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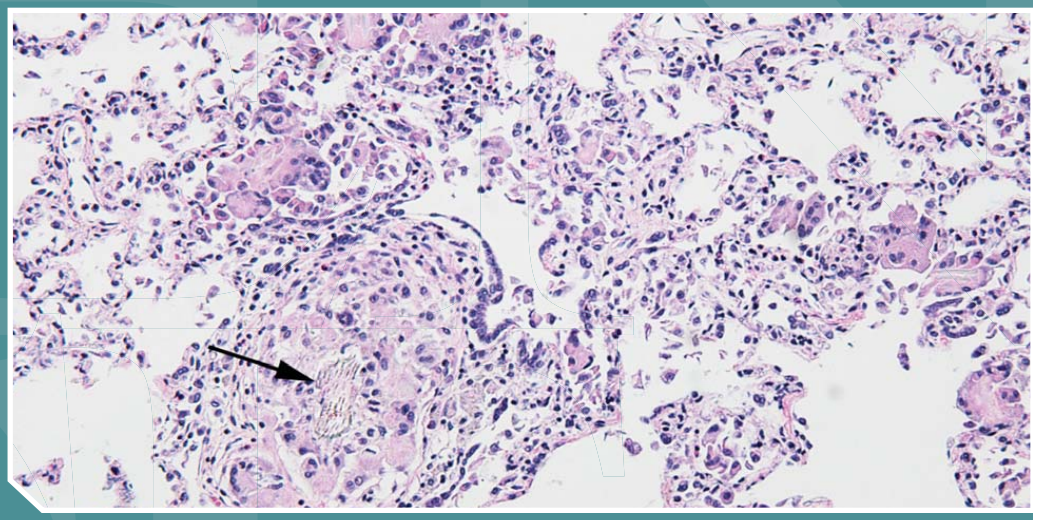
United States
Environmental Protection
Agency

Review and Design of Low Dose *Bacillus anthracis* Inhalation Exposures

MEETING REPORT



Office of Research and Development
National Homeland Security Research Center

EPA/600/X-12/001

Review and Design of Low Dose *Bacillus anthracis* Inhalation Exposures

MEETING REPORT

United States Environmental Protection Agency
Cincinnati, Ohio 45268

Disclaimer

This report was prepared as a summary of the presentations and discussions held at the U.S. Environmental Protection Agency (EPA) technical meeting, *Review and Design of Low Dose Bacillus anthracis Inhalation Exposures* (July 27-28, 2011). This report captures the main points and highlights of the meeting; it is not a complete record of all detailed discussions, nor does it embellish, interpret, or enlarge upon matters that were incomplete or unclear. At this meeting, EPA was not seeking consensus decisions but rather hoped to solicit the views of individual experts to help the Agency develop its future research agenda.

The EPA through its Office of Research and Development's National Homeland Security Research Center contracted the preparation of this report under contract number EP-W-09-011 to RESOLVE, Inc. It has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. This draft report will be submitted through the EPA clearance process after initial meeting attendee reviews have been conducted.

Questions concerning this document or its application should be addressed to:

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Contents

List of Figures

Foreword

Following the terrorist events of 2001, the U.S. Environmental Protection Agency's (EPA) mission was expanded to account for critical needs related to homeland security. Presidential Directives identified EPA as the primary federal agency responsible for the country's water supplies and for decontamination following a chemical, biological, and/or radiological attack. EPA's National Homeland Security Research Center (NHSRC) program is focused on conducting research and delivering products that improve the capability of the Agency to carry out its homeland security responsibilities.

NHSRC is responsible for delivery of reports and databases with information on the health effects of contaminants. Reliable dose-response data are critical to assessing the human health risks from exposure to microorganisms originating from intentional and unintentional releases. Such releases can result in contamination of buildings, drinking water systems, outdoor areas, or food. However, dose-response data for biological threat agents in the low-dose range are very limited. To bridge this critical data gap, advanced methods, animal studies, and other approaches must generate credible low-dose data to support the development of acceptable, scientifically defensible response and remediation actions.

In July 2011**,** EPA NHSRC sponsored a *Review and Design of Low-Dose Bacillus anthracis Inhalation Exposures* meeting to review the research done to date and to identify gaps that future research should address regarding low-dose exposures. This effort brought together many organizations across the country, including EPA's program offices, federal government agencies and laboratories, academia, and the private sector. Participants of the conference shared knowledge, explored differing opinions, and expanded understanding of the current state of research for low-dose exposure and future research needs. This report represents a summary of the presentations and discussions during the meeting. We value your comments as we move one step closer to achieving our homeland security mission and our overall mission of protecting human health and the environment. Please send any comments to Sarah Taft, 26 W. Martin Luther King Drive, MS NG16, Cincinnati, OH 45268, 513-569-7037, Taft.Sarah@epa.gov.

Jonathan G. Herrmann, P.E., BCEE
Director, National Homeland Security Research Center

Acronyms and Abbreviations

B. anthracis	*Bacillus anthracis*
BclA	*Bacillus* collagen-like protein of *anthracis*
CDC	Centers for Disease Control and Prevention
CRP	C-reactive protein
DHS	Department of Homeland Security
DOD	Department of Defense
EPA	U.S. Environmental Protection Agency
FBI	Federal Bureau of Investigation
IgM	Immunoglobulin M
LF	lethal factor
MALT	mucosa-associated lymphoid tissue
NHSRC	National Homeland Security Research Center
NZW	New Zealand White rabbits
ORD	Office of Research and Development
PA	protective antigen

Acknowledgments

The EPA National Homeland Security Research Center would like to thank all the meeting participants who attended the technical meeting. The EPA would like to acknowledge Charles Haas, Margaret Coleman, James Estep, Paul Hinderliter, and Mary Alice Smith who took on the role of reporters for the small group discussions. The EPA would also like to acknowledge the presenters, Tonya Nichols, Jonathan Herrmann, Kevin Teichman, Stephen Morse, Christopher Russell, Sarah Taft, Anne Boyer, Conrad Quinn, and Crystal Briscoe.

The EPA also thanks the meeting facilitators from RESOLVE and Rocky Mountain Collaborative Solutions for coordinating a successful meeting.

Executive Summary

Along with its federal partners, the U.S. Environmental Protection Agency's (EPA) National Homeland Security Research Center (NHSRC) conducts research in the area of microbial risk assessment. The aim is to improve our understanding of the human health effects of exposure to microbial agents, including *Bacillus anthracis* (*B. anthracis*). Better knowledge of these effects will support first responders and decision makers during a biothreat event.

Although there have been studies of the effects of *B. anthracis*, previous research was focused on high-level exposures. Such research yields little insight into the potential impact of repeated low-level exposures, which is important to understand when making decisions on whether and when to reoccupy a building that has been the site of a biothreat incident. EPA, in partnership with the Department of Defense (DOD) and the Centers of Disease Control and Prevention (CDC), decided to conduct research into the human health effects of acute and multiple low dose exposures to *B. anthracis.*

Upon completion of the initial studies, EPA consulted with other technical experts in the field on the interpretation of the results and their recommendations for future studies. The Agency convened representatives of federal government agencies, private research institutions, and academia, in Cincinnati, Ohio, July 27 – 28, 2011, to discuss the results of the studies of acute and multiple low dose *B. anthracis* inhalation exposure in rabbits. The objectives of the meeting were to:

- Review the preliminary acute and multiple low dose exposure studies.
- Identify strengths and weaknesses of the current approach.
- Design follow-on subchronic low dose exposure studies.

There were 42 attendees; in addition, there were three participants from Research Triangle Park, North Carolina, and two from Washington, D.C., via video-teleconference link. The meeting focused on science and recommendations for future studies rather than on policy concerns. At this meeting, EPA was not seeking consensus decisions but rather hoped to solicit the views of individual experts to help the Agency develop its future research agenda.

Jonathan Herrmann and Tonya Nichols from the EPA NHSRC, Kevin Teichman from the EPA Office of Research and Development (ORD), Stephen Morse from the CDC, and Christopher Russell from Department of Homeland Security (DHS) opened the meeting by providing the background and context for the research. Sarah Taft of the EPA NHSRC then gave an overview of EPA's acute and multiple low dose exposure studies. Anne Boyer, CDC National Center for Environmental Health, and Conrad Quinn, CDC National Center for Immunization and Respiratory Diseases, gave presentations summarizing the CDC studies seeking to identify biomarkers of *B. anthracis* infection. An extensive question-and-answer session with the meeting participants followed the presentations.

The meeting participants then broke into smaller groups for in-depth discussion of the studies. The participants received a set of questions to facilitate discussions in the small groups. A reporter from each group presented the highlights and conclusions from each group's deliberations back to the plenary. The content of this report is drawn entirely from the information in the presentations and meeting discussion sessions.

The following are brief summaries of some of the discussion highlights:

- Overall, participants agreed that the multiple low dose study addressed an important gap in the current research. None of the meeting participants identified other existing multiple daily inhalation low dose exposure studies for EPA to consider in the assessment of the subchronic and chronic health effects of anthrax.
- Meeting participants identified the sample population as a concern for EPA's multiple low dose study design, due to the small sample number and the homogenous composition of the sample. In addition, many noted that except for mortality, the data from the multiple low dose study do not show any other obvious adverse effects of *B. anthracis* in the rabbits. Participants stated that no clear conclusions on adverse effects could be drawn from the studies for the following combination of reasons: the data as presented do not convey the temporal dimension for each observed response, the study period was not long enough, and there were an insufficient number of sick survivors.

Dr. Taft opened the second day of the meeting with a presentation on the options for future EPA studies, emphasizing the need to prioritize activities in light of limited resources. Dr. Taft would

like to receive participants' input on what those priorities should be. Dr. Crystal Briscoe of Batelle provided further context with a presentation on the pathogenesis of *B. anthracis*. At the conclusion of the meeting, EPA indicated that the next steps will be to develop a two-to-three year plan for an additional study. The Agency will take the ideas and suggestions raised during the meeting into account and welcomes additional input from the participants and readers of this report. Please send any comments to Sarah Taft, 26 W. Martin Luther King Drive, MS NG16, Cincinnati, OH 45268, 513-569-7037, Taft.Sarah@epa.gov.

The meeting participants once again broke into smaller groups on the second day to have discussions about options for future studies. The participants received a second set of discussion questions regarding future studies, and a reporter from each group presented the highlights and conclusions from each group's deliberations back to the plenary.

The following are brief summaries of some of the discussion highlights:

- Participants offered suggestions for several activities EPA could carry out in the short-term, prior to beginning a next round of studies. Specifically, the Agency could perform further pathologies and histologies on the existing tissue samples. Another suggestion was that EPA should do additional modeling before progressing to a next round of studies. The modeling could explore simulated time intervals and dose levels to better predict internal doses and infection, illness, severity of illness, and fatality.
- Prior to conducting another round of studies, it would be useful to arrive at a better characterization of the retained dose in the rabbits, as well as an improved understanding of the effects of multiple doses in the rabbit system and the risks of different portals of entry.
- When planning future studies, EPA should consider the real-life scenarios that the study results would inform, and, in particular, the risks associated with different types and levels of exposure.
- With regard to future studies, participants offered two main recommendations for altering the study design and methodology: (1) increasing the sample size; and (2) lengthening the monitoring period post-exposure. Some suggested that EPA design future studies to take into account population variability, including age, relative health, and gender.

Opening Comments

Representatives from the U.S. Environmental Protection Agency (EPA) National Homeland Security Research Center (NHSRC), the EPA Office of Research and Development (ORD), the Centers for Disease Control and Prevention (CDC), and Department of Homeland Security (DHS) provided opening comments to the technical meeting. The detailed meeting agenda is provided in Appendix A.

Jonathan Herrmann
EPA NHSRC

The EPA NHSRC is doing pioneering work in the area of microbial risk assessment. In its research program, the NHSRC is working with other federal agencies, including DHS and CDC. The Agency is particularly interested in the topic of this meeting - addressing the human health effects of microbial exposure and of *B. anthracis*.

Kevin Teichman
EPA Office of Research and Development

To the best of EPA ORD's knowledge, there is no other research into low dose multiple daily exposures for *B. anthracis*. For that purpose, the microbial research program would seek to address the following key questions:

- How do we provide crucial site-specific information after a biothreat release has occurred so people can know when it is safe to occupy spaces?
- What is the variability and susceptibility in the human population?
- Can we extrapolate from animal studies?
- Are there biomarkers that can indicate infection?
- In the absence of data, when can we use models to fill in the data gaps?

Dr. Teichman asked the experts in attendance to help review the preliminary study results and conclusions and give guidance that will improve future work. Dr. Teichman also spoke from the perspective of a parent whose child was present during the ricin attack at the U.S. Senate building, highlighting the urgency of finding answers that will allow informed decision making during biothreat events.

Stephen Morse
CDC National Center for Emerging and Zoonotic Infectious Diseases

CDC has a longstanding working relationship with EPA, involving both joint research and interaction between the agencies on an emergency response network. When a biothreat event takes place, leaders will need to make decisions about whether to shelter or evacuate. They will also need to estimate the associated risks of clinical disease. "Zero risk" does not exist, and there are a number of issues involved in making reoccupation decisions, such as the insufficient understanding of the effects of multiple low dose exposures to spores. Through ongoing research, CDC hopes to generate the highest quality scientific data possible. Such data will help to develop risk-based approaches that support cleanup goals and subsequent reoccupation decisions. Dr. Morse's presentation slides are provided in Appendix D.

Christopher Russell
DHS Science and Technology Directorate

Since 2003, EPA and DHS have undertaken a number of interagency exercises and joint projects in the DHS microbial response recovery portfolio. It is of utmost importance to understand the link between the environmental presence of a biothreat and the public health risk. The need to provide policymakers with information about the risks is also paramount.

Presentations Session I

Preliminary Acute and Multiple Low Dose Exposure Studies

Sarah Taft, Ph.D.
EPA NHSRC

In EPA's studies of acute and multiple daily low dose *B. anthracis* exposures in New Zealand White (NZW) rabbits (EPA 2011a, 2011b), the research team's main goal was to understand the human health effects of the microbial agents in order to support first responders, stakeholders, and decision makers in the case of an event. Understanding "how clean is clean" is critical to developing cleanup goals, detection limits, and treatment technologies. The team is taking a risk assessment approach to examine exposure pathways and make the connection to dose-response. When looking at the historical dose-response data, the team found that most of the research focused on very high acute exposure doses, and concluded that there was very limited inhalation *B. anthracis* dose-response data in the low dose range and for multiple daily exposure doses.

NHSRC, in partnership with the Department of Defense and CDC, therefore conducted low dose exposure studies to determine whether the NZW rabbits would survive low dose exposures and, if so, what effects they would experience. The team began with an acute exposure study, and then conducted a multiple daily exposure study. In the acute study, all of the rabbits in the highest exposure group died, while none of those in the control or the two lowest exposure groups died (Figure 1). The NZW rabbits in the lowest dose groups experienced minimal suppurative inflammation and multi-nucleated giant cells in the lungs (Figure 2). A few bacteria were also detected in the lungs during the histological examination of those receiving the higher of the two inhalation doses. It was unclear whether the appearance of the multi-nucleated giant cells indicated an adverse effect or a sign of immune response.

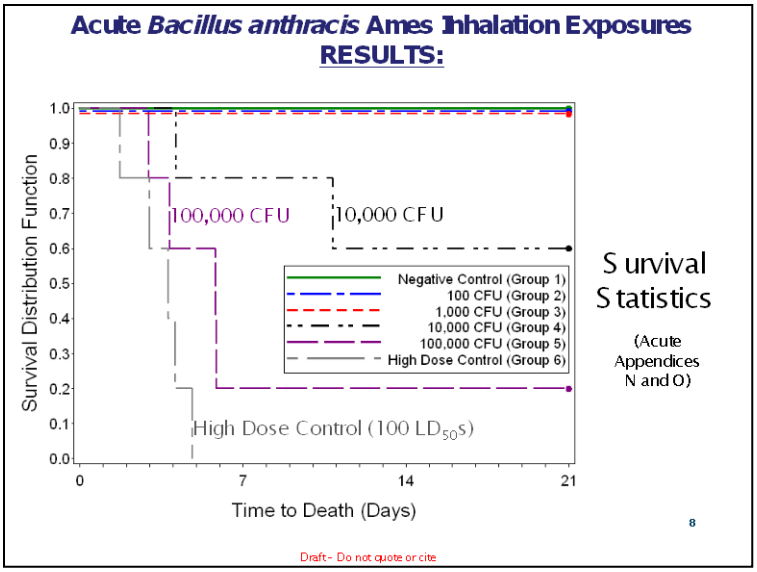

Figure 1. Acute exposure study survival statistics from Dr. Taft's slide 8.

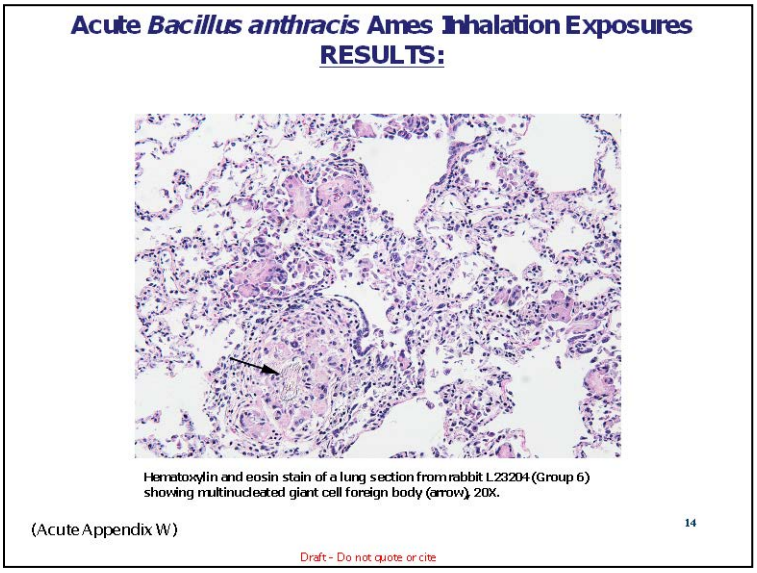

Figure 2. Multi-nucleated giant cells in lung of rabbit from acute exposure study from Dr. Taft's slide 14.

In the multiple daily exposure study, NZW rabbits in the two highest dose groups died (Figure 3). The team was only able to detect responses in the animals that died. There were, however, two rabbits in the highest dose groups with similar patterns – both became seriously ill but recovered, and both experienced seroconversion. The team was able to detect very few

measurable effects in the surviving groups, again noting the presence of the multi-nucleated giant cells.

