemic disease, with death occurring within two to four days
482 post-challenge (8, 18), as shown with guinea pigs in this study. Histopathological
483 findings in these models include necrotizing lymphadenitis, splenitis, pneumonia,
484 vasculitis, hemorrhage, congestion, and edema in multiple tissues (18). In NHPs,
485 pathology findings typically include congestion, edema, fibrin, hemorrhage, necrosis,
486 infiltrates of acute inflammatory cells, and vasculitis in the lungs, lymph nodes, spleen,

and meninges. In addition, the brain, adrenal glands, mesentery, liver, gastrointestinal tract, mediastinum, and urogenital organs are commonly affected in NHPs (14, 15, 18, 24).

This study showed close similarities between the natural history of experimental inhalation anthrax disease in guinea pigs exposed to aerosolized *B. anthracis* spores under the standard conditions (200 LD_{50}, Ames strain, nose-only exposure, etc.) and that in the established rabbit and NHP models. By demonstrating the similarity of the pathophysiology and pathology of pulmonary anthrax disease in guinea pigs to that in humans, this study supports the use of this model as an alternative small animal model for the assessment of efficacy of anthrax countermeasures.

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706 **Table 1. Natural History Animal Group Designation and Study Endpoints**

Group	No. of Animals per Group	Terminal Endpoint (Hours or days after aerosol exposure)	Telemetry	CBC	Bacteremia	qPCR	PA ELISA	Clinical Chemistry/ CRP
1	4 (2M, 2F)	24±1 hours		X	X	X	X	X
2	4 (2M, 2F)	30±1 hours		X	X	X	X	X
3	4 (2M, 2F)	36±1 hours		X	X	X	X	X
4[a]	4 (2M, 2F)	48±1 hours		X	X	X	X	X
5[a]	4 (2M, 2F)	60±1 hours		X	X	X	X	X
6[a]	4 (2M, 2F)	72±1 hours		X	X	X	X	X
7[a]	4 (2M, 2F)	96±1 hours		X	X	X	X	X
8[b]	9 (4M, 5F)	Day 14	X					
9	4 (2M, 2F)	Day 0 (Control)		X	X	X	X	X

707 [a] Animals in these groups died prior to the scheduled terminal endpoint.
708 [b] Animals in Group 8 implanted with telemetry units were not bled.
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711 **Table 2. Aerosol Challenge and Mortality Results**

Iteration	Dosage Group	No. of Animals	Estimated Inhaled Dosage (spores/animal)	No. of Animals Survived
I	1	8	3.6×10^4	5
	2	8	5.3×10^4	5
	3	8	4.8×10^4	5
	4	8	4.1×10^4	3
	5	8	6.1×10^4	4
	6	8	1.1×10^5	2
	7	8	3.1×10^5	0
	8	8	7.7×10^5	0
II	9	8	6.6×10^3	8
	10	8	7.6×10^3	6
	11	8	1.5×10^4	5
	12	8	2.3×10^4	4
	13	8	4.4×10^4	4
III	14	8	6.4×10^3	6
	15	8	1.0×10^4	8
	16	8	2.0×10^4	6
	17	8	4.4×10^4	5
	18	8	1.0×10^5	5
	19	8	2.6×10^5	1
	20	8	5.7×10^5	1

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714 **Table 3. Estimated LD_{50} and LD_{90} for Each Iteration.**

Iteration	Estimated LD_{50} (spores/animal)		Estimated LD_{90} (spores/animal)	
	LD_{50}	95% Confidence Interval	LD_{90}	95% Confidence Interval
I	5.27×10^4	(3.09×10^4 , 7.80×10^4)	1.79×10^5	(1.06×10^5 , 1.78×10^6)
II	3.13×10^4	(1.69×10^4 , 6.83×10^6)	2.01×10^5	(5.74×10^4 , 1.55×10^{14})
III	7.63×10^4	(3.69×10^4 , 1.94×10^5)	8.54×10^5	(2.92×10^5 , 1.36×10^7)
I, II, and III	5.01×10^4	(3.44×10^4 , 7.54×10^4)	4.51×10^5	(2.35×10^5 , 1.47×10^6)

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719 **Table 4. Summary of Mortality Results**

