Abstract # 035(B)

Determination of the 50% Lethal Dose (LD_{50}) of *Bacillus anthracis* in Mice Following Inhalation Exposure

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Abstract

Background: The 50% lethal dose of *Bacillus anthracis* Ames was determined in BALB/c mice. Eight groups of mice, consisting of five males and five females each, were exposed to USP Water For Injection or *Bacillus anthracis* Ames by nose-only inhalation.

Methods: Varying aerosol concentrations were delivered to the breathing zone of the mice corresponding to inhaled doses of 0 CFU (Group 1), 3.1E+02 CFU (Group 2 females), 3.4E+02 CFU (Group 2 males), 2.8E+03 CFU (Group 3 females), 3.1E+03 CFU (Group 3 males), 5.4E+03 CFU (Group 4 females), 6.4E+03 CFU (Group 4 males), 3.2E+04 CFU (Group 5 females), 3.7E+04 CFU (Group 5 males), 9.1E+04 CFU (Group 6 females), 1.0E+05 CFU (Group 6 males), 3.8E+05 CFU (Group 7 females), 4.4E+05 CFU (Group 7 males), and 1.2E+06 CFU (Group 8 females), 1.3E+06 CFU (Group 8 males). Animals were observed for up to 15 days, and body weights and temperatures were collected. Blood samples were collected at the time of euthanasia or when animals were found dead. Collected sera were analyzed for the presence of *Bacillus anthracis*.

Results: Twenty-seven of 80 mice, including 14 males and 13 females, died prior to scheduled euthanasia. No statistical differences were identified in survivability between male and female dose groups; however, comparisons between male and female survivability were not possible for Groups 1, 2, and 4 since 100% survival was the outcome for both samples. Probit analyses projected the LD_{50} to be 7.5E+04 CFU for male mice and 1.3E+05 CFU for female mice. The gender neutral LD_{50} was 9.4E+04 CFU. Clinical signs of anthrax were observed in male and female mice within 24 hours following exposure; however, no clinical signs were observed after 72 hours. The administration of *Bacillus anthracis* Ames had no effect on body weights of mice.

Conclusions: In summary, exposure of mice to *Bacillus anthracis* by inhalation exposure was highly pathogenic and an LD_{50} similar to that reported in the literature was observed.

Introduction

Bacillus anthracis Ames (BAA) is the etiological agent of anthrax and poses a significant threat as a bioterrorism weapon. Following inhalation and deposition in the lungs, BAA spores are transported by the alveolar macrophages into lymphatic system where they multiply and produce lethal toxins. The development of an animal model to test therapeutic and vaccine candidates against inhalation anthrax requires the determination of the inhalation 50% lethal dose (LD_{50}). Eight groups of five male and five female BALB/c mice were exposed to aerosolized phosphate buffered saline (PBS) or BAA spores by nose-only inhalation for 30 minutes. Our objective was to determine the 50% lethal dose (LD_{50}) of BAA in mice following aerosol challenge in a radial nose-only inhalation exposure system.

Methods

Challenge Material:

Bacillus anthracis Ames (BAA) spores were prepared by growing cultures in DSM (Difco Sporulation Media) to nutrient exhaustion. The sporulated culture was harvested by centrifugation, washed with USP-WFI, purified using Renografin-60 (Bracco; Princeton, NJ) density gradients and washed with USP-WFI to remove trace amounts of Renografin-60. The spores were then resuspended in USP-WFI, observed by phase contrast microscopy (Figure 1), and tested for endotoxin using the limulus amebocyte lysate assay (Charles River Laboratories; Wilmington, MA). Spore number (CFU/mL) was determined by plating on Tryptic Soy Agar (TSA) with 5% sheep's blood plates (Becton Dickson; Franklin Lakes, NJ). Prior to exposure, the spore suspension was serially diluted in USP-WFI to achieve concentrations of 0, 3.0E+05, 3.0E+07, 1.0E+08, 5.0E+08, 1.0E+09, 3.5E+09, and 5.0E+09 CFU/mL.

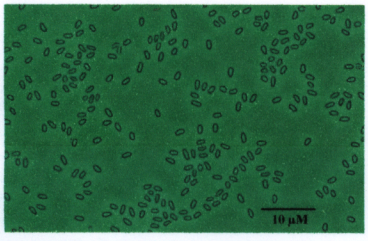

Figure 1. Purified BAA Spores. Phase contrast microscopy photograph of purified BAA spores.