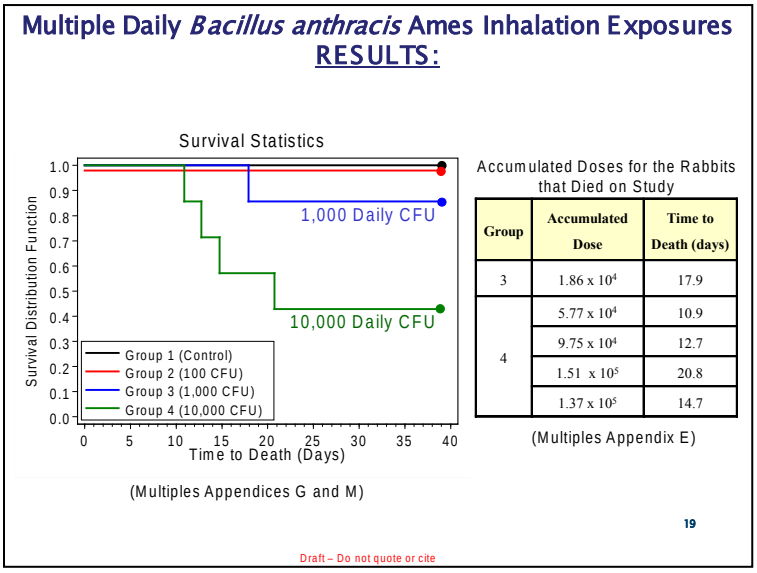

Figure 3. Multiple daily exposure study survival statistics from Dr. Taft's slide 19.

During the discussion, meeting attendees posed questions on the methodology and results of the studies, as well as on the behavior of *B. anthracis,* and made suggestions for future studies. Dr. Taft's presentation slides are provided in Appendix D, and her responses to the questions and participants' suggestions are summarized below.

Methodology

- **Inhalation chambers:** The inhalation chambers were muzzle-only rather than full body chambers.
- **Length of study:** The post-exposure observation period of time between the last inhalation exposure dose and euthanasia was 21 days. If possible, the team would like to conduct future studies for a longer period, perhaps 60 days.
- **Particle size:** The team measured the particle size of the microbes in the aerosol and found that they were around one micron. There are graphs in the study appendix containing the data on the exact distribution of the aerosol sizes.
- **Severity categories of effects:** The pathology categories used were no effect, minimal, mild, moderate, and marked.

- **Potential contamination:** Although there was no explanation for the appearance of a spore on a plate in the control group, it was potentially due to contamination during the sample characterization. It is likely that any contamination occurred in the lab after the actual exposures.
- **Clinical observations:** A chart of daily clinical observations is included in the study appendix. The team used professional judgment rather than a scoring system for the observations.
- **Liver enzymes:** The team looked at the C-reactive protein (CRP) in the multiple low dose study, but did not do a full clinical chemistry because the analysis of the liver enzymes in the acute study was not complete when the team designed the multiple low dose study. In retrospect, there were very slight increases in liver enzymes that could be considered in a future study.
- **Rinse step:** It was noted that it might be beneficial to rinse the impinger instrument with a sterile wash to get a more accurate environmental spore concentration measurement.
- **Health of rabbits:** The NZW rabbits were from Covance (Princeton, NJ) and were categorized as pathogen-free, although they are only tested for certain pathogens. The study team tested separately for *Bordetella bronchiseptica*. They also examined all the responses for a match to *Bordetalla* status but did not find a correlation. It did not appear to affect the results in terms of survival outcome. In the multiple low dose study, all the rabbits were negative for *Bordetalla.*
- **Blood collection:** In the first study, some rabbits had malfunctioning ports, so the team moved them to the control group. The team instead collected blood from the ear. Dr. Taft acknowledged that this was a problem for the acute study. For the multiple low dose study, researchers first performed a pilot study on port placement to identify an alternative location for the ports.
- **Irradiated spores for control group:** In preliminary studies, the team used the irradiated killed spores as a control group and observed lung foci. They therefore decided to repeat the use of irradiated spores in the next study to observe the difference between the virulent and non-virulent spores.

- **Baseline:** The telemetry results obtained a week before the first challenge were averaged for the baseline measurements. Many of the blood parameters were within the normal ranges established by historical studies and assays specifications.
- **Frequency of monitoring:** The team monitored the NZW rabbits at least twice daily. At times, they found the rabbits dead and would attempt to get a terminal bleed, but sometimes it was not possible to do so. Although the team did not always know exactly when the animal died, they can make a good estimate because telemetry data was taken every 15 minutes.
- **Variability in animals:** A participant commented on the enormous complexity of gut interactions, noting that some of the variability between animals and studies could be due to factors outside of the respiratory system entirely.

Behavior of B. anthracis

- **Deposition of spores:** The EPA team did not look at deposition in these studies, although it had been done in previous work co-funded by EPA at the Lovelace Respiratory Research Institute (Albuquerque, NM). This research indicated that one would normally expect to see five percent of the inhaled viable deposited dose in the lungs, although the studies used high spore dosages. In the current studies, the team estimated the inhaled dose based on real-time plethysmography monitoring in the chamber and the breathing rate.

- **Latency of anthrax:** There are hypotheses that *B. anthracis* can remain dormant in the lungs 60 to 90 days before it germinates. There are estimates from the Sverdlovsk 1979 accidental anthrax release of an approximate ten day incubation period (Brookmeyer et al., 2005), but the data are uncertain because symptoms in victims occurred up to six weeks after reported exposure (Barlett et al., 2002). Extrapolating that data to what is known about particle retention in various species, the clearance estimate extends out to 100 days or more. There are some reports of animals having died up to 100 days post exposure (Cieslak et al., 1999). Animals can only achieve such long survival windows if researchers intervene, however. The 1956 Henderson study showed that spores may remain in the lungs for up to a year after exposure. They were inert in the presence of

antibiotics; however, if the animals were taken off the antibiotics, some of them experienced infection many days after exposure.

- **Tolerable/no effect level:** There are studies indicating a tolerable or no effect level for *B. anthracis.* These studies (Albrink et al., 1960; Brachman et al., 1960; Dahlgren et al., 1960; Brachman et al., 1962; Cohen and Whalen 2007) are retrospective epidemiologic studies conducted in occupational settings (mills importing contaminated goat hair from hyperendemic regions). These studies estimated inhaled doses from 500-700 spores per day.

Results

- **Timeframe of parameters:** The summary tables indicate whether there were changes or measures above normal values on any of the study days. In most cases, the parameters became detectable two days before the animal died.
- **Seroconversion:** There was no detectable seroconversion in the acute exposure study. In the multiple low dose study, the team did not observe seroconversion in the survivors at the lower doses. For the two animals that did seroconvert, anthrax toxin-specific antibodies were detected between days 18 and 25. The rabbits continued to receive exposures after they became ill on day 18. The team began detecting the changes in the parameters, including the white blood cell count, for these rabbits when they got sick; the team then detected seroconversion two days later. Time course charts and antibody measurements for the surviving rabbits are included in the report.
- **Bacteremia:** Bacteremia was detected in the rabbits' blood right before they died or around day 18 for the survivors in the multiple exposure study. The surviving rabbits began to look sick around the time bacteremia was detected.
- **Cumulative or peak effect:** How the multiple doses interact and whether they are independent or dependent of each other is unknown.
- **Pathology:** While the summary presentation focused on the surviving animals and the dose groups with minimal effects, the researchers also collected pathology data for all the animals that died during the study. The findings are consistent with anthrax disease in rabbits include hemorrhage, necrosis, respiratory failure, and heart failure.

Future studies

- **Other tissues:** Participants suggested looking at other tissues in future studies, including the liver, spleen, and upper respiratory system. Dr. Taft informed the group that around 5,000 tissues were preserved from the study for future investigation.

Anthrax Lethal Factor Quantification: Summary for EPA Spore Exposure Dose Studies
Anne E. Boyer, Ph.D.
CDC National Center for Environmental Health

In comparing spore exposure dose studies in non-human primates and the studies of NZW rabbits, Dr. Boyer concluded that the anthrax toxin lethal factor (LF) assay is an effective assay at detecting infection because LF was detected in all the animals that died, as well as in one rabbit that became ill but recovered. In eight of ten rabbits that died, anthrax toxin protective antigen (PA) was detected later in the course of the infection and at higher levels than LF. Therefore, for spore exposures that may lead to illness and death, LF provided the earliest and most consistent measure of illness. Although LF levels in early illness appear to correlate with spore dose, greater numbers are needed to confirm this observation (Figure 4). Dr. Boyer's presentation slides are provided in Appendix D.

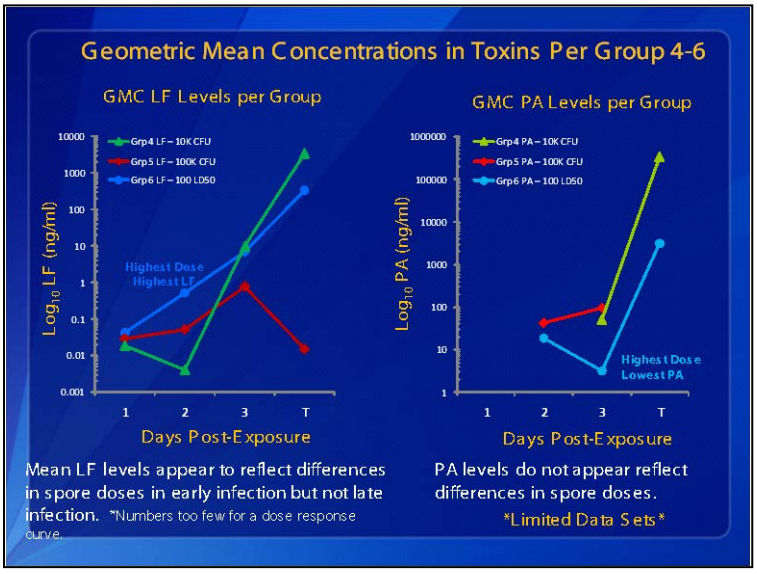

Figure 4. Geometric mean concentrations of lethal factor (LF) and protective antigen (PA) for acute exposure study from Dr. Boyer's slide 17.

Questions and Answers

Dr. Boyer responded to meeting participants' questions as follows:

- **Length of study:** The study of non-human primates lasted 35 days and the animals were not terminated at the end, but moved on to other studies.
- **Sensitivity of assays:** LF might be observed earlier due to a combination of the sensitivity of the assay used to detect it and the kinetics of the toxin in the blood. PA might be taken up more rapidly and intracellularly, preventing researchers from detecting it as quickly.
- **Triphasic kinetics:** Referring to the triphasic kinetics of LF, a participant asked whether the distribution of LF had been examined. Dr. Boyer responded that the LF results were primarily in the blood, not what has been taken up in cells. She has not looked at other species, so it is an open question and researchers are just beginning to look at LF in other areas.
- **LF results for survivors:** The LF results for survivors were all negative in the first study. In the second study, there was one positive for LF and PA in one of the animals that became sick.

Spore Carbohydrate Antigens: Markers of Aerosol Exposure to *Bacillus anthracis* Ames in Animal Models

Conrad P. Quinn, Ph.D.
CDC National Center for Immunization and Respiratory Diseases

CDC's research was driven by the need for biomarkers of disease and exposure that will better inform responses in an emergency event. The objective of the study was to provide high-specificity, high-sensitivity tests to identify personnel who are exposed to *B. anthracis*. Dr. Quinn's research lab is investigating the host's immune responses to the *Bacillus* collagen-like protein of *anthracis* (BclA) on the spore coat to serve as a biomarker of exposure. The NZW rabbit serra from both the acute and multiple exposure low dose studies were tested for antibodies to BclA, but showed no obvious dose-response relationship (Figure 5). Given that both live and killed spores interact with the mucosa-associated lymphoid tissue of the host, spore carbohydrate antigens have potential as a biomarker for spore contamination in asymptomatic individuals. The outstanding issue is to determine how to measure that response. Dr. Quinn's presentation slides are provided in Appendix D.

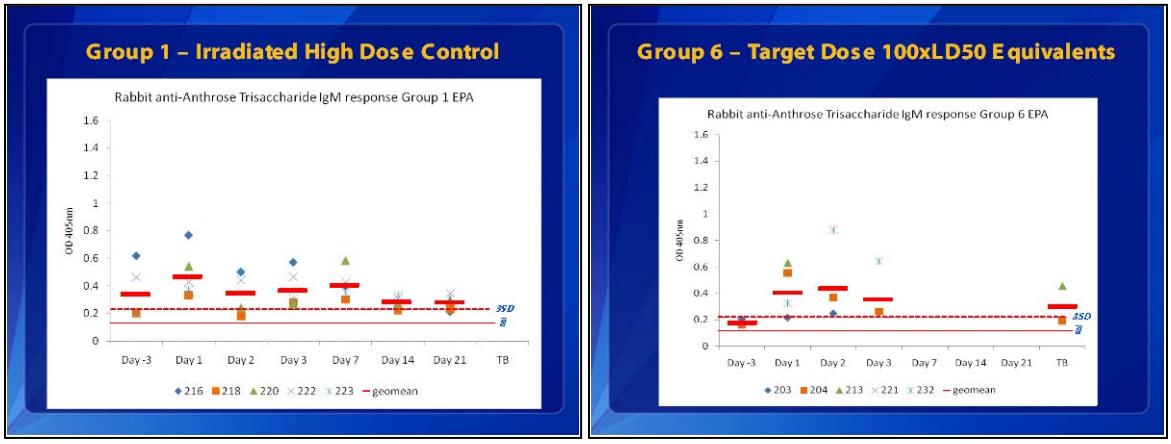

Figure 5. Rabbit anti-BclA IgM responses for the positive and negative control groups in the acute exposure study from Dr. Quinn's slides 16 and 21.

Questions and Answers

Dr. Quinn then responded to participants' questions:

- **Strain-specific responses:** It is likely that there are animal strain-specific responses; therefore, it is important to keep an open mind when reviewing the data.
- ***B. subtilis* BclA:** A participant mentioned that BclA might not be specific for *B. anthracis* and also part of the spore coats of *B. subtilis* which can be present in high concentrations as part of the rabbit natural flora. Dr. Quinn acknowledged that although the antigen was once thought to be exclusive to *B. anthracis*, that may not be the case, and BclA of *B. subtilis* could also be contributing factors to the data results.

Small Group Discussions I

This section of the report draws out some common themes that emerged from the plenary discussion and in the small group discussions from the first day related to the study design, methodology, and results of the low dose exposure studies. In the small groups, participants were given a set of discussion questions (see Appendix B).

Study Design and Methodology

None of the meeting participants identified other multiple daily inhalation exposure studies for EPA to consider in the assessment of the subchronic and chronic health effects of *B. anthracis* exposure, though some offered ideas of other studies that could help EPA better design future studies. These included:

- toxicokinetics and toxicodynamics studies
- models or examples that can be adopted from the chemical field
- the biomarkers in the 2009 Sela-Abramovich paper (although not a low dose study, it could still be useful)
- epidemiological studies of those exposed to *B. anthracis*

For the study design, meeting participants identified the sample population as a concern, due to the small sample number and the homogenous composition of the sample. EPA could design future studies to take into account population variability, such as age, relative health, and gender. Despite the resource limitations EPA faces in designing these studies, participants recommended an increased sample number not only to improve statistical relevance, but also to provide animals for additional controls (e.g., process control, non-spore negative control), test groups (e.g., increase the number of dosage levels, increase the number of low dose doses), and for sacrificial animals prior to death.

Furthermore, EPA could include or consider in future studies some additional variables that could affect the study outcome:

- activity level of the study animals
- exposure to different particle sizes

- different portals of entry
- rinsing the sampler between experiments
- using different spore strains

During the small group discussions meeting attendees raised a number of questions related to the study design and data presented, including:

- What is meant by "deep lung"?
- What are the definitions for "infection" and "illness"? What other endpoints were measured for which no data have been reported?

Although the studies measured the exposure dose, they did not determine the retained dose. Several individuals suggested that in future studies, EPA determine the relative deposition of *B. anthracis* in various regions of the respiratory tract.

Some additional questions that individuals raised included:

- Do other factors enter into estimating risk from multiple exposures?
- How are biomarkers linked to adverse effects?
- When does infection lead to an illness?

Results from the NHSRC Studies

There was some discussion on what type of effect qualifies as "adverse." Some participants believed that any effect or alteration in the study animal could be considered adverse as it is not a normal response. One small group observed that even a transient response may provide a window of vulnerability for a more adverse response, and that adversity could also be a function of the period of time a study animal is affected by low-level alterations. Some other participants noted that effects, while potentially adverse, are not necessarily so and could instead be transient, reversible, or adaptive (e.g., increased heart rate). The preliminary studies did not identify a measurable adverse endpoint for pre-illness, only some indicators post-illness.

Participants were unable to draw conclusions on adverse effects from the studies for a variety of reasons, including:

- The data presented do not convey the temporal dimension for each observed response.
- The study period was not long enough.

- An insufficient number of endpoints were measured.
- There were an insufficient number of sick survivors.

Several individuals suggested that the raw data need to be analyzed further to determine each observed response's temporal dimension. Some noted that the presence of the multi-nucleated cells possibly represented an adverse effect, but the data were insufficient to determine if (a) these multi-nucleated cells were adverse, or (b) if these multi-nucleated cells were the direct result of *B. anthracis* infection. One meeting participant expressed the opinion that as these cells were not normal, they should be further investigated to determine if they are adverse, and if they are an effect that could be seen in humans.

A few groups added that, except for mortality, there was no obvious dose-response for any adverse effect. Some did list factors that appeared to be indicative of anthrax disease; these included temperature, bacteremia, toxemia, seroconversion, and PA levels. One group also observed that there was some indication of a dose-response in the lung pathology, but it was not an effect that can be correlated with *B. anthracis* exposure. One group observed that the most promising biomarker appears to be LF, noting it appeared to be sensitive, and the least invasive to measure.

Presentations Session II

Future Multiple Daily Low Dose *Bacillus anthracis* Ames Inhalation Exposures in the Rabbit Designs
Sarah Taft, Ph.D.
EPA NHSRC

The ultimate goal of the multiple low dose exposure studies and future follow-on studies is to extrapolate from the rabbit dose-response to humans. Researchers are currently conducting a significant amount of physiological modeling work to estimate human responses to different doses. In the case of a biothreat event, that information would inform on-the-ground responders and decision makers on the probability of exposure, disease, and mortality in light of site-specific characteristics.

The EPA is developing a two-to-three year plan for further study and activities. Given that EPA has limited resources for conducting further studies, the Agency must prioritize which studies it will carry out. From the Agency's point of view, the multiple exposure studies are the most relevant. Given that there are no standard protocols for microbial health effects testing for multiple inhalation exposure, the Agency plans to first look at the existing standards and codes for testing chemical toxicity. EPA is still in the early planning stages for future animal studies, and welcomes further ideas and input on prioritizing its activities. Please send any comments to Sarah Taft, 26 W. Martin Luther King Drive, MS NG16, Cincinnati, OH 45268, 513-569-7037, Taft.Sarah@epa.gov. Dr. Taft's second set of presentation slides can be found at the end of Appendix D.

Pathogenesis of *Bacillus anthracis*
Crystal Briscoe, Ph.D.
Battelle

To give context to the discussion of the inhalation exposure study results, Dr. Briscoe gave a brief overview of the pathogenesis of *B. anthracis*, drawing on a paper by N.A. Twenhafel (Twenhafel 2010) to illustrate her points. In order to interpret results of the studies, the framework, outlined in Lewis et al. (2002) circulated to participants prior to the meeting, is applicable. The significance of the multi-nucleated giant cells and macrophages is an outstanding question that is important to investigate further.

Small Group Discussions II

On the second day, the meeting participants once again broke into smaller groups to discuss options for future studies. The participants received a second set of discussion questions (see Appendix B) regarding the future studies, and a reporter from each group presented the highlights and conclusions from each group's deliberations back to the plenary. This section of the report draws out some common themes that emerged from the plenary discussion and the small group discussions.

Short-Term Activities

In their discussions for follow-on to the NHSRC studies, the groups offered suggestions of activities EPA should undertake prior to designing future studies that could help to inform the future study designs. The meeting participants suggested that EPA first look at the data and samples collected from the acute and multiple low dose studies to determine if any effects were overlooked. Several groups observed that the Agency had existing samples from the NZW rabbits used in the exposure studies and could perform further pathology and histology characterization on those tissues. One group suggested EPA then uses the results from those additional examinations to compare what is known about anthrax pathology in other animal models. Another group noted that additional pathology could help identify the most sensitive target organs.

The meeting participants also recommended EPA further analyze the existing data from the preliminary rabbit studies in order to determine if any other effects were overlooked. One group suggested including LF into the summary table of effects. Further data analysis could inform future study design as well. One group suggested that from the existing data, EPA could identify the suite of endpoints, including invasive measures, pathology, presence of organism and graded clinical observations, and assign each study animal a severity score. The score would allow for a cross-study categorical regression-assisted estimate of the dose associated with non-lethal response.

Participants identified other studies that EPA could use to inform future studies. One group suggested the Agency look at studies that measure the kinetics of infection in different species to help with the interspecies extrapolation of the data. Another group suggested EPA look at the available human data, such as the Doolan study of human cellular immune reactions in multiple exposure groups in and outside the Hart Senate Office Building, and the isolated perfused lung and cadaver and virtual lung studies/projects. Several groups suggested that EPA also compare the results from the current rabbit studies to other previous animal studies.

Some of the groups also recommended EPA conduct additional modeling before progressing to additional studies, in order to explore simulated time intervals and dose levels that will better predict internal doses and infection, illness, severity of illness, and fatality. One group suggested EPA compare the existing models and data, and use those to predict the study results, which researchers can, in turn, use to improve and validate the model.

In order to inform future studies, groups advised that it would be useful to arrive at a better characterization of the retained dose in the NZW rabbits, as well as an improved understanding of the effects of multiple doses in the rabbit system and the risks of different portals of entry.

Furthermore, to help determine future study design and methodology, EPA should first consider these questions:

- What questions do the studies seek to answer?
- What are the data quality objectives?

Some groups suggested that when planning future studies, EPA should consider the real-life scenarios that the study results would inform, and in particular the risks associated with different types (e.g., ingestion, cutaneous, inhalation) and levels (most likely low-level following an attack) of exposure. While inhalation exposure may be the most dangerous infection pathway, other routes may be more likely and pose more risk.

Finally, one group noted that there are other federal agencies that might be interested in the outcome of future studies and could collaborate and share expenses with EPA.

Future Studies

In addition to the short-term activities detailed above, the groups also generated ideas for future follow-up studies. The groups identified objectives that EPA should pursue with these studies, including:

- Identify reliable and sensitive biomarkers for infection, not just physiological and hematologic responses.
- Develop a better characterization of the disease model, responses, and natural history of anthrax in experimental animals.
- Determine differences between species and responding groups to better extrapolate to the human model.

The groups expressed two principal suggestions for characteristics of study design and methodology: (1) increase sample size; and (2) lengthen the monitoring period post-exposure.

(1) A number of groups noted that a larger sample size would better enable EPA to determine an obvious dose response and account for any outliers. As mentioned previously, groups identified additional control groups that would be useful to include in future studies, which would increase the sample size. The meeting participants discussed the exact number of experimental animals to include in each control and dose group. One group suggested at least three to five animals per gender, per group, per sample collection, with additional sacrificial animals. Another group concluded there should be a minimum of 15 animals if sequential lavages are performed on a subset. A number of individuals suggested that EPA perform a statistical analysis (i.e., a statistical power determination) to inform study size.

Another recommendation related to increasing sample size was that of increasing the variability of the study population, particularly to determine the susceptibility of vulnerable populations. One group suggested that EPA design future studies to predict the dose response for such populations, such as the elderly, neonates, and the immuno-compromised. Another group noted that EPA can use animal models, such as guinea pig and rabbit, to identify additional relevant "uncertainty factors" that may affect susceptibility – such as age, underlying health status, vaccinations, and stress. In addition,

studies with immunocompetent and immunocompromised animal models could inform inter- and intra-species extrapolations for more sensitive subpopulations. Increasing the population variability would call for a substantial increase in the total sample size to balance that variability.

(2) Each group observed that the study animals should be monitored for a longer period of time following the last exposure dose, citing ranges between 30 and 90 days. One group suggested that EPA expose study animals to doses that were lethal in the 15-day study over a period of 60 to 90 days in order to estimate a level of clearance for the spores. The goals of the exercise would be to determine if there is any immune or adaptive response to the infection and to gain more information on latent infections.

With regard to the existing assays, two groups suggested that the C-reactive protein (CRP) test does not appear to be useful, and could be excluded from future studies. Another group disagreed, however, stating that none of the markers should be dropped. The group noted that although some assays were not particularly sensitive in the preliminary studies, they could prove useful with a larger sample size or with a different animal model.

Individual participants identified a number of additional assays and tests that EPA could incorporate into future studies:

- additional low-cost diagnostic tests such as LF and BclA
- vehicle and route control to help determine the relevance of multi-nucleated cells
- *in vitro* or *in vivo* tests to determine whether gene expression or other molecular assessments could be used
- re-exposing surviving animals 90 days post-exposure

The groups expressed the view that future studies should include a greater number of, and more involved, pathology of the study animals. As anthrax is a systemic disease, a full pathology and histopathology examination should be carried out. Other groups identified specific tissues which should be tested, including the spleen, lung lymphoid tissues, hematology, heart, and liver. One group added that it was important to include the pathology of animals that were not sick although they had been exposed at low doses. Several groups suggested including sacrificial animals in each study group for the purpose of conducting pathology examinations throughout the study to

seek early evidence of subclinical or active infection and to learn more about how the disease progresses. One group suggested EPA consider engineering green fluorescent protein into the organism to aid in detection, similar to the engineered strain developed in Glomski et al. (2007a-c) for use in the murine model.

The ultimate objective of these studies would be to extrapolate to the human model. Specific suggestions to incorporate this goal into the study design included using dynamic models for pathogens and designing mechanistic and target organ assays to identify specific markers, which would be linked to animal to human studies. While the rabbit model was adequate for certain endpoints, meeting participants suggested EPA also consider what information could be obtained from other animal models and *in vitro* studies with human tissues (e.g., Ruthel et al., 2004) in order to determine which species might be best for extrapolation to humans.

Conclusions and Next Steps

This report was prepared as a summary of the presentations and discussions held at the EPA NHSRC *Review and Design of Low Dose Bacillus anthracis Inhalation Exposures* meeting, July 27 – 28, 2011. Participants at the meeting reviewed the preliminary acute and multiple low dose exposure studies, identified strengths and weaknesses of the current study approach, and offered input to the design of follow-on subchronic low dose exposure studies. This report captures the main points and highlights of the meeting, but does not embellish, interpret, or enlarge upon matters that were incomplete or unclear. EPA was not seeking consensus decisions but rather hoped to solicit the views of individual experts to help the Agency develop its future research agenda. Although EPA hosted this meeting, the discussions presented here actually benefit the mission of many different agencies and organizations and will advance the science forward.

In terms of next steps, EPA is developing a two-to-three year plan for further study and activities. The Agency is still in the early planning stages for future animal studies and welcomes further ideas and input. Please send any comments to Sarah Taft, 26 W. Martin Luther King Drive, MS NG16, Cincinnati, OH 45268, 513-569-7037, Taft.Sarah@epa.gov.

References

Albrink WS, Brooks SM, Biron RE, Kopel M. 1960. Human Inhalation Anthrax. A Report of Three Fatal Cases. American Journal of Pathology 36(4):457-471.

Barlett JG, Inglesby TV, Borio L. 2002. Management of Anthrax. Clinical Infectious Diseases 35(7):851-858.

Brachman PS, Plotkin SA, Bumford FH, Atchison MM. 1960. An Epidemic of Inhalation Anthrax: the First in the Twentieth Century II. Epidemiology. American. Journal of Hygiene 72:6-23.

Brachman PS, Gold H, Plotkin SA, Fekety FR, Werrin M, Ingraham NR. 1962. Field Evaluation of a Human Anthrax Vaccine. American Journal of Public Health 52(4):632-645.

Brookmeyer R, Johnson E, Barry S. 2005. Modeling the Incubation Period of Anthrax. Statistics in Medicine 24:531-542.

Cieslak TJ, Eitzen EM. 1999. Clinical and Epidemiological Principles of Anthrax. Emerging Infectious Diseases 5(4):552-555.

Cohen ML, Whalen T. 2007. Implications of Low Level Human Exposure to Respirable *B. anthracis*. Applied Biosafety 12(2):109-115.

Dahlgren CM, Buchanan LM, Decker HM, Freed SW, Phillips CR, Brachman PS. 1960. *Bacillus anthracis* Aerosols in Goat Hair Processing Mills. American Journal of Hygiene 72(1):24-31.

Dixon TC, Meselson M, Guillemin J, Hanna PC. 1999. Anthrax. New England Journal of Medicine 341(11):815-826.

Doolan DL, Freilich DA, Brice GT, Burgess TH, Berzins MP, Bull RL, Graber NL, Dabbs JL, Shatney LL, Blazes DL, Bebris LM, Malone MF, Eisold JF, Mateczun AJ, Martin GJ. 2007. The US Capitol Bioterrorism Anthrax Exposures: Clinical Epidemiological and Immunological Characteristics. Journal of Infectious Diseases 195(2):174-84.

Glomski IJ, Corre JP, Mock M, Goossens PL. 2007a. Cutting Edge: IFN-Gamma-Producing CD4 T Lymphocytes Mediate Spore-Induced Immunity to Capsulated *Bacillus anthracis*. The Journal of Immunology 178(5):2646-2650.

Glomski IJ, Corre JP, Mock M, Goossens PL. 2007b. Noncapsulated Toxinogenic *Bacillus anthracis* Presents a Specific Growth and Dissemination Pattern in Naïve and Protective Antigen-Immune Mice. Infection and Immunity 75(10):4754-4761.

Glomski IJ, Piris-Gimenez A, Huerre M, Mock M, Goossens PL. 2007c. Primary Involvement of Pharynx and Peyer's Patch in Inhalational and Intestinal Anthrax. PLoS Pathogens 3(6):699-708.

Henderson DW, Peacock S, Belton FC. 1956. Observations of the Prophylaxis of Experimental Pulmonary Anthrax in the Monkey. Journal of Hygiene 54(1): 28-36.

Lewis RW, Billington R, Debryune E, Camer A, Lang B, Carpanini F. 2002. Recognition of Adverse and Nonadverse Effects in Toxicity Studies. Toxicologic Pathology 30(1): 66-74.

Ruthel G, Ribot WJ, Bavari S, Hoover TA. 2004. Time-Lapse Confocal Imaging of Development of *Bacillus anthracis* in Macrophages Journal of Infectious Diseases 189 (7): 1313-1316.

Sela-Abramovich S, Chitlaru T, Gat O, Grosfeld H, Cohen O, Shafferman A. 2009. Novel and Unique Diagnostic Biomarkers for *Bacillus anthracis* Infection. Applied and Environmental Microbiology 75 (19): 6157-6167.

Twenhafel NA. 2010. Pathology of inhalational anthrax animal models. Veterinary Pathology 47(5): 819-830.

U.S. Environmental Protection Agency (EPA). 2011a. Acute Low Dose *Bacillus anthracis* Ames Inhalation Exposures in the Rabbit. Cincinnati, Ohio: U.S. Environmental Protection Agency, Office of Research and Development, National Homeland Security Research Center. EPA/600/R-11/075.

U.S. EPA. 2011b. *DRAFT* Multiple Daily Low Dose *Bacillus anthracis* Ames Inhalation Exposures in the Rabbit. Cincinnati, Ohio: U.S. Environmental Protection Agency, Office of Research and Development, National Homeland Security Research Center. EPA/600/R-11/145.

Appendix A: Meeting Agenda

AGENDA

Review and Design of Low Dose *Bacillus anthracis* Inhalation Exposures

Andrew W. Breidenbach Environmental Research Center (Rooms 130/138), Cincinnati, OH
July 27 and 28, 2011

Due to renovations, the Main Lobby Entrance directly across from the hotel is closed, so please come around to the back side of the building and enter via the Annex Entrance.

Meeting Goals:

- Review preliminary acute and multiple low dose exposure studies
- Identify strengths and weaknesses of current approach
- Design follow-on subchronic low dose exposure studies

Wednesday, July 27

8:00 a.m. Registration and Coffee. Registration desk is located outside of Room 130.

9:00 a.m. Introductions and Opening Comments

- Tonya Nichols, EPA National Homeland Security Research Center
- Jonathan Herrmann, EPA National Homeland Security Research Center
- Kevin Teichman, EPA Office of Research and Development
- Stephen Morse, CDC National Center for Emerging and Zoonotic Infectious Diseases
- Christopher Russell, DHS Science and Technology Directorate

10:00 a.m. Agenda and Logistics Review

- Kristi Parker Celico, Meeting Facilitator, Resolve

10:05 a.m. Preliminary acute and multiple low dose exposure studies

- Sarah Taft, EPA National Homeland Security Research Center
- Anne Boyer, CDC National Center for Environmental Health
- Conrad Quinn, CDC National Center for Immunization and Respiratory Diseases

10:45 a.m. Coffee Break

11:00 a.m.	Question and Answer Period
12:30 p.m.	Lunch Break
1:45 p.m.	Small Groups Discussion
	Group 1 Panelist: Margaret Coleman, Coleman Scientific
	Group 2 Panelist: Charles Haas, Drexel University
	Group 3 Panelist: Mary Alice Smith, University of Georgia
	Group 4 Panelist: Paul Hinderliter, Battelle Memorial Institute
3:00 p.m.	Coffee Break
3:15 p.m.	Continued Small Groups Discussion
4:00 p.m.	Report Backs from Small Groups and Discussion of Next Steps
5:00 p.m.	Adjourn for day
6:30 p.m.	Group Dinner at Kingsgate Marriott's Caminetto

Thursday, July 28

Please note that hotel check-out time is at 12 noon.

8:00 a.m.	Coffee
8:30 a.m.	Summary of First Day Discussions
8:45 a.m.	Options for Follow-on Studies
9:00 a.m.	Small Groups Discussion
10:00 a.m.	Coffee Break
10:15 a.m.	Continued Small Groups Discussion
11:00 a.m.	Report Backs from Small Groups
12:00 p.m.	Lessons Learned and Next Steps
12:30 p.m.	Adjournment of meeting

Appendix B: Small Group Questions

Small Group Day I Discussion Questions:

Are there any additional multiple daily inhalation exposure studies that should be considered in the assessment of the subchronic/chronic health effects of *B. anthracis*?

What are the weaknesses or gaps in the design and/or methodology for the rabbit exposure studies?

Utilizing the Lewis et al. (2002) framework for evaluating adverse effects, which of the measured physiological responses in the multiple daily inhalation exposure rabbit study would you consider to be effects of *B. anthracis* infection/illness and to be adverse?

To determine if an effect is due to *B. anthracis* infection/illness:

Is there an obvious dose-response?

Is the effect due to findings in one or more rabbits that could be considered outliers?

Is the effect within normal biological variation (i.e. within the range of historical control or reference values)? (What are the most appropriate designs to capture variation?)

Is there a lack of biological plausibility (i.e. lack of direct causal-connection of *B. anthracis* infection and measured responses)?

To determine if an effect is adverse:

Could there be any alteration in the general function of the organ/tissue affected?

Is the response adaptive?

Is the response transient?

Is the effect isolated or independent (i.e., changes in other parameters usually associated with the effect of concern are not observed)?

Is the effect a consequence of the rabbit model and/or associated study design?

Do you believe risk is a function of dose for anthrax disease? Or, is inhalational anthrax more dose-independent?

Small Group Day II Discussion Questions:

Based on yesterday's data review for the preliminary studies, what measurable endpoint and associated assay is the most important to determine if there are any adverse effects from multiple low doses of *B. anthracis*?

Are there any of the tests or assays used in the preliminary study that you would not recommend using in the next study?

What additional characterization tests or assays would you recommend for the next study?

How much pathology and histology should be conducted?

What is the most appropriate exposure design and duration to evaluate subchronic/chronic effects of *B. anthracis* inhalation exposure in the rabbit model?

How many rabbits are required per dose group to achieve greater statistical power and decrease confidence intervals in subsequent dose-response modeling?

How long should the rabbits be monitored after the last exposure dose?

What other considerations should be examined?

What *in vitro* tests could be conducted to help decrease the uncertainty from the rabbit to human interspecies data extrapolation?

Appendix C: List of Participants

Femi Adeshina
EPA/ORD/NHSRC

Roy Barnewall
Battelle Memorial Institute

Ed Barth
EPA/ORD/NRMRL

Anne Boyer
HHS/CDC

Eletha Brady-Roberts
EPA/ORD/NHSRC

Crystal Briscoe
Battelle Memorial Institute

Wayne Cascio
EPA/ORD/NHEERL

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Peg Coleman
Coleman Scientific Consulting

Jason Comer
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Judy Day
University of Tennessee

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Conrad Quinn
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Chris Russell
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Alan Sasso
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Gregory Sayles
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Sarah Taft
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Sheila Van Cuyk
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Jay Zhao
EPA/ORD/NCEA

Contractor Support

Kristi Parker Celico
Rocky Mountain Collaborative Solutions

Dana Goodson
RESOLVE

Debbie Lee
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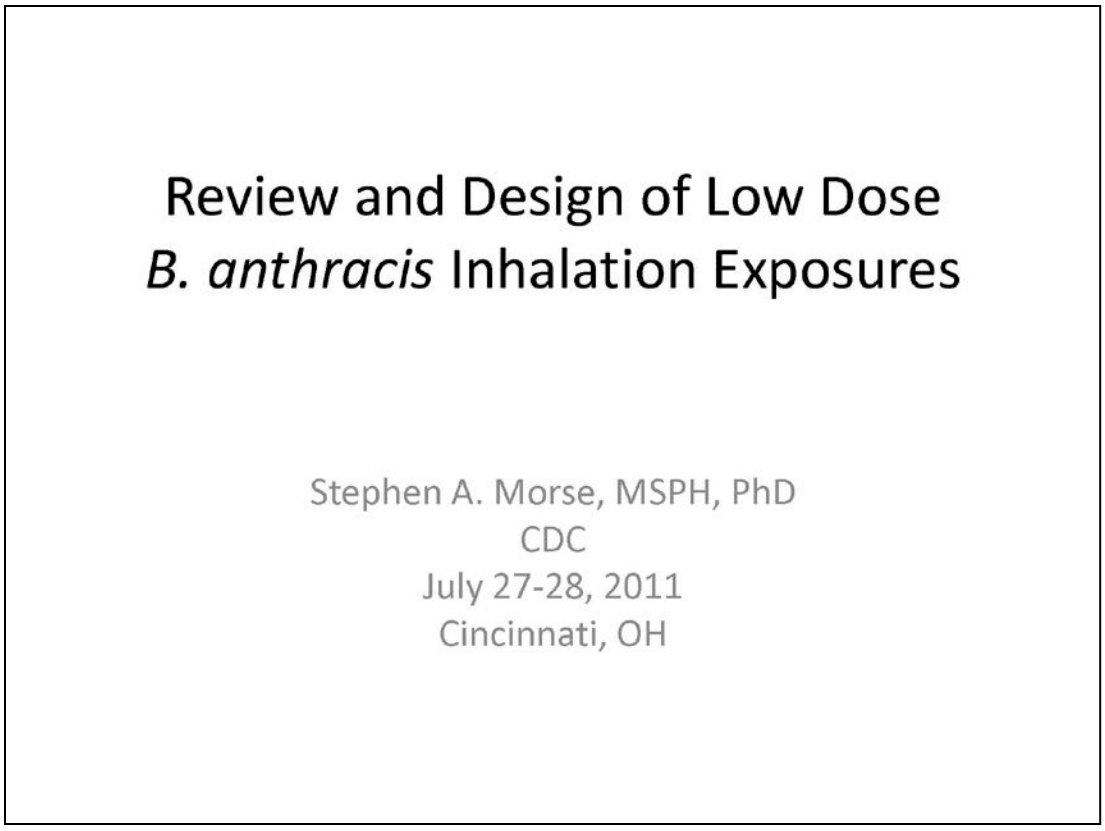
Appendix D: Slide Presentations

Introductions and Opening Comments

Stephen Morse, CDC National Center for Emerging and Zoonotic Infectious Diseases



Where We've Been

- EPA and CDC have a long-standing working relationship.
- Historically, through work with NCEH/ATSDR on environmental (health) issues. Bolstered by co-location of ATSDR field staff in EPA regional offices.
- Anthrax events of 2001-2002 strengthened relationship of EPA with CDC's infectious disease component.
- MOUs for collaborative research and response activities.
- Meetings to promote interagency dialogue and consensus (e.g., "Janus" Meeting in March, 2008).
- Clearance strategy meeting at CDC, June 29-30, 2011.

Thoughts

- Once an event has been recognized, a decision will be made whether to shelter in place or evacuate.
- Following an actual event, public health leadership, the media, policy makers, and the public will want some estimate (most likely numerical) of the risk of clinical disease (inhalation, cutaneous, gastrointestinal) associated with sheltering in place or re-occupancy.
- Role of public health is not to make a declaration of "risk free" or "safe", but to provide an evaluation of risk of re-occupancy, where risk will be characterized in terms of risk of clinical disease, and to recommend strategies to reduce risk (e.g., prophylaxis, vaccination, PPE).

CDC Mantra

"Base all public health decisions on the highest quality scientific data openly and objectively derived."

Walter R. Dowdle, Ph.D.
Joseph Mountain Lecture, 1990

More Thoughts

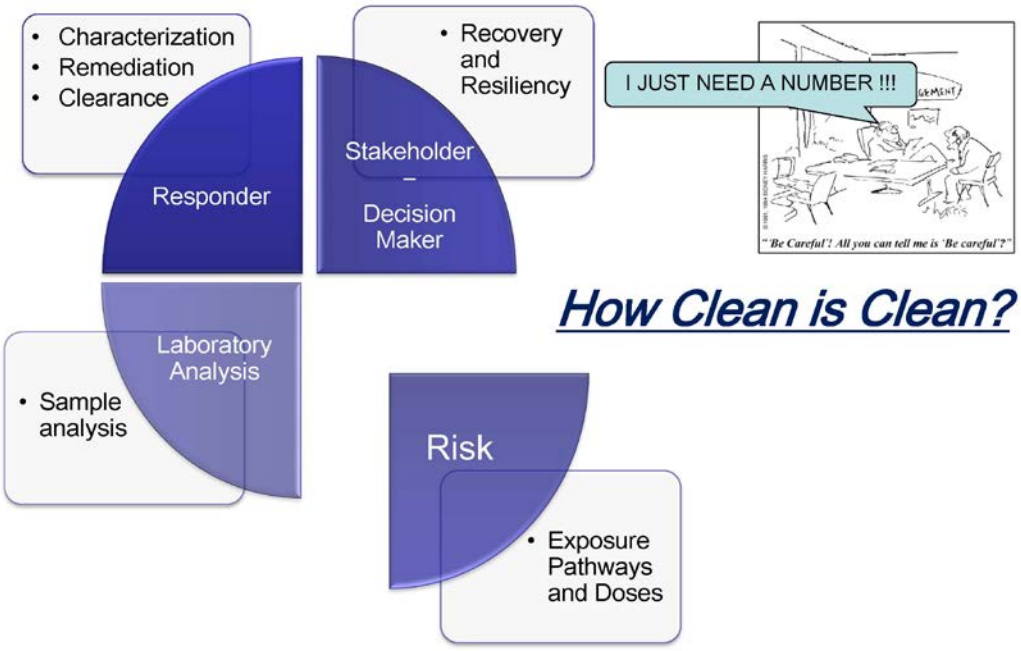
- There is no such thing as "zero risk."
- Cannot accurately estimate risk of exposure or disease based on surface samples.
- Failure to detect viable spores by culture after decontamination doesn't mean there are zero spores present.
- In a wide area release, it will be impossible to eliminate all outdoor spores.
- We do not fully understand the effects of single or multiple low dose exposures to spores.
- Medical surveillance will be necessary for a long period of time after re-occupancy.

Relevance to CDC

- Facilitate the development of risk-based approaches to support cleanup goals and subsequent reoccupation decisions.
- Provide information for public health decision makers and the public.
- Provide information for public health risk reduction strategies.

Preliminary acute and multiple low dose exposure studies

Sarah Taft, EPA National Homeland Security Research Center



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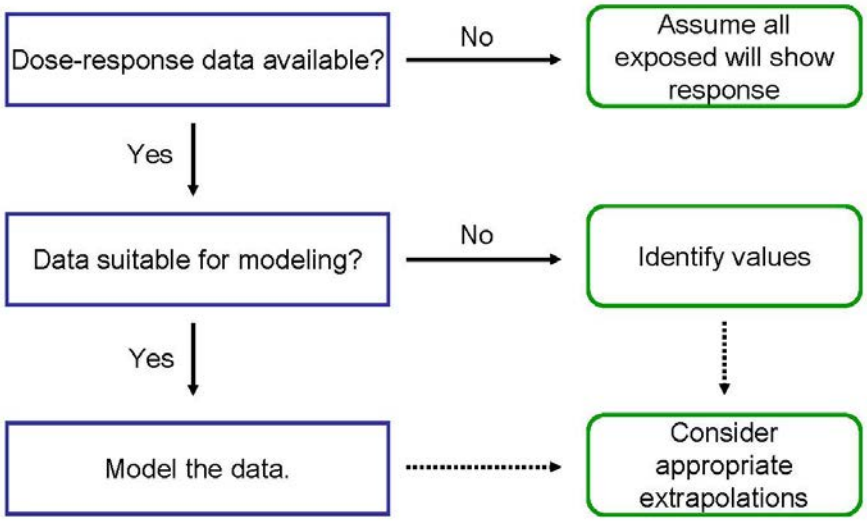
EPA United States Environmental Protection Agency

Estimating Concentrations of Concern for Biologicals



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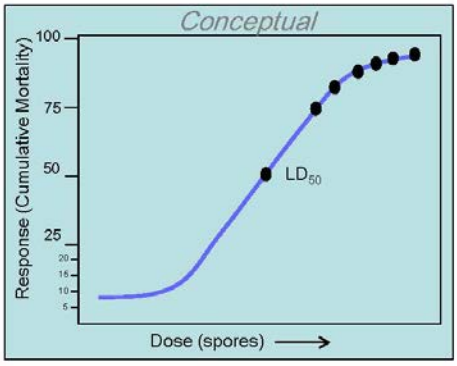
EPA United States Environmental Protection Agency

Anthrax Dose-Response Data

- Historical dose-response data
  - Extremely high doses to test countermeasures or weapons potentials
  - Great inconsistencies
    - Spore characterization
    - Exposure assumptions
  - Only acute exposures



No usable inhalation Bacillus anthracis dose-response relationship in low dose range.

3

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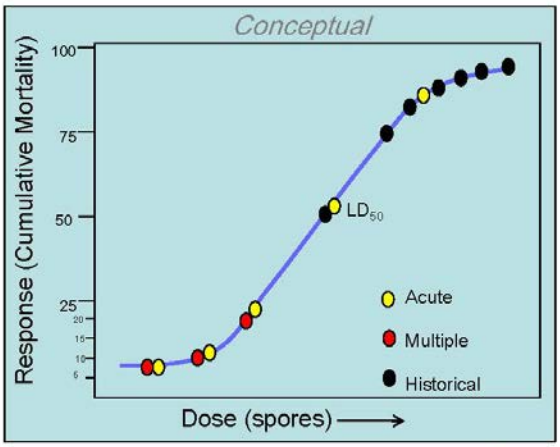
EPA United States Environmental Protection Agency

Low Dose Rabbit Exposures

- ***Objective***: to generate dose-response estimates by modeling the survival of rabbits following low-dose *Bacillus anthracis* exposures
- Acute and Multiple daily low dose exposures to examine any potential adverse responses
  - Telemetry – continuous monitoring
  - Hematology and clinical chemistry
  - Assays for bacteria and toxins
  - Pathology and histology

Battelle The Business of Innovation



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Acute *Bacillus anthracis* Ames Inhalation Exposures
METHODS:

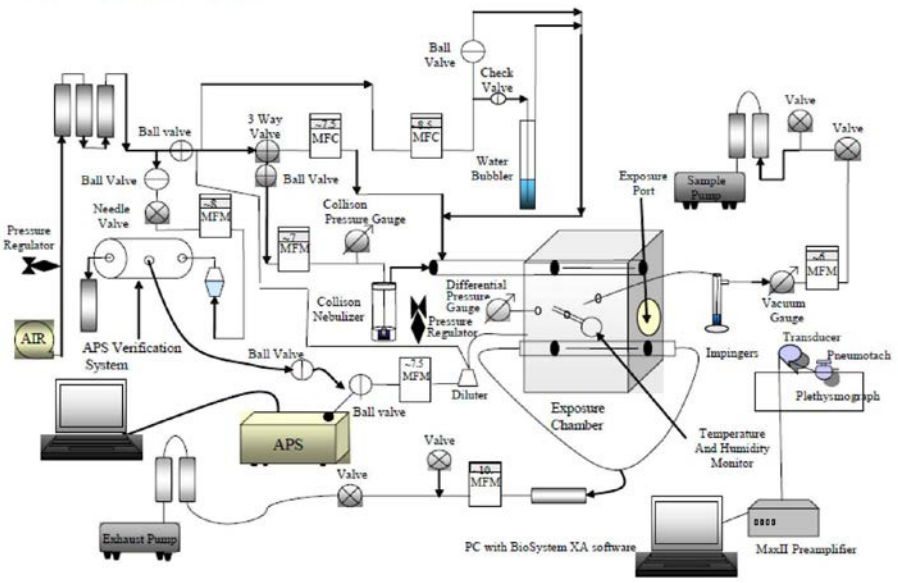
- Male New Zealand White (NZW) rabbits > 3.5 kg with vascular access ports and D70-PCT telemetric devices (Acute Appendix A)
  - All tested for *Bordetella bronchiseptica* (Acute Appendix C)
  - Randomized by weight
- Spray factor testing performed to ensure aerosol system was capable of achieving low aerosol doses (Acute Appendix D)
- During aerosol challenges, rabbits placed in plethysmography chambers and aerosol challenged via Collison nebulizer (Acute Appendix E)
  - Negative control group challenged with equivalent of 100 x LD_{50} of gamma irradiated spores (LD_{50} = 1.05 x 10^5 CRU based on Zaucha et al., 1998)

Group	Targeted Inhaled Dose (CFU)	No. of Spore Challenges	No. of Rabbits
1 (Negative control*)	100 x LD_{50}†	1	5
2	100	1	5
3	1000	1	5
4	10,000	1	5
5	100,000	1	5
6 (High dose positive control)	100 x LD_{50}†	1	5

*Negative control = irradiated spores
† LD_{50} = 1.05 x 10^5

Acute *Bacillus anthracis* Ames Inhalation Exposures
METHODS:

Figure 1. Exposure System Diagram

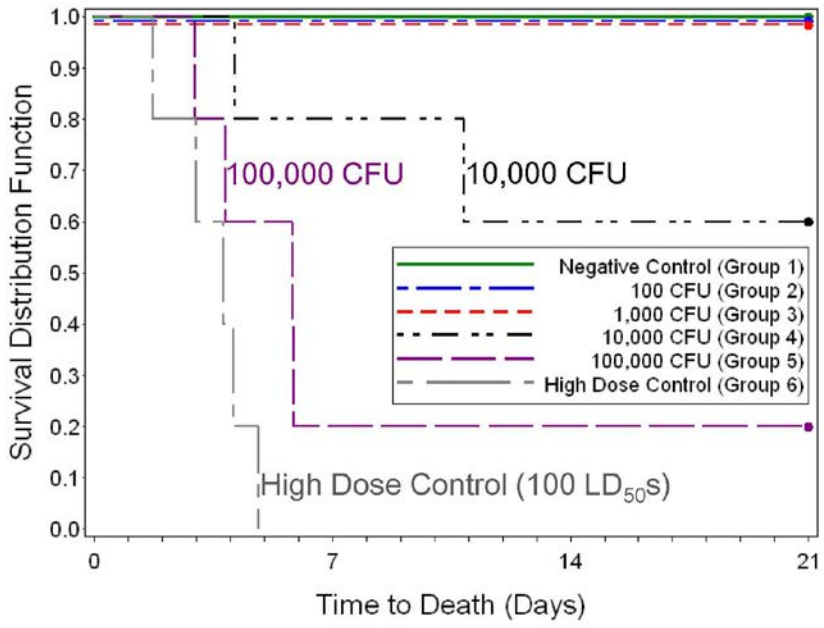


Acute *Bacillus anthracis* Ames Inhalation Exposures
METHODS:

- Total inhaled dose was calculated: $InD = (C \times V)(S \times T)^{-1}(TATV)$
  - *InD* = Inhaled dose (CFU)
    C = Impinger concentration (CFU/mL)
    V = Impinger sampler volume (mL)
    S = Sampling rate (6 L/min)
    T = Exposure time (min)
    TATV = Total accumulated tidal volume (L)
- Rabbits monitored 3 weeks post challenge:
  - Telemetry (30 seconds every 15 minutes)
    - Body temperature
    - Electrocardiogram activity
    - Heart rate
    - Respiratory pressure
  - Clinical observations and body weights (Acute Appendices L and M)
  - Blood characterizations (Acute Appendix G for Blood Draw Times)
    - Hematology
    - Clinical chemistry
    - Protective Antigen (PA) ELISA
    - Bacteremia
      - Culture
      - qPCR
    - Seroconversion
      - Anti-PA IgG ELISA
      - Toxin neutralization assay
    - Necropsy
    - Histopathology

7

Acute *Bacillus anthracis* Ames Inhalation Exposures
RESULTS:



Survival Statistics

(Acute Appendices N and O)

8

Acute *Bacillus anthracis* Ames Inhalation Exposures RESULTS:

Animal ID	Dose Group	Inhaled Dose (CFU)	Heart Rate[a]	Respiration Rate[a]	Body Temp.[a]	Neutrophil Levels[b]	CRP[b]	AST[b]	PA Toxin	Bacteremia	Time to Death (days)
L23235		1.76×10^4	↑	↑	↑	↑	↑	↔	-	+	11
L23205		2.73×10^4	↔	↑	↔	↔	↔	↔	-	-	NA
L23225	4	2.59×10^4	↑	↔	↑	↑↓	↑	↔	+	+	4
L23231		2.41×10^4	↑	↔	↔	↓	↔	↔	-	-	NA
L23207		3.19×10^4	↔	↔	↔	↓	↑	↔	-	-	NA
L23201		1.78×10^5	↑↓	↑	↑	↑	↑	↑	+	+	4
L23234		2.96×10^5	↑	↑	↑	↑	↑	↑	+	+	6
L23212	5	3.29×10^5	↔	↔	↔	↓	↑	↔	-	-	NA
L23200		2.19×10^5	↓	↑	↑	↓	↔	↔	-	+	3
L23214		3.54×10^5	↑	↑	↑	↑	↑	↑	+	+	6
L23204		5.95×10^6	↑	↑	↑	↑	↑	↔	+	+	4
L23203		8.86×10^6	↑	↑	↑	↑	↑	↔	+	+	5
L23213	6	7.29×10^6	↑	↑	↑	↔	↑	↔	+	+	3
L23221		8.88×10^6	↑	↑	↑	↔	↑	↔	-	+	2
L23232		1.04×10^7	↑	↑	↑	↓	↑	↔	+	+	4

↑ = Increases in a parameter
↓ = Decreases in a parameter
↔ = No change in the parameter
+ = Positive for bacteremia culture or toxemia
CFU = Colony forming units
AST = Aspartate Aminotransferase

- = Negative for bacteremia culture or toxemia
[a] = Changes based on baseline
[b] = Changes base on normal ranges
NA = Not applicable
CRP = C-reactive protein
PA = Protective Antigen

9

Draft – Do not quote or cite

Acute *Bacillus anthracis* Ames Inhalation Exposures RESULTS:

Animal ID	Dose Group	Inhaled Dose (CFU)	Heart Rate[a]	Respiration Rate[a]	Body Temp[a]	Neutrophil Levels[b]	CRP[b]	AST[b]	PA Toxin	Bacteremia	Time to Death (days)
L23220		0	↔	↑↓	↔	↑↓	↑	↔	-	-	NA
L23216		1.00×10^1	↑	↑	↔	↔	↑	↔	-	-	NA
L23218	1	0	↑	↔	↔	↔	↑	↔	-	-	NA
L23223		0	↔	↔	↔	↔	↔	↔	-	-	NA
L23222		0	↔	↔	↔	↑	↑	↔	-	-	NA
L23215		3.22×10^2	↔	↔	↔	↓	↑	↔	-	-	NA
L23206		2.98×10^2	↔	↓	↔	↓	↑	↔	-	-	NA
L23210	2	2.18×10^2	↔	↓	↔	↓	↑	↔	-	-	NA
L23219		3.21×10^2	↔	↓	↔	↓	↑	↔	-	-	NA
L23211		2.73×10^2	↑	↑	↔	↓	↑	↔	-	-	NA
L23217		1.48×10^3	↓	↓	↔	↔	↔	↔	-	-	NA
L23230		2.02×10^3	↑	↓	↔	↓	↑	↔	-	-	NA
L23228	3	2.23×10^3	↔	↔	↔	↓	↑	↑	-	-	NA
L23227		2.32×10^3	↔	↔	↔	↓	↔	↔	-	-	NA
L23229		2.24×10^3	↔	↑	↔	↓	↑	↔	-	-	NA

↑ = Increases in a parameter
↓ = Decreases in a parameter
↔ = No change in the parameter
+ = Positive for bacteremia culture or toxemia
CFU = Colony forming units
AST = Aspartate Aminotransferase

- = Negative for bacteremia culture or toxemia
[a] = Changes based on baseline
[b] = Changes base on normal ranges
NA = Not applicable
CRP = C-reactive protein
PA = Protective Antigen

10

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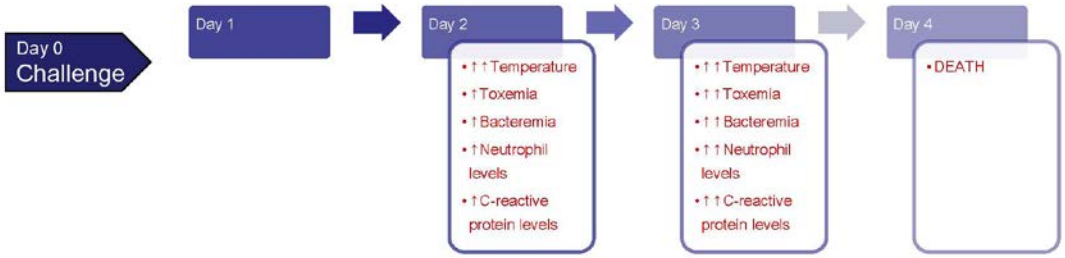
Acute *Bacillus anthracis* Ames Inhalation Exposures
RESULTS:

Time Course of Detectable Physiological Responses before Death



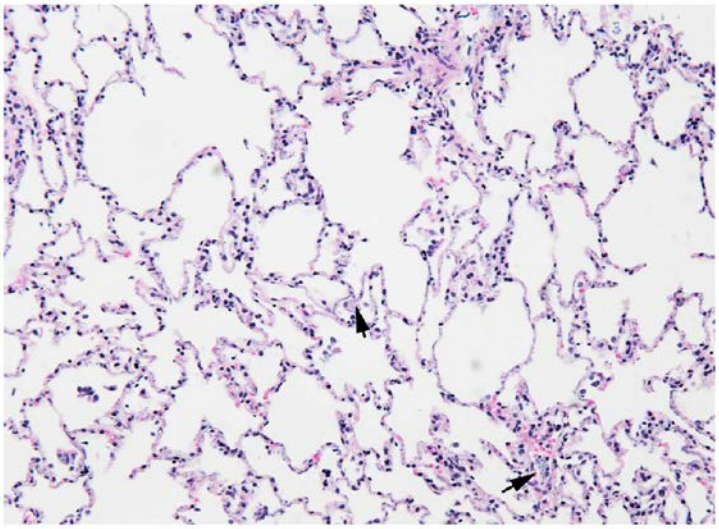
11

Pathology of Rabbit Tissues (Acute Appendix W)

Group Number	Animal Number	Microscopic Findings
1 100 LD_{50} (Irradiated)	L23218	Lung: Multi-nucleated giant cells, minimal
	L23222	Lung: Multi-nucleated giant cells, minimal
2 100 CFU	L23215	Lung: Inflammation, suppurative, minimal Lung: Multi-nucleated giant cells, minimal
	L23206	Lung: Inflammation, suppurative, minimal
	L23219	Lung: Inflammation, suppurative, minimal Lung: Multi-nucleated giant cells, minimal
3 1000 CFU	L23217	Lung: Inflammation, suppurative, moderate Lung: Multi-nucleated giant cells, moderate Lung: Perivascular eosinophils, moderate
	L23230	Lung: Inflammation, suppurative, minimal
	L23228	Lung: Inflammation, suppurative, minimal Lung: Multi-nucleated giant cells, minimal
	L23227	Lung: Bacteria (bacilli), minimal
	L23229	Lung: Inflammation, suppurative, minimal

12

Acute *Bacillus anthracis* Ames Inhalation Exposures
RESULTS:



Hematoxylin and eosin stain of lung alveoli showing interstitial inflammation, and intravascular and interstitial *B. anthracis* bacilli (arrow) for rabbit L23225 (Group 4), 20X.

(Acute Appendix W)

13

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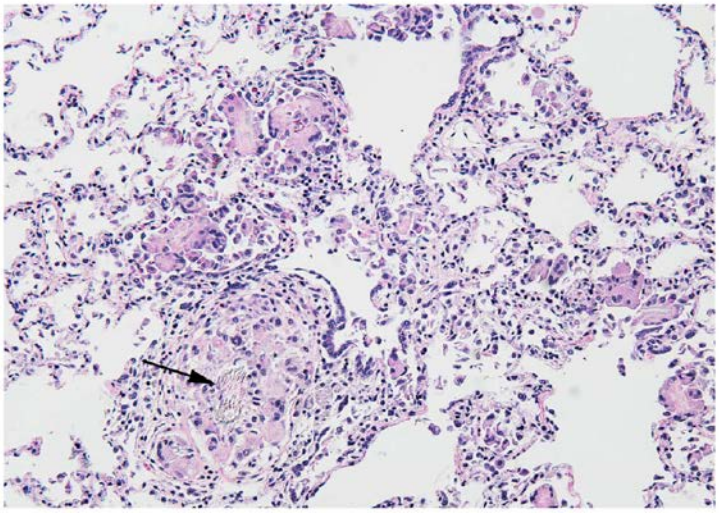
Acute *Bacillus anthracis* Ames Inhalation Exposures
RESULTS:



Hematoxylin and eosin stain of a lung section from rabbit L23204 (Group 6) showing multinucleated giant cell foreign body (arrow), 20X.

(Acute Appendix W)

14

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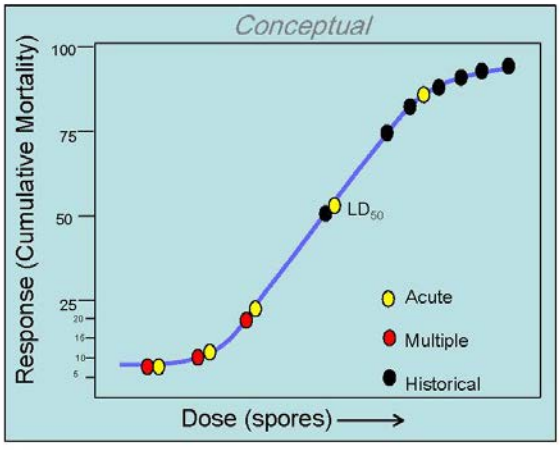
EPA United States Environmental Protection Agency

Summary of Acute Low Dose *Bacillus anthracis* Ames Inhalation Exposures in the Rabbit

- Average Inhaled Doses per Group (CFU):
  1. Negative Controls = 0/5 Died
  2. **286 = 0/5 Died**
     - Minimal suppurative inflammation in the lungs
     - Multi-nucleated giant cells in the lungs
  3. **2.06×10^3 = 0/5 Died**
     - Moderate suppurative inflammation in the lungs
     - Multi-nucleated giant cells in the lungs
     - Minimal bacilli bacteria in the lungs
  4. 2.54×10^4 = 2/5 Died
  5. 2.75×10^5 = 4/5 Died
  6. 8.27×10^6 = 5/5 Died

EPA United States Environmental Protection Agency

Acute → Multiple Daily Exposures



Multiple Daily *Bacillus anthracis* Ames Inhalation Exposures METHODS:

- Male New Zealand White (NZW) rabbits ~ 2.7 kg with vascular access ports and D70-PCT telemetric devices (Multiples Appendix A)
  - All tested for *Bordetella bronchiseptica* (Multiples Appendix C)
  - Randomized by weight (Multiples Appendix D)
- During aerosol challenges, rabbits placed in plethysmography chambers and aerosol challenged via Collison nebulizer (Multiples Appendix E)
  - Negative control group challenged with 10,000 gamma irradiated
  - Rabbits were challenged once each day for 5 straight working days (Monday-Friday) each week for 3 consecutive weeks

Group	Targeted Inhaled Daily Spore dose (CFU)	Number of Spore Challenges	# of Rabbits
1 (Negative control)	10,000 irradiated	15	5
2	100	15	7
3	1,000	15	7
4	10,000	15	7

17

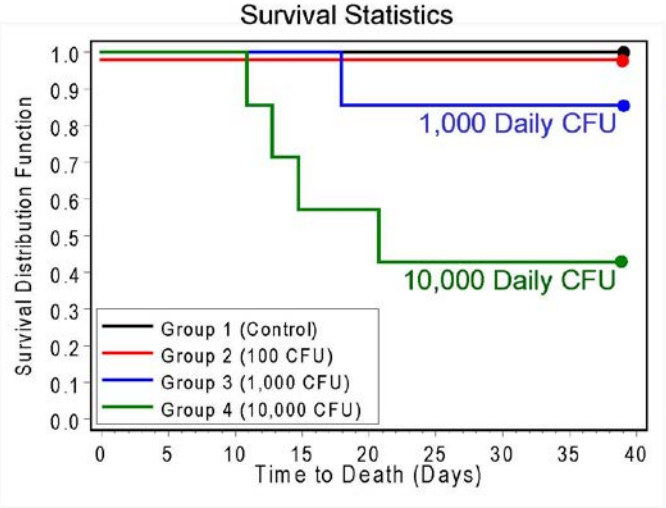
Multiple Daily *Bacillus anthracis* Ames Inhalation Exposures METHODS:

- Total inhaled dose was calculated: $InD = (C \times V)(S \times T)^{-1}(TATV)$
  - *InD* = Inhaled dose (CFU)
    C = Impinger concentration (CFU/mL)
    V = Impinger sampler volume (mL)
    S = Sampling rate (6 L/min)
    T = Exposure time (min)
    TATV = Total accumulated tidal volume (L)
- Rabbits monitored 3 weeks post last challenge:
  - Telemetry (30 seconds every 15 minutes)
    - Body temperature
    - Electrocardiogram activity
    - Heart rate
    - Respiratory pressure
  - Clinical observations and body weights (Multiples Appendices H, K, and L)
  - Blood characterizations (Multiples Appendix I for Blood Draw Times)
    - Hematology
    - C-reactive protein
    - Protective Antigen (PA) ELISA
    - Bacteremia
      - Culture
      - qPCR
    - Seroconversion
      - Anti-PA IgG ELISA
      - Toxin neutralization assay
    - Necropsy
    - Histopathology

18

Multiple Daily *Bacillus anthracis* Ames Inhalation Exposures RESULTS:

Survival Statistics



(Multiples Appendices G and M)

Accumulated Doses for the Rabbits that Died on Study

Group	Accumulated Dose	Time to Death (days)
3	1.86×10^4	17.9
4	5.77×10^4	10.9
	9.75×10^4	12.7
	1.51×10^5	20.8
	1.37×10^5	14.7

(Multiples Appendix E)

19

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Multiple Daily *Bacillus anthracis* Ames Inhalation Exposures RESULTS:

Group	ID	Mean Daily Inhaled Dose (CFU)	Sum of Doses (CFU)	Heart Rate[a]	Resp. Rate[a]	Body Temp[a]	WBC[b]	Neut. Levels[b]	CRP[b]	Bacteremia	PA Toxin	Time to Death (days)
3	14	7.38×10^2	1.11×10^4	↔	↔	↔	↑	↔	↔	-	-	NA
	11	1.12×10^3	1.68×10^4	↔	↔	↔	↑↓	↑	↑	-	-	NA
	2	1.35×10^3	2.02×10^4	↑	↑	↑	↔	↔	↔	+	+	17.9
	8	1.40×10^3	2.10×10^4	↔	↔	↔	↑	↔	↑	-	-	NA
	12	1.30×10^3	1.95×10^4	↔	↔	↔	↔	↔	↑	-	-	NA
	18	1.24×10^3	1.85×10^4	↔	↔	↔	↑	↔	↔	-	-	NA
	32	1.89×10^3	2.83×10^4	↔	↔	↔	↔	↔	↔	-	-	NA
4	6	6.41×10^3	5.77×10^4	↑	↑	↑	↑	↑	↑	-	-	10.9
	33	9.75×10^3	9.75×10^4	↑	↑	↑	↔	↔	↑	+	-	12.7
	27	1.08×10^4	1.51×10^5	↑	↔	↑	↑	↔	↑	+	-	20.8
	31	1.25×10^4	1.37×10^5	↑	↔	↑	↔	↑	↑	+	+	14.7
	39	1.44×10^4	2.16×10^5	↔	↔	↔	↑	↔	↑	-	-	NA
	21	1.32×10^4	1.98×10^5	↔	↔	↑	↔	↑	↑	-	+	NA
	38	1.27×10^4	1.91×10^5	↑	↑	↑	↑	↑	↑	+	+	NA

↑ = Increases in a parameter
↓ = Decreases in a parameter
↔ = No change in the parameter
\+ = Positive for bacteremia culture or toxemia
CFU = Colony forming units
CRP = C-reactive protein
\- = Negative for bacteremia culture or toxemia
[a] = Changes based on baseline
[b] = Changes base on normal ranges
NA = Not applicable
WBC = White blood cells
PA = Protective Antigen

20

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Multiple Daily *Bacillus anthracis* Ames Inhalation Exposures RESULTS:

Group	ID	Mean Daily Inhaled Dose (CFU)	Sum of Doses (CFU)	Heart Rate[a]	Resp. Rate[a]	Body Temp[a]	WBC[b]	Neut. Levels[b]	CRP[b]	Bacteremia	PA Toxin	Time to Death (days)
1	40	0	0	↔	↔	↔	↔	↓	↑	-	-	NA
	7	0	0	↔	↔	↔	↓	↓	↑	-	-	NA
	5	0	0	↔	↔	↔	↑	↔	↔	-	-	NA
	9	0	0	↔	↔	↔	↔	↔	↑	-	-	NA
	37	0	0	↔	↔	↔	↓	↓	↑	-	-	NA
2	13	3.85×10^2	5.78×10^3	↔	↔	↔	↑	↔	↑	-	-	NA
	34	3.17×10^2	4.76×10^3	↔	↔	↔	↑	↔	↑	-	-	NA
	25	2.79×10^2	4.19×10^3	↔	↓	↔	↑	↔	↑	-	-	NA
	15	3.17×10^2	4.76×10^3	↔	↔	↔	↔	↔	↔	-	-	NA
	30	2.72×10^2	4.07×10^3	↔	↑	↔	↔	↔	↔	-	-	NA
	28	2.34×10^2	3.51×10^3	↔	↔	↔	↔	↔	↔	-	-	NA
	19	2.32×10^2	3.48×10^3	↔	↔	↔	↓	↔	↑	-	-	NA

↑ = Increases in a parameter
↓ = Decreases in a parameter
↔ = No change in the parameter
\+ = Positive for bacteremia culture or toxemia
CFU = Colony forming units
CRP = C-reactive protein
\- = Negative for bacteremia culture or toxemia
[a] = Changes based on baseline
[b] = Changes base on normal ranges
NA = Not applicable
WBC = White blood cells
PA = Protective Antigen

21

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Pathology of Rabbit Tissues

(Multiples Appendix U)

Group	Animal Number	Microscopic Findings
1 Control	5	Lung: Perivascular eosinophils, minimal.
	9	Lung: Perivascular eosinophils, minimal.
2 100 CFU	13	Lung: Perivascular eosinophils, minimal.
	34	Lung: Multinucleated giant cells, mild.
	15	Lung: Perivascular eosinophils, minimal.

22

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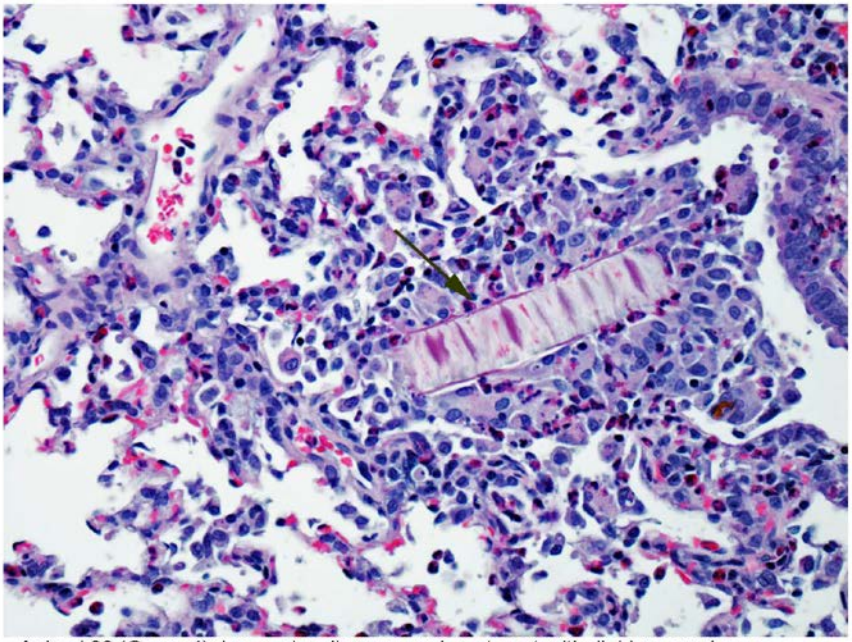
Multiple Daily *Bacillus anthracis* Ames Inhalation Exposure



Animal 38 (Group 4): Lung, alveoli; pyogranulomatous (epithelioid macrophages, lymphocytes, and neutrophils) inflammatory reaction to a foreign body (arrow). Hematoxylin and Eosin Stain. 40X.

23

(Multiples Appendix U)

EPA United States Environmental Protection Agency

Summary of Multiple Daily Low Dose *Bacillus anthracis* Ames Inhalation Exposures in the Rabbit

1. Negative Controls = 0/5 Died
2. 291 Daily (4.37×10^3 Total) = 0/7 Died
   - Multi-nucleated giant cells in the lungs
3. 1.22×10^3 Daily (1.93×10^4 Total) = 1/7 Died
4. 1.17×10^4 Daily (1.5×10^5 Total) = 4/5 Died

EPA
United States
Environmental Protection
Agency

Acknowledgements

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  - Sarah Taft
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  - Gregory Stark
  - Andrew Lair
  - Michael Taylor
  - Stephanie Hines
  - Zachary Willenberg
  - Michael Etheridge
  - Kevin Tordoff
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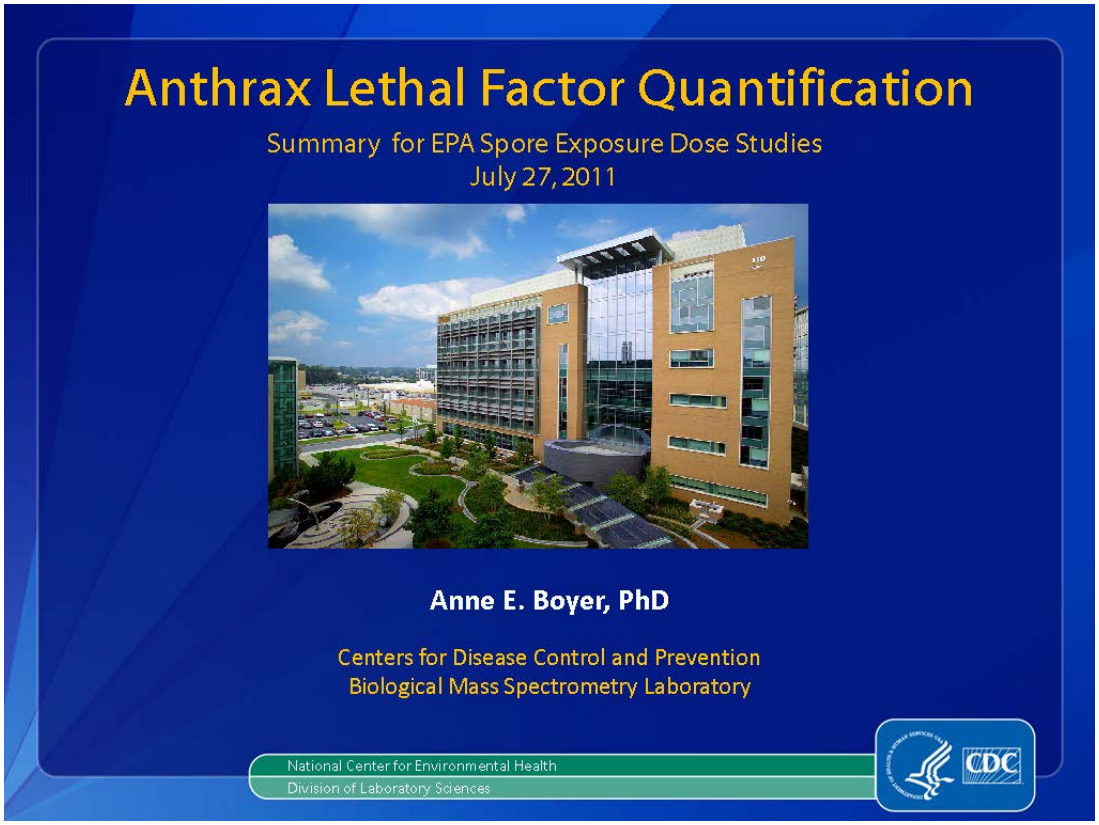
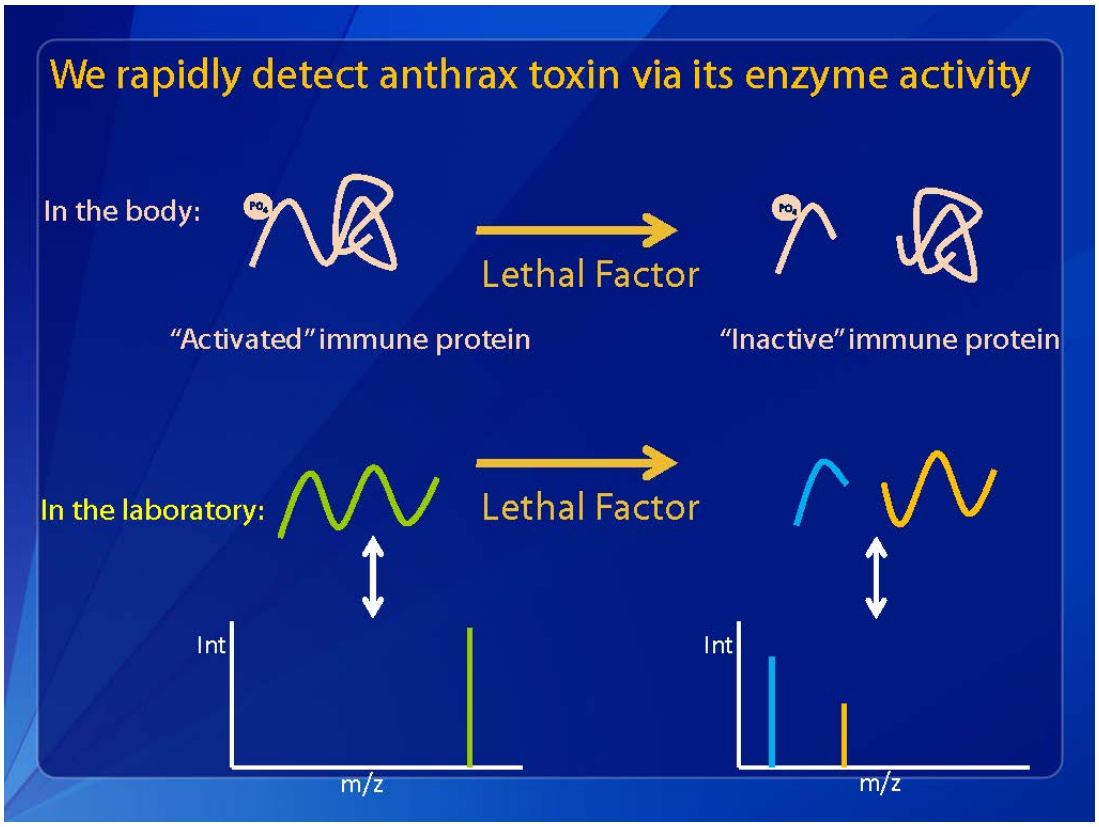
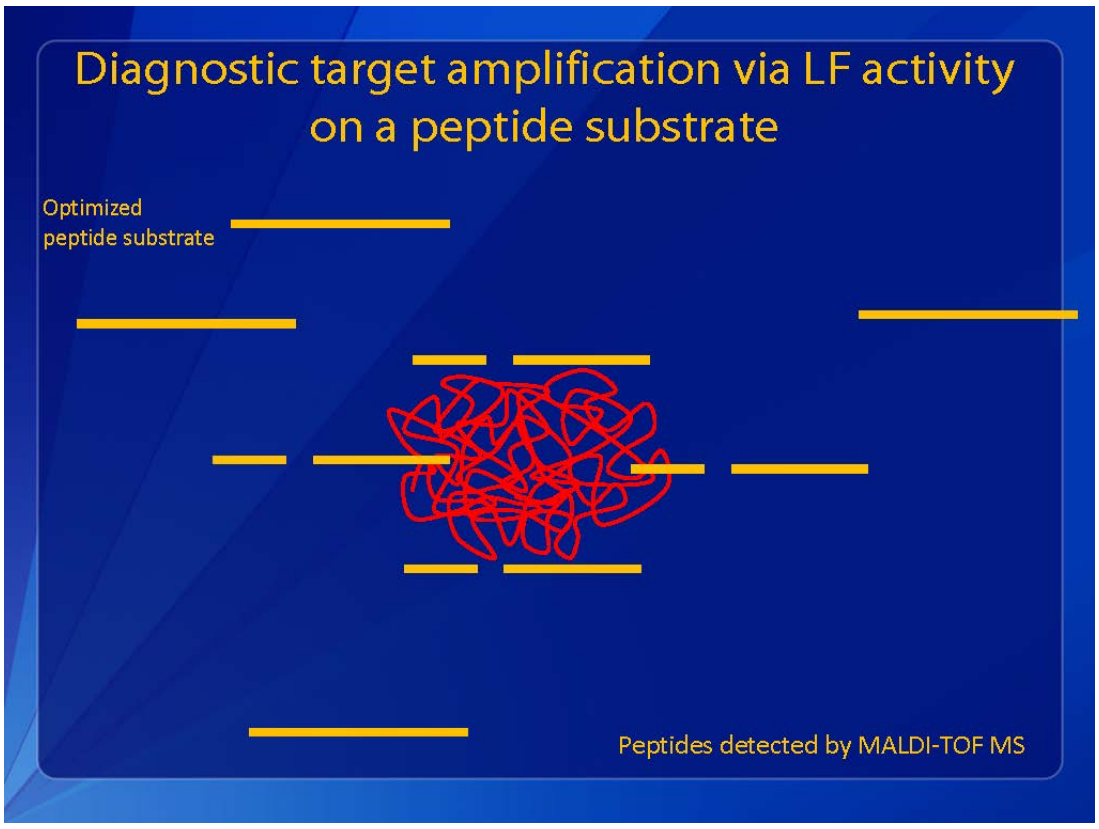
  - Daniel Wolfe
  - Bradford Gutting
- NIAID
- Numerous reviewers…

25

Anne Boyer, CDC National Center for Environmental Health




We rapidly detect anthrax toxin via its enzyme activity
In the body:
Lethal Factor
"Activated" immune protein
"Inactive" immune protein
In the laboratory:
Lethal Factor
Int
m/z
Int
m/z


Diagnostic target amplification via LF activity on a peptide substrate
Optimized peptide substrate
Peptides detected by MALDI-TOF MS

LF MS Limits of Detection

LF-CT Peptide	$3S_0$ (ng/mL)
200 µl 4h analysis	0.025
200 µl 18h analysis	0.005

PA ELISA at 2 ng/ml: LF is up to 400 times lower detection than ELISA

LF Method Fully Validated and CLIA Compliant Lab
High Precision, Accuracy, 100% Sensitivity/Specificity
Quarterly Proficiency Testing

Inhalation Anthrax in NHP

- 24 rhesus macaques exposed to 200 LD50 Ames Spores by inhalation
- 6 treated with Cipro 2xBID for 2 wks
- Treatment commenced at 48 and 72 h post-exposure

LF MS: Early and Consistent Diagnosis

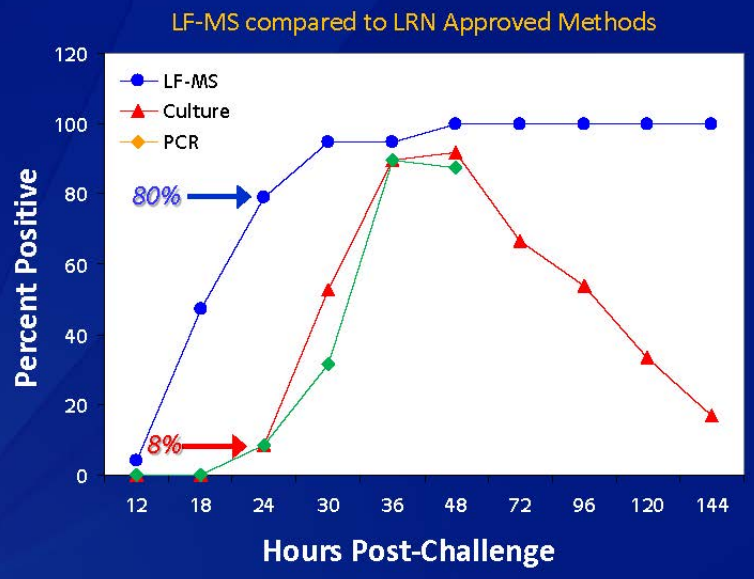
ID	Day 0 12 Hr	Day 0 18 Hr	Day 1 24 Hr	Day 1 30 Hr	Day 1 36 Hr	Day 2 48 Hr	Day 3 72 Hr	Day 4	Day 5	Day 6	Day 7
10N	-	-	+	+	+	+					
0434	-	-	-	+	+	+					
142N	-	-	+	+	+	+	+				
4206	-	-	+	+	+	+	+				
R050217	+	+	+	+	+	+	+				
R03130	-	+	+	+	+	+	+				
03E094	-	+	+	+	+	+	+				
04E005	-	-	+	+	+	+	+				
R04124	-	-	+	+	+	+	+	+			
0475	-	-	+	+	+	+	+	+			
03E006	-	NA	+	NA	NA	+	+	+			
03E052	-	NA	+	NA	NA	+	+	+			
03E073	-	NA	-	NA	NA	+	+	+			
16N	-	+	+	+	+	+	+	+	+		
89N	-	-	+	+	+	+	+	+	+		
03E042	-	NA	-	NA	NA	+	+	NA	+		
03E058	-	NA	+	NA	NA	+	+	+	+	NA	NA
03E101	-	-	-	-	+	+	+	+	+	+	S
Treated with Ciprofloxacin											
R050003	-	+	+	+	+	+	+				
4208	-	+	+	+	+	+	+	+	+	+	
04E067	-	+	+	+	+	+	+	+	+	+	S
004062	-	+	+	+	+	+	+	+	+	+	S
412	-	-	-	-	-	+	+	+	+	+	S
04E083	-	+	+	+	+	+	+	+	+	+	S

Culture and LF+

LF+

LF Assay: Not subject to immune- or antibiotic-based clearance of microorganism

Greater percent positives for LF-MS before and after Ciprofloxacin treatment



Inhalation Anthrax in NZW Rabbits

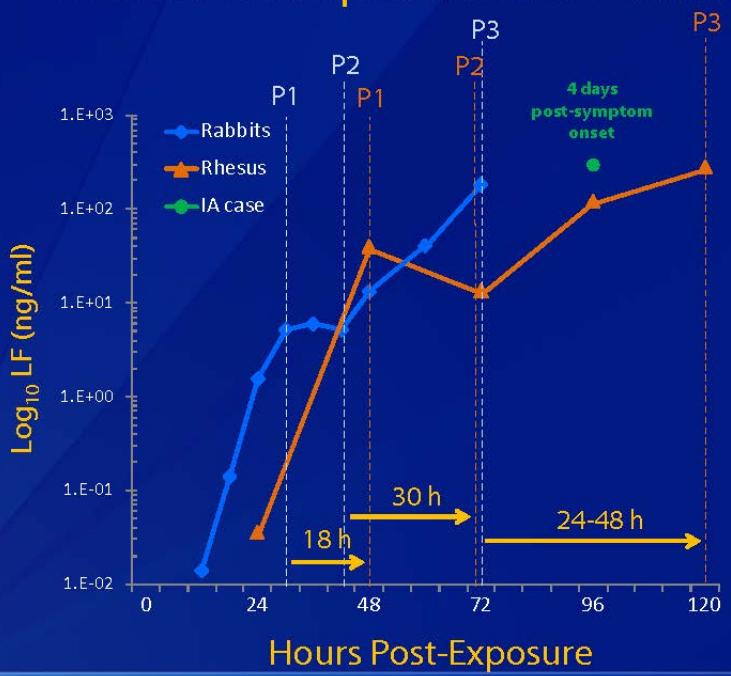
By Battelle (Primary - Gabriel Meister) Sponsored by NIH in 2007

12 NZW Rabbits

Exposed to 200 LD50 Ames Spores by Inhalation

Sample collection at 6 h intervals

Triphasic Kinetics of toxemia in Rhesus macaques and NZW Rabbits



Comparison of PA and LF toxins in study groups 4, 5, and 6

In total exposure resulted in 11 infections in 15 animals

Group 4: 2 of 5 infected and died

Group 5: 4 of 5 infected and died

Group 6: 5 of 5 infected and died

PA ELISA detection in NZW rabbits that died after receiving variable spore doses

Animal ID	Spore Dose (LD50)	Day-3	Day 1	Day 2	Day 3	Day 4	Day 7	Time of Death (day)
203	84.363	BD	BD	<LLOQ	<LOD			5
204	56.664	BD	BD	BD		T		4
213	69.423	NA	BD		T			3
221	84.571	BD	BD	NA	NA			2
232	98.795	NA	BD	BD	<LLOQ			4
200	2.090	BD	BD	BD	BD-T			3
201	1.694	BD	BD					4
214	3.367	BD	BD		NA			6
234	2.817	BD	BD	BD				6
225	0.247	BD	BD	BD		T		4
235	0.168	BD	BD	BD	BD	NA	BD	11
% Positive		0	0	40	60	100	0	

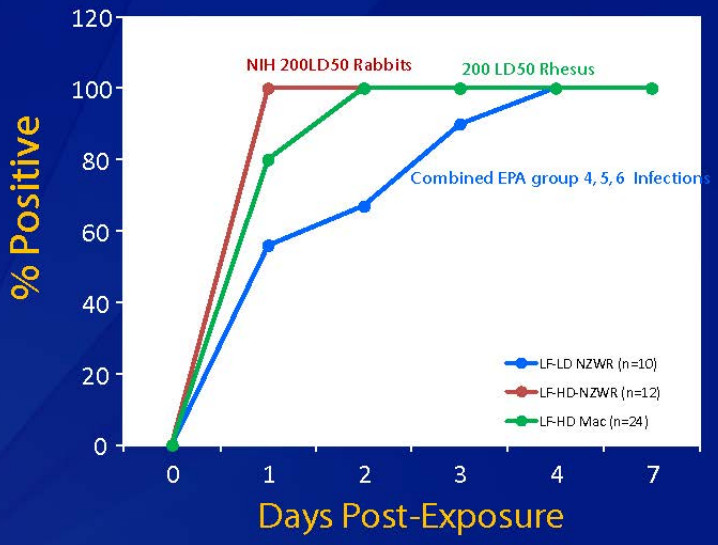
12 definitive positive results for PA

LF-MS detection in NZW rabbits that died after receiving low spore doses

Animal ID	Spore Dose (LD50)	Day-3	Day 1	Day 2	Day 3	Day 4	Day 7	Time of Death (day)
203	84.363	BD	BD					5
204	56.664	BD				T		4
213	69.423	NA		NA	T			3
221	84.571	BD	NA	NA	NA			2
232	98.795	NA						4
200	2.090	BD	BD	BD	T			3
201	1.694	BD						4
214	3.367	BD	BD					6
234	2.817	BD	BD	BD				6
225	0.247	BD				T		4
235	0.168	BD	BD	BD	BD	NA		11
% Positive		0	56	67	90	100	100	

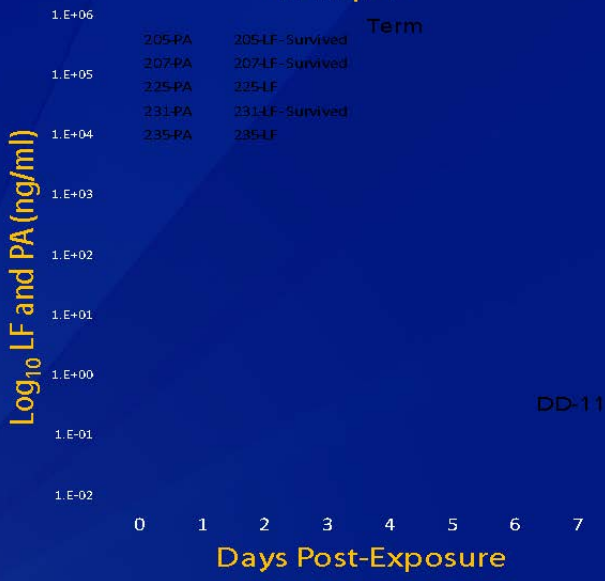
23 definitive positive results for LF

Comparison of Percent LF Positive Between Studies



LF and PA in EPA Variable Dose Study

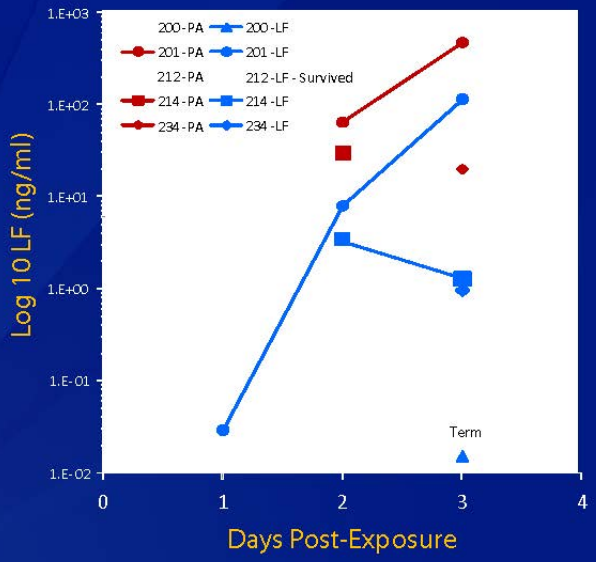
Group 4



Three Survivors
Two Deaths

LF and PA in EPA Variable Dose Study

Group 5

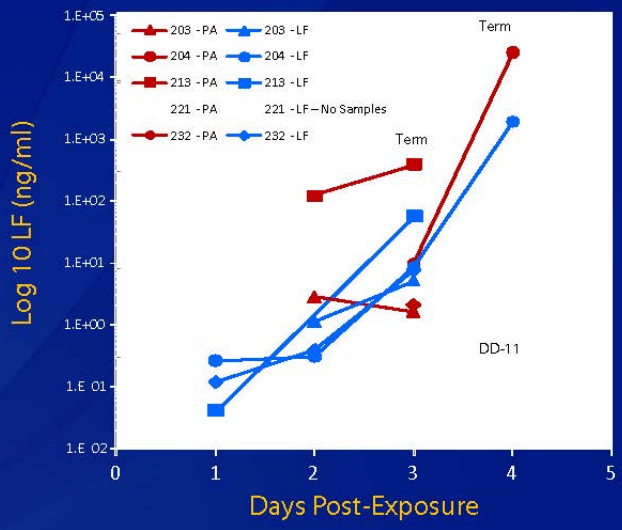


One Survivor
Four Deaths

LF and PA in EPA Variable Dose Study

Group 6 Dose

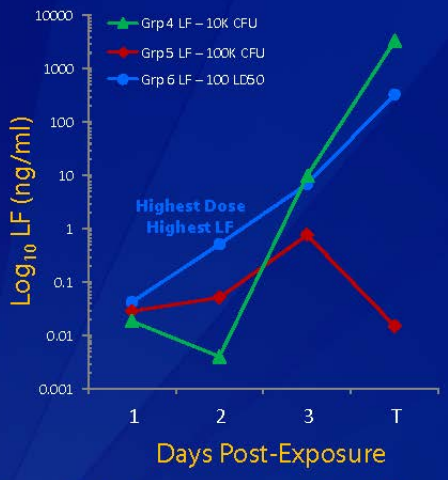
77.37 ± 0.101 LD50



No Survivors

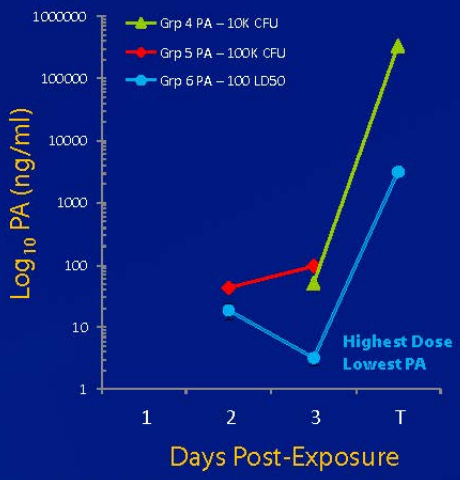
Geometric Mean Concentrations in Toxins Per Group 4-6

GMC LF Levels per Group



Mean LF levels appear to reflect differences in spore doses in early infection but not late infection. *Numbers too few for a dose response curve.

GMC PA Levels per Group



PA levels do not appear reflect differences in spore doses.

Limited Data Sets

Summary

- LF was detected earlier (day 1 and 2) and more consistently than PA.
- LF was detected in all 10 animals (with available sample) that died = 100 % sensitivity
- PA was detected later than LF in 8 of 10 non-survivors = 80% sensitivity.
- In late infection, PA levels were usually higher than LF.
- Mean LF levels appear to reflect differences in spore doses in early infection but not late infection. *Numbers too few for a dose response curve.
- PA levels do not appear reflect differences in spore doses.

Conclusions

- For spore dose exposures that may lead to infection and death, LF determination by MS provided the earliest and most consistent measure of infection.
- LF levels in early infection may correlate with spore dose (greater numbers are needed to confirm this correlation).

Discussion Questions which LF results may help answer:

To determine if an effect is due to B. anthracis infection/illness:

Is there an obvious dose-response?

Yes, trend observed for LF at the early stages (day 1 and day 2). As infection progresses to late infection LF levels did not correlate with initial dose. Greater numbers needed for statistical confidence.

Is the effect due to findings in one or more rabbits that could be considered outliers? *No*

Is the effect within normal biological variation (i.e. within the range of historical control or reference values)? (What are the most appropriate designs to capture variation?)

Yes. Need to increase the sample sizes.

Is there a lack of biological plausibility (i.e. lack of direct causal-connection of *B. anthracis* infection and measured responses)?

No, there is not a lack of plausibility. All were infected, all died, all that died had measureable LF (when samples were available).

To determine if an effect is adverse:

Is the response adaptive?

No, for LF the response is causal and directly due to infection.

Is the response transient?

Not typically, for LF the response is continuous after first detection unless treatment (when administered)clears LF below the detection limit (7-12 days post-treatment).

Is the effect isolated or independent (i.e. changes in other parameters usually associated with the effect of concern are not observed)?

For LF, it may be independent in early infection but later other diagnostic tests corroborate the early LF findings. For example on Day 1 only LF was positive in groups 4, 5, 6. By day 3 post-exposure most animals that died had 3-4 positive diagnostic targets (LF, PA, culture, PCR).

Acknowledgments

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Not pictured: Anne Boyer, Renato Lins, Susan Kuklenyik, Don Wang, Jon Bundy, Bryan Parks, Jakub Baudys, and Maria Solano

Battelle Biomedical Research and NIH
Gabriel Meister
Judith Hewitt
Ed Nuzum

Conrad Quinn and Team
NCIRD/CDC

Alex Hoffmaster and Team
NCZVED/CDC

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E-mail: cdcinfo@cdc.gov Web: http://www.cdc.gov

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

National Center for Environmental Health
Division of Laboratory Sciences

Conrad Quinn, CDC National Center for Immunization and Respiratory Diseases

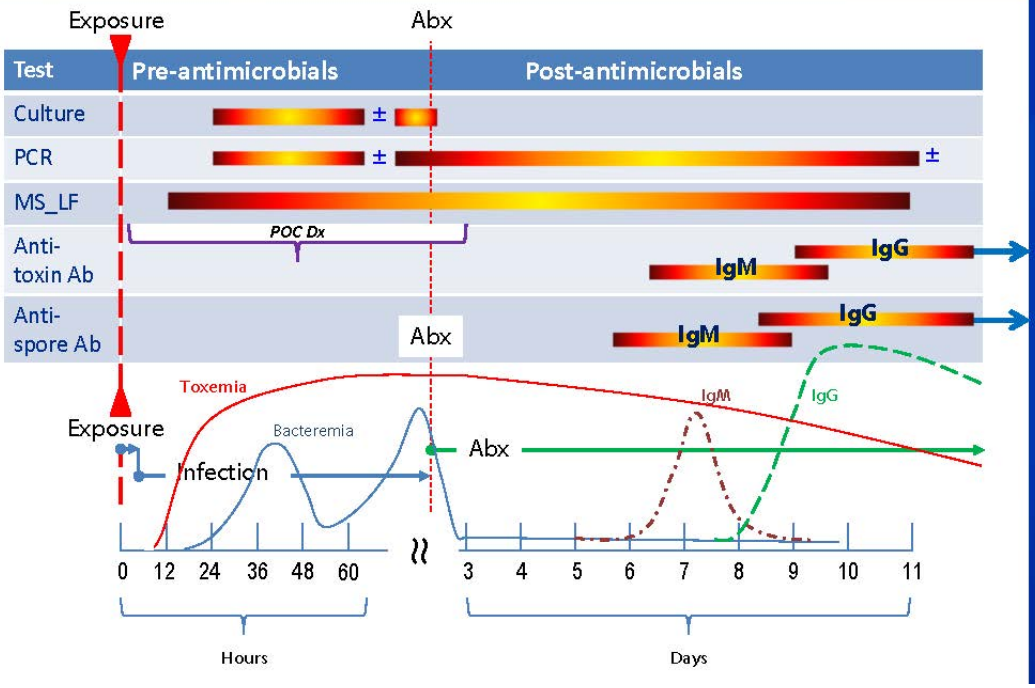
Priority Targets in Anthrax Emergency Response

- **Develop methodologies for determining the risk of potential anthrax contamination, duration of contamination, and requisite precautions to minimize risk**
- **Develop field-based reliable, rapid diagnostic and clinical tools for detecting anthrax and countermeasure effectiveness**

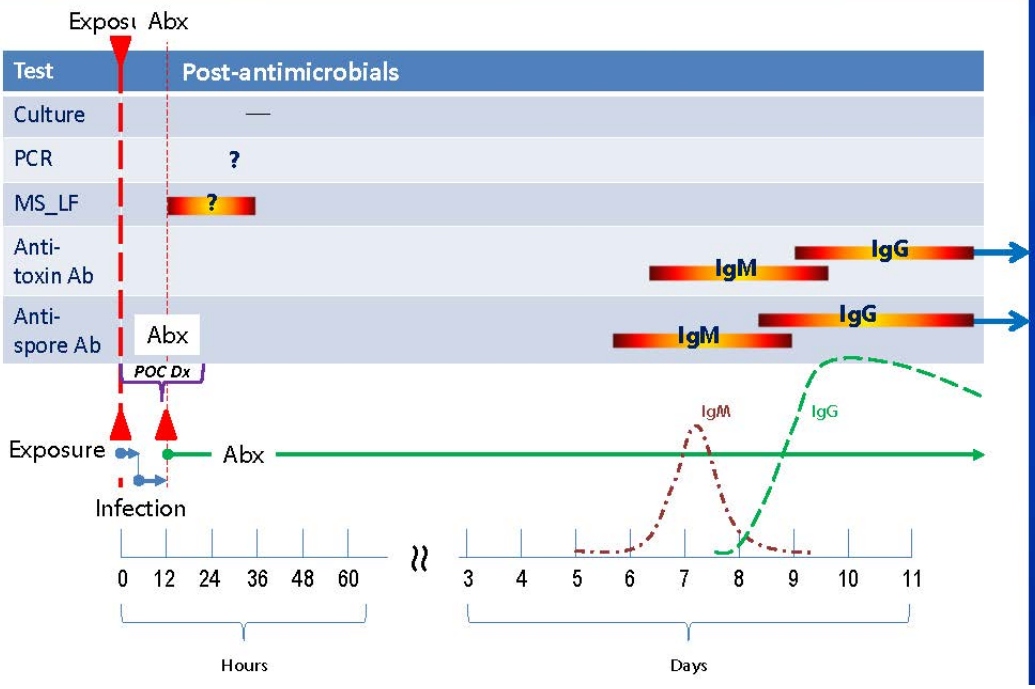
Inhalation Anthrax Clinical Diagnostics Scenarios

- **Exposure – Infection – Treatment**
  - Presentation of clinical disease
- **Exposure – Infection – Intervention**
  - Post-event, exposed/contaminated, infection but pre-symptoms
- **Post-exposure, pre-infection**
  - Post-event, exposed/contaminated, infection prevented

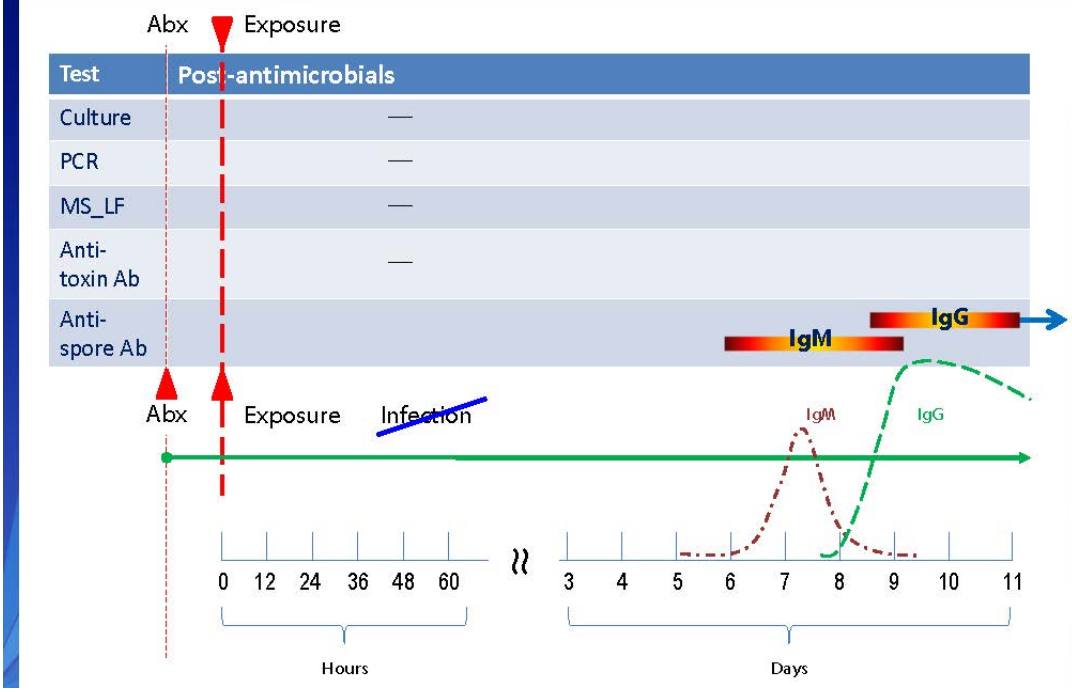
Exposure – Infection – Treatment



Exposure – Infection – Intervention



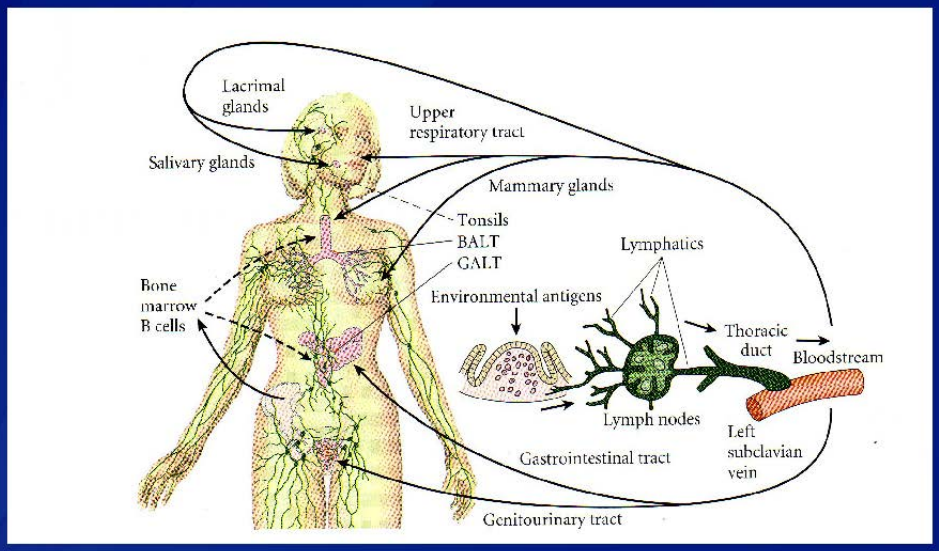
Post-exposure – Pre-infection



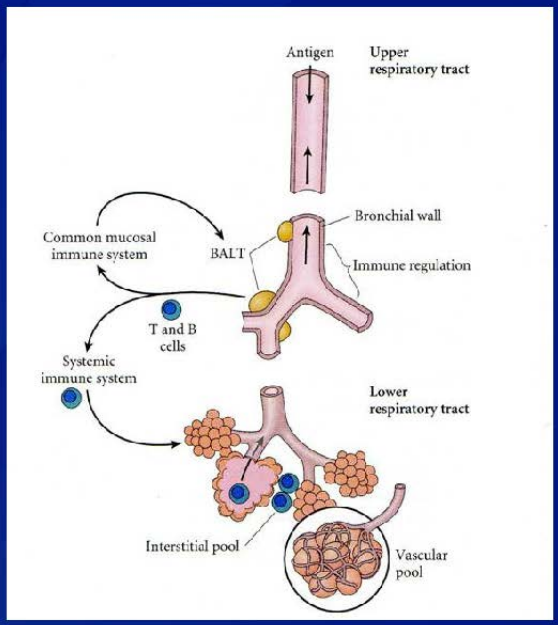
Anthrax Exposure Zone Diagnostics

- **Objective**
  - To provide a high specificity, high sensitivity test(s) to identify personnel exposed and at risk of *B. anthracis* infection
  - 'Hot Zone' diagnostic test to confirm spore exposure
  - Applicable to vaccinated and non-vaccinated personnel
  - Not negated by antibiotics intervention
- **Hypothesis**
  - Inhalation exposure to *B. anthracis* spores may initiate a detectable & marker specific host response
- **Approach**
  - Detection of antibody responses to spore surface antigens
  - IgM and IgG
  - Independent of infection