Animal ID	Group	Time of Scheduled Terminal Endpoint	Time to Death from Median Challenge Time (Hours:Minutes)	Scheduled Euthanasia/Moribund Euthanasia/Found Dead
136F	1	24±1 hours	27:24	Scheduled Euthanasia
138F	1	24±1 hours	27:25	Scheduled Euthanasia
172M	1	24±1 hours	27:36	Scheduled Euthanasia
188M	1	24±1 hours	27:51	Scheduled Euthanasia
144F	2	30±1hours	30:02	Scheduled Euthanasia
145F	2	30±1hours	30:17	Scheduled Euthanasia
178M	2	30±1hours	30:29	Scheduled Euthanasia
179M	2	30±1hours	30:34	Scheduled Euthanasia
141F	3	36±1 hours	36:00	Scheduled Euthanasia
148F	3	36±1 hours	36:06	Scheduled Euthanasia
175M	3	36±1 hours	36:18	Scheduled Euthanasia
183M	3	36±1 hours	36:23	Scheduled Euthanasia
140F	4	48±1 hours	47:45	Found Dead
153F	4	48±1 hours	48:17	Scheduled Euthanasia
181M	4	48±1 hours	48:21	Scheduled Euthanasia
185M	4	48±1 hours	48:27	Scheduled Euthanasia
139F	5	60±1 hours	59:44	Found Dead
149F	5	60±1 hours	54:22	Moribund Euthanasia
173M	5	60±1 hours	51:42	Found Dead
182M	5	60±1 hours	59:44	Found Dead
150F	6	72±1 hours	45:55	Found Dead
151F	6	72±1 hours	66:10	Found Dead
171M	6	72±1 hours	72:04	Scheduled Euthanasia
176M	6	72±1 hours	54:01	Found Dead
142F	7	96±1 hours	48:08	Moribund Euthanasia
147F	7	96±1 hours	59:44	Found Dead
184M	7	96±1 hours	51:42	Found Dead
187M	7	96±1 hours	70:45	Found Dead
131F	8	Day 14	51:41	Found Dead
132F	8	Day 14	60:13	Moribund Euthanasia
133F	8	Day 14	59:44	Found Dead
134F	8	Day 14	59:59	Found Dead
135F	8	Day 14	54:01	Found Dead
166M	8	Day 14	48:01	Moribund Euthanasia
167M	8	Day 14	54:01	Found Dead
168M	8	Day 14	66:10	Found Dead
169M	8	Day 14	54:01	Found Dead
143F	9	Day 0	Not challenged	Scheduled Euthanasia
146F	9	Day 0	Not challenged	Scheduled Euthanasia
174M	9	Day 0	Not challenged	Scheduled Euthanasia
186M	9	Day 0	Not challenged	Scheduled Euthanasia

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722 **Table 5. Summary of the Time to Abnormal Results for All Telemetry Parameters**

Parameter	No. Abnormal/ No. of Animals	Proportion Abnormal (Exact 95% Confidence Interval)	Geometric Mean Time to Abnormality in Hours (95% Confidence Interval)[a]
Activity	0/9	0.00 (0.00, 0.34)	--[b]
Temperature	8/9	0.89 (0.52, 1.00)	46.16 (34.75, 57.56)
Heart Rate	7/9	0.78 (0.40, 0.97)	44.32 (26.78, 61.86)
Mean Arterial Pressure	9/9	1.00 (0.66, 1.00)	52.48 (48.01, 56.95)
Pulse Pressure	7/9	0.78 (0.40, 0.97)	51.75 (45.15, 58.35)
Diastolic Pressure	9/9	1.00 (0.66, 1.00)	52.37 (49.44, 55.29)
Systolic Pressure	9/9	1.00 (0.66, 1.00)	53.92 (50.23, 57.62)

723 [a] These calculations only include animals that showed abnormal parameters.
724 [b] No calculations were performed since the parameter was normal for all animals.
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Table 6. Summary of Hematology Changes in Infected Animals Compared to Unchallenged Controls

Parameter	Dunnett's Two-Sided P-values for Comparisons to the Unchallenged Control Group (Group 9), by Challenge Group				
	Group 1 (24 hours)	Group 2 (30 hours)	Group 3 (36 hours)	Group 4 (48 hours)	Group 6* (72 hours)
RBC†	-	-	-	-	++ ↓
Hemoglobin	-	-	-	-	+ ↓
Hematocrit†	-	-	-	-	+++ ↓
MCV	-	-	-	-	++ ↑
MCH	-	-	-	-	+++ ↑
MCHC	+ ↑	-	++ ↑	-	+++ ↑
RDW	-	-	-	-	+++ ↑
Platelet Count	-	-	-	-	+++ ↑
MPV†	-	-	-	-	-
WBC†	-	-	-	+ ↑	+++ ↑
Heterophil Count†	-	-	-	++ ↑	+++ ↑
Lymphocyte Count	-	-	-	-	+++ ↑
N/L Ratio†	-	-	-	++ ↑	+++ ↑
Monocyte Count	-	-	-	-	+++ ↑
Eosinophil Count†	-	-	-	-	-
Basophil Count	-	-	-	-	+++ ↑

* Comparisons between group 6 and the unchallenged control group (group 9) were based on one observation in group 6.

† Indicates that values for this parameter were log-transformed for the analysis.