Methods Continued

Inhalation Exposure Procedure

A Collison three-jet nebulizer (BGI, Inc.; Waltham, MA) generated the aerosol. The aerosol was conditioned with an in-line mixer (In-Tox Products, LLC; Albuquerque, NM (ITP)), dried with passive dilution air, and directed through the stainless steel aerosol delivery line into a multi-port radial nose-only exposure chamber with isoaxial sample collection ports (ITP). Flows were calibrated using a primary flow calibration device (DryCal DC-Lite) (BIOS International; Butler, NJ). Flows were maintained using gas flow controllers (Alicat Scientific, Inc.; Tucson, AZ). The chamber environment was monitored with a 5800 Intelligent Oxygen Monitor (Hudson RCI; Durham, NC) and a Traceable Memory Humidity/Temperature Meter (Fisher Scientific; Pittsburgh, PA).

The entire exposure system (Figure 2), with the exception of the vacuum pump and compressed air source, was placed inside a Class III Biosafety Cabinet (The Baker Company; Sanford, ME). All downstream flow lines encompassed high-efficiency particulate absorbing (HEPA) filters, and the Chamber pressure was maintained slightly negative relative to the biosafety cabinet. Prior to conducting exposures, the inhalation system was characterized for spatial and temporal uniformity as well as for stability

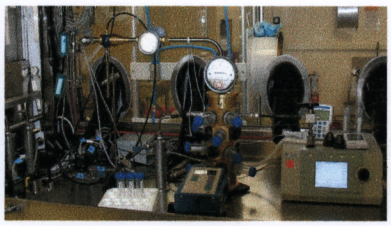

Figure 2. The inhalation exposure system. A. Aerodynamic Particle Sizer Diluter, B. Aerodynamic Particle Sizer, C. 24-port plenum, D. Mixer, E. Stainless steel impinger, F. Collison 3-Jet nebulizer, and G. Gas Flow Controllers.

Study Design

Eighty BALB/c mice 40 males and 40 females were assigned to groups one through eight randomly to achieve five males and five females in each group of ten mice. Passive integrated transponders (IPTT-300 Chip; Biomedic Data Systems Inc.) were implanted in each mouse, and the mice were tube trained twice in nose-only restraint tubes prior to exposure. Mice were placed in nose-only restraint tubes and connected to the exposure chamber using Positive Flow-By™ nose cones (In-Tox Products, LLC; Albuquerque, NM). The nebulizer was filled with the BAA suspension and operated at a constant pressure. The mice were exposed according to the exposure outline (Table 1).

Group Number	Target Inhaled Dose (CFU/mL)	Target LD_{50} Equivalents	Target Aerosol Concentration (CFU/L)	Number of Mice
1	0	0	0.0E+00	5M / 5F
2	3.5E+03	0.1	2.9E+03	5M / 5F
3	1.8E+04	0.5	1.5E+04	5M / 5F
4	3.5E+04	1.0	2.9E+04	5M / 5F
5	1.8E+05	5.0	1.5E+05	5M / 5F
6	3.5E+05	10.0	2.9E+05	5M / 5F
7	1.8E+06	50.0	1.5E+06	5M / 5F
8	3.5E+06	100.0	2.9E+06	5M / 5F

Table 1. Exposure Outline

Aerosol Characterization

The concentration of challenge material in the exposure atmosphere was determined using the stainless steel impinger (SSI) (In-Tox Products, LLC, Albuquerque, NM) samples which were collected from the breathing zone of the animals during the exposure. Aerosol concentration was reported as colony forming units per liter of air (CFU/L). The aerosol particle size distribution was determined by time-of-flight analysis using the Aerosol Particle Sizer (APS) (TSI, Inc.; Shoreview, MN (TSI). Each group of animals were exposed for 30 minutes.

Results

Bioaerosol Characterization

Aerosol Concentration

Liquid impinger samples were collected from the exposure plenum during each exposure. Impinger plate counts were used to calculate aerosol concentration. Guyton's formula (1) was used to estimate mouse minute ventilation. Aerosol concentration, mouse minute ventilation, and exposure time were used to calculate inhaled dose. Group mean aerosol concentrations and mean inhaled doses are given in Table 2.

Group Number	Nebulizer Concentration (CFU/mL)	Impinger Concentration (CFU/mL)	Aerosol Concentration (CFU/mL)	Inhaled Dose (CFU)
1	0.0E+00	0.0E+00	0.0E+00	0.0E+00
2	3.0E+05	2.7E+03	5.6E+02	6.8E+02
3	3.0E+07	2.6E+04	5.0E+03	6.0E+03
4	1.0E+08	5.6E+04	1.0E+04	1.2E+04
5	5.0E+08	3.0E+05	5.9E+04	7.0E+04
6	1.0E+09	9.0E+05	1.6E+05	2.0E+05
7	3.5E+09	2.8E+06	7.0E+05	8.4E+05
8	5.0E+09	9.1E+06	2.1E+06	2.6E+06

Table 2. Mean Aerosol Concentration and Mean Inhaled Dose

Particle Size Distribution

A single APS sample was collected at the midpoint of each exposure (t = 15 minutes). The mean mass median aerodynamic diameter of the BAA challenge aerosol was 0.9 ± 0.0 µm and the mean geometric standard deviation was 1.5 ± 0.5. The mean count median aerodynamic diameter was 0.8 ± 0.1 µm and the mean geometric standard deviation was 1.2 ± 0.1. Group particle size distribution data is presented in Table 3. A representative APS depicting BAA aerosol mass distribution is shown in Figure 3.