Human Mucosal Immune System
NALT – (BALT) – GALT



BALT Organization in Rodents & Rabbits



Targeting a Novel Spore Carbohydrate Antigen

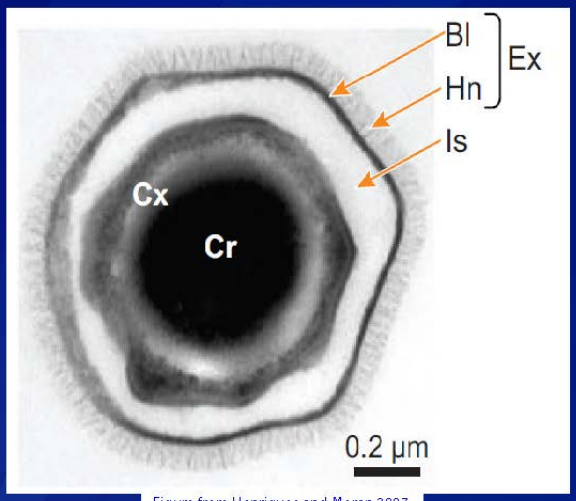


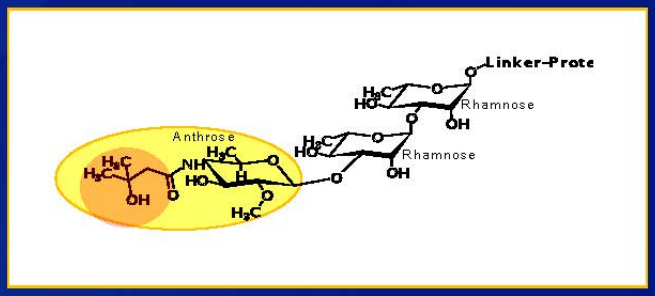
Figure from Henriques and Moran 2007

- *B. anthracis* BclA is an exosporium protein

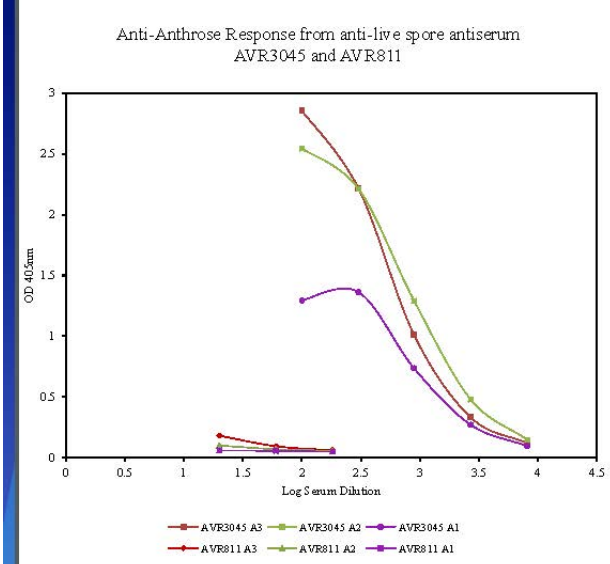
- Cr=spore core
- Cx= cortex peptidoglycan layer
- Is=interspace (area between the exosporium and the coat)
- Ex=exosporium (Bl basal layer and the hirsute nap)

Spore Oligosaccharide is Specific & Antigenic

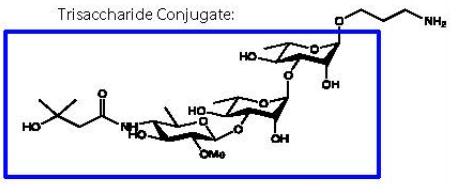
- **Glycoprotein BclA is an important constituent of the exosporium of Bacillus anthracis spores**
- **BclA is substituted with an oligosaccharide composed of a ß-L-rhamnoside substituted with a terminal saccharide, 2-Omethyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-d-glucopyranose – '*anthrose*'**
- **'Anthrose' is antigenic and exposed on the surface of *B. anthracis* spores**
- **Synthetic trisaccharide analogues retain the antigenic structure**
- **Antigenic region is localized to a specific terminal groups of the oligosaccharide**
- **The 4''-(3-methylbutyryl)-moiety is an important structural motif of the saccharide epitope**



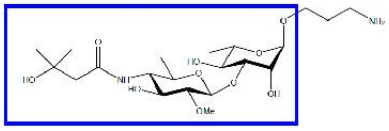
Anthrose Containing Mono- and Disaccharides Retain Antigenicity



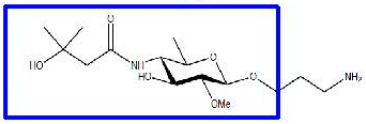
Trisaccharide Conjugate:



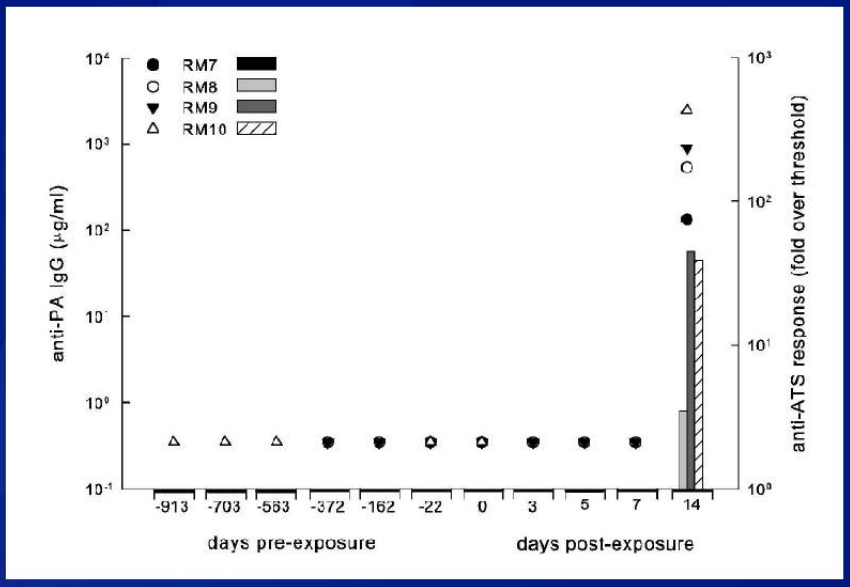
Disaccharide Conjugate:



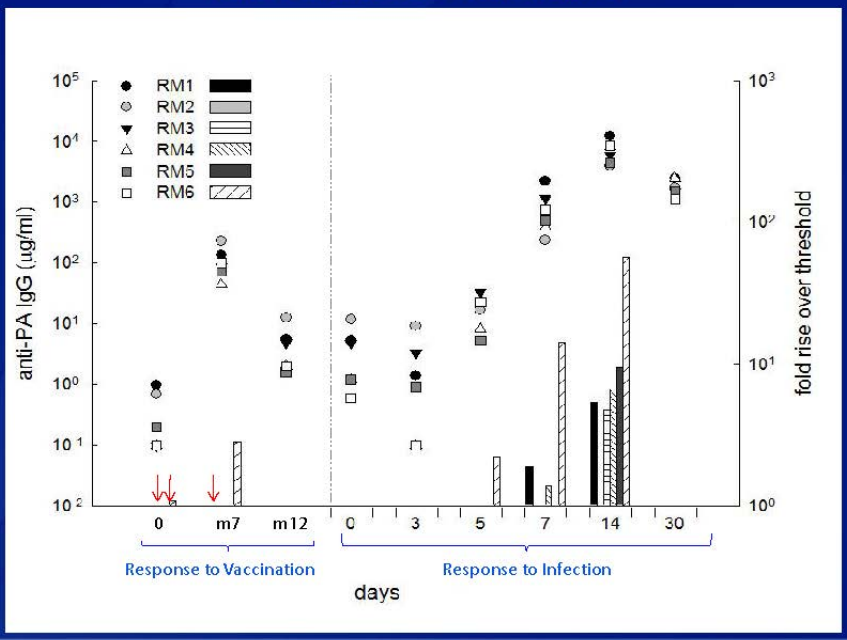
Monosaccharide Conjugate:



Spore aerosol exposure elicits anti-ATS responses in Rhesus macaques

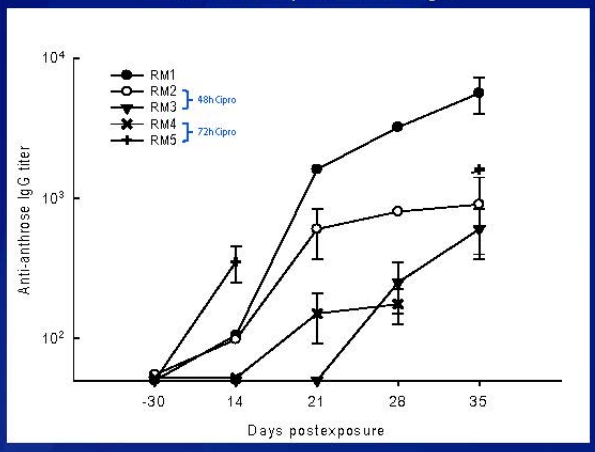


Infection, but not AVA vaccination elicits Anti-ATS IgG Responses Rh. Macaques



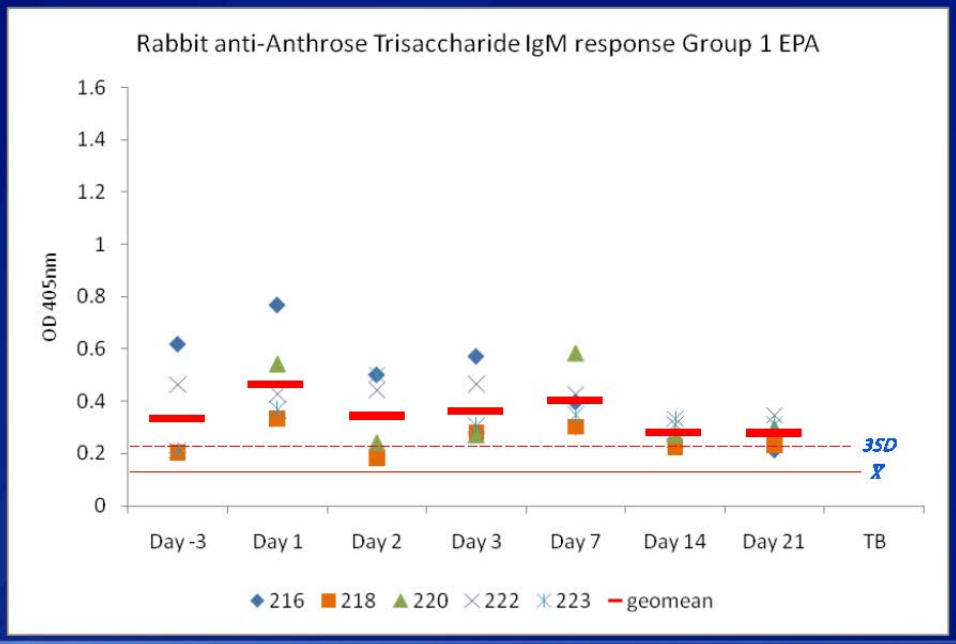
Spore aerosol exposure elicits anti-ATS responses in Ciprofloxacin Treated Rh. macaques

Rhesus macaque anti-ATS IgG

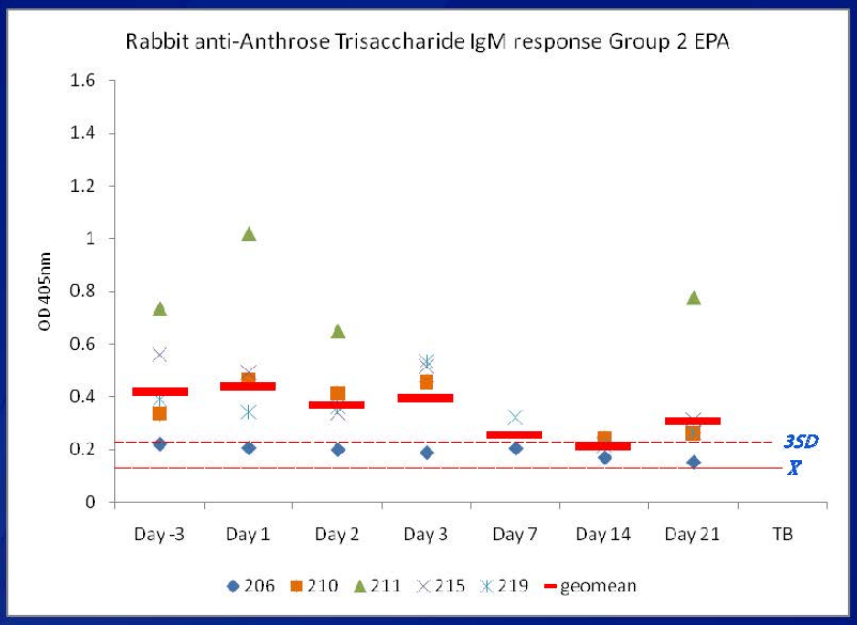


- **Infection confirmed by MS-LF and culture**
- **NHP rescued at 48h and 72 h with ciprofloxacin**
- **Anti-ATS IgG responses were independent of AVA vaccination and antibiotic intervention**
- **NHP**
  - DSP – 92.1%
  - DSN – 86.7%
- **Human**
  - DSP – 99.5% (Ag inhibition assay)
  - DSN – not yet determined

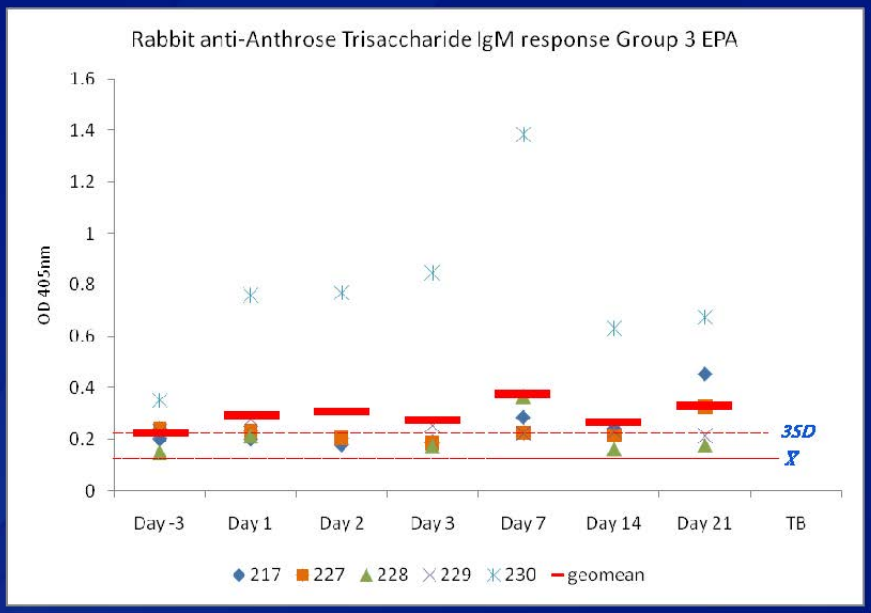
Group 1 – Irradiated High Dose Control



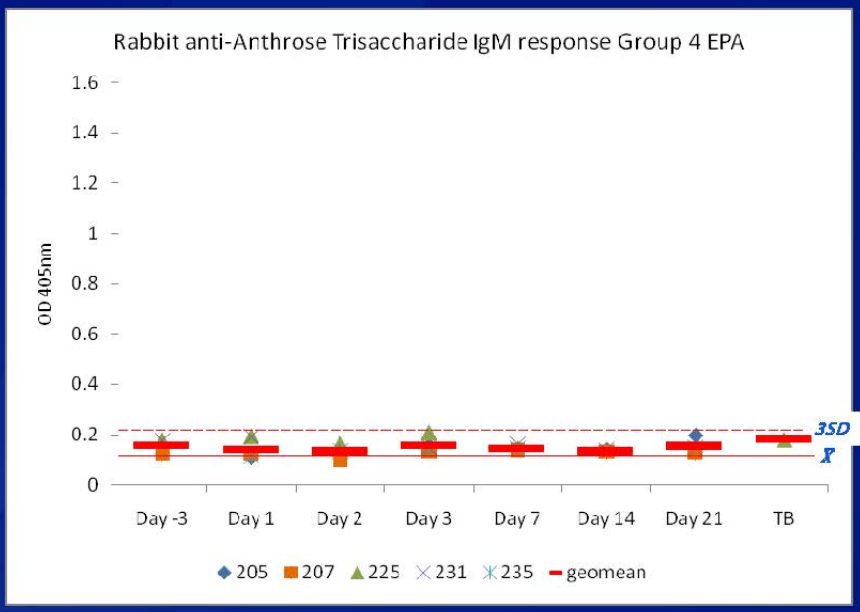
Group 2 – Target Dose 100 CFU



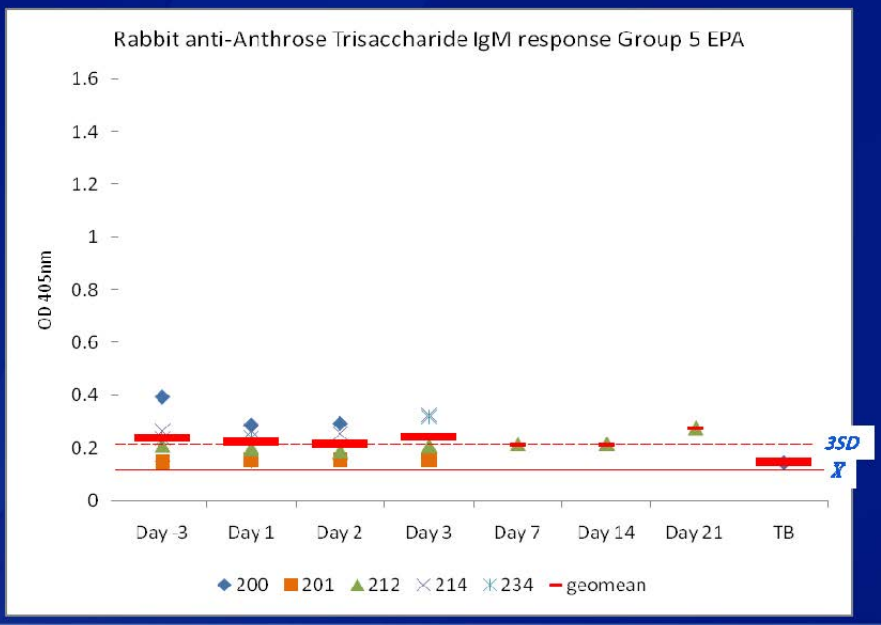
Group 3 – Target Dose 1000 CFU



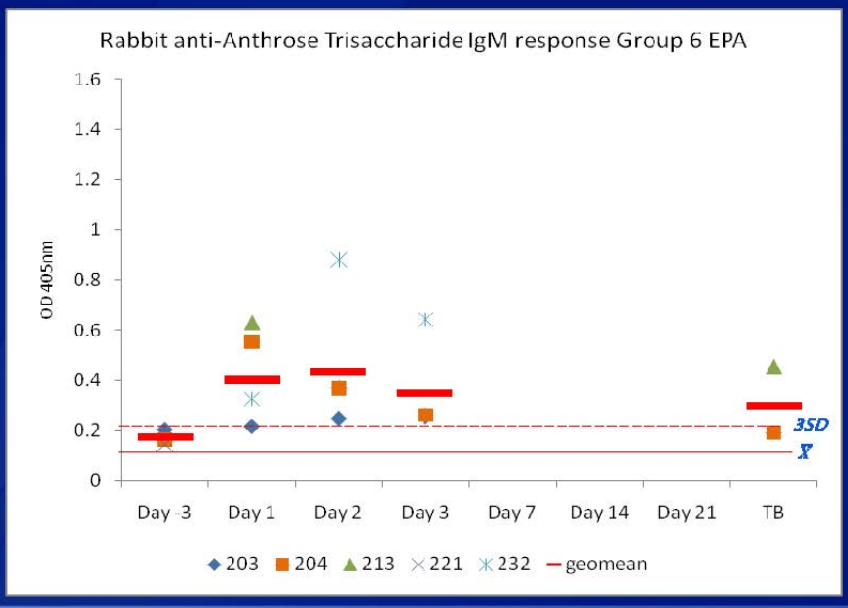
Group 4 – Target Dose 10,000 CFU



Group 5 – Target Dose 100,000 CFU