\- Group mean (or geometric mean for log-transformed parameters) was not significantly different ($p > 0.05$) compared to the unchallenged control group (group 9)

\+ Group mean (or geometric mean for log-transformed parameters) was significantly different ($p < 0.05$) compared to the unchallenged control group (group 9)

++ Group mean (or geometric mean for log-transformed parameters) was significantly different ($p < 0.01$) compared to the unchallenged control group (group 9)

+++ Group mean (or geometric mean for log-transformed parameters) was significantly different ($p < 0.001$) compared to the unchallenged control group (group 9)

↑ Group mean (or geometric mean for log-transformed parameters) was significantly greater than that in the unchallenged control (group 9)

↓ Group mean (or geometric mean for log-transformed parameters) was significantly lower than that in the unchallenged control (group 9) at the 0.05 level.

757 **Table 7. Aerosol Anthrax Spore LD_{50} in Laboratory Animals and Humans**
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Species	Route	Strain	LD_{50}	Reference
Guinea pig	IM	Ames	1.0×10^2	(6)
Guinea pig	Intranasal instillation	Ames	6.0×10^5	(48)
Guinea pig	Whole body aerosol exposure	NH6 and Vollum1B	$6.5 \times 10^2 – 7.0 \times 10^4$	(47)
Guinea pig	Nose only; Aerosol	Ames	5.0×10^4	Current Study
Rabbit	Muzzle	Ames	1.1×10^5	(8)
Common marmoset	Head only aerosol exposure	Ames	1.5×10^3	(24)
Cynomolgus Macaque	Head only aerosol exposure	Ames	$6,2 \times 10^4$	(15)
Rhesus Macaque	Head only aerosol exposure	Ames	5.5×10^4	(13)
Human (Estimates)	Aerosol	--[a]	$2.0 \times 10^3 – 5.5 \times 10^4$	(49-51, 65)

759 [a] Modeling is based in part on the isolate from the 1979 accidental spore release in Sverdlovsk, USSR.
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Figure Legends

Figure 1. Dose-Lethality Curve Using the Log-Transformed Estimated Inhaled Dosage as the Predictor Variable.
The LD50 was determined in three iterations. Groups of animals were exposed to various doses with challenge dose levels in the second and third iteration based on the survival results from the previous iteration(s). In each iteration, guinea pigs were monitored for mortality twice daily for up to 21 days. The predicted curve from the
probit model using data from all three iterations is plotted with a solid line while the observed lethalities are displayed as symbols by iteration. In addition, the upper and lower 95% confidence bounds on the predicted curve are provided as dashed lines. Two horizontal reference lines correspond to 50 percent and 90 percent mortality. The LD50 for all three iterations combined was estimated at 5.01 x 104 spores per animal with a 95% confidence interval of (3.44 x 104, 7.54 x 104) spores per animal.

Figure 2. Changes in Telemetric Parameters Post-Challenge.
Nine challenged animals were surgically implanted with a telemetry transmitter and were monitored for clinical signs of disease. The mean value for each parameter measured by telemetry was computed for every 15 minutes for 24 hours (00:00, 00:15,…, 23:45) prior to challenge (baseline). Data collected post-challenge was then baseline adjusted according to the associated clock time. In these figures, mean differences that were significantly greater than zero are indicated by a black square, while mean differences that were significantly less than zero are indicated by a black
triangle. The parameters monitored included temperature (**A**), heart rate (**B**), mean arterial pressure (**C**), pulse pressure (**D**), diastolic pressure (**E**) and systolic pressure (**F**).

Figure 3. Bacteria and Toxin Levels Post-Challenge.
(**A**) Quantitative bacteremia: 100 µL of whole blood was plated in triplicate on tryptic soy agar (TSA). In addition, 10-fold serial dilutions were performed by transferring 100 µL of whole blood or previous dilution into 900 µL of PBS. For each dilution prepared, 100 µL was plated in triplicate on TSA. Plates were incubated at 37°C for 16-24 hours, bacterial colonies enumerated, and the corresponding concentration (cfu/mL) calculated. (**B**) Protective Antigen (PA) levels as measured by ELISA. Plates were read 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 samples were determined by computer interpolation from the plot of the reference standard curve data.

Figure 4. Histopathological Findings.
Tissue samples were preserved in 10% neutral buffered formalin, paraffin-embedded, processed to slides and stained with hematoxylin and eosin. The pictures above are of representative histopathological findings. (**A**) Lymph node sinuses and extravascular tissues are filled with blood (hemorrhage) and only remnants of lymphoid follicles remain (arrows). HE 4X. (**B**) Brain, meninges: Blood vessel contains large rod-shaped bacteria consistent with anthrax bacilli (arrows). HE 10X. (**C**) Alveoli filled with fibrin and edema (arrows) including pulmonary intravascular B. anthracis (arrow heads). HE 40X. (**D**) Renal vessels contain anthrax bacilli (arrows). HE 40X. (**E**) Spleen shows sinusoidal anthrax bacilli and degenerate and viable heterophils (arrows) HE 40X. (**F**) Heart: Anthrax bacilli within coronary vessels (arrows). HE 10X.
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