Group Number	MMAD (µm)	MMAD GSD	CMAD (µm)	CMAD GSD
1	0.953	1.580	0.633	1.300
2	0.933	1.170	0.894	1.190
3	0.909	1.180	0.863	1.160
4	0.887	1.400	0.846	1.140
5	0.887	1.180	0.845	1.150
6	0.898	2.210	0.837	1.160
7	0.929	1.150	0.899	1.120
8	0.948	2.270	0.859	1.180
Mean	0.918	1.518	0.835	1.175
Stdev	0.026	0.470	0.084	0.055
%CV	2.874	30.981	10.124	4.684

Table 3: Aerodynamic Particle Sizer Results

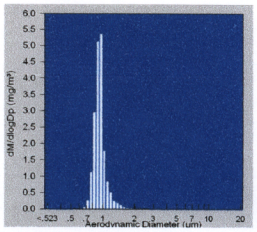

Figure 3: BAA Aerosol Mass Distribution

Animal Survival Data

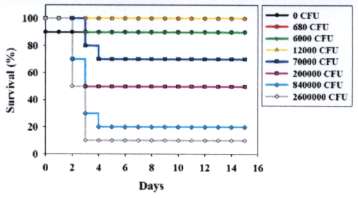

Figure 4. Mouse Mortality Data. Ninety percent of the mice in Group 8 died by Day 3.

Results Continued

Probit Analysis

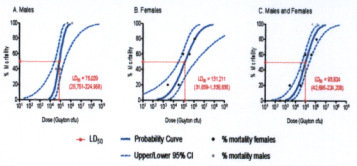

Figure 5. Probit Analysis of Murine Data. Statistical differences between the dose groups were evaluated using probit analysis. Independent variables for the probit analysis were dose and sex, and the dependent variable was survival. The combined LD_{50} in BALB/c mice was 9.4E+04 CFU.

Discussion

Inhalation anthrax is the most severe form of the disease and has the most rapid onset. Once inhaled, *Bacillus anthracis* Ames (BAA) spores germinate and release several toxins which cause internal bleeding, swelling, and tissue necrosis. There are usually two stages of inhalation anthrax. Stage one can last from hours to a few days and presents with flu-like symptoms such as fever, fatigue, and malaise. Stage two usually develops suddenly. Symptoms include fever, shortness of breath, and shock (2). Male and female mice were exposed to concentrations of *Bacillus anthracis* Ames spores ranging from 0.0E+00 to 2.1E+06 CFU/L in a nose-only inhalation exposure system. The 50% lethal dose was projected to be 9.4E+04 CFU for male and female mice collectively. Separate analyses for male mice and female mice projected 50% lethal doses of 7.5E+04 CFU and 1.3E+05 CFU, respectively. These are similar to previously reported values of 3.4E+04 CFU in BALB/c mice exposed by whole-body inhalation and 2.0E+05 CFU in A/J mice exposed by nose-only inhalation (3, 4).

The results of this study suggest a collective inhalation 50% lethal dose in BALB/c mice following nose-only inhalation exposure of *Bacillus anthracis* Ames spores of 9.4E+04 CFU.

Conclusions

- **Impinger analysis demonstrated that the actual aerosol concentration of the BAA spores was similar to the projected targets.**
- **Aerodynamic particle sizer analysis demonstrated the particle size distribution was similar for all groups.**
- **Probit analysis shows that BALB/c male and female mice combined LD_{50} of *Bacillus anthracis* Ames spores following nose-only inhalation exposure of was 9.4E+04 CFU.**

References

1. Guyton, A.C. Measurement of the Respiratory Volumes of Laboratory Animals. Am J Physiol, 150, 70-77, 1947.
2. Logan NA. *Bacillus anthracis*, *Bacillus cereus*, and other aerobic endospore-forming bacteria. Topley and Wilson's Microbiology & Microbial infections, Bacteriology Vol 2. ed. Borriellow, SP, Murray, PR, Funke G. p. 932.
3. Heine HS, et al. 2007. Determination of Antibiotic Efficacy Against *Bacillus anthracis* in a Mouse Aerosol Challenge Model. Antimicrob. Agents Chemother. 2007 April; 51(4): 1373-1379.
4. Loving CL, Kennett M, Lee GM, Grippe VK, Merkel TJ. 2007. Infection and Immunity. 2007, p. 2689-2698.

Acknowledgements

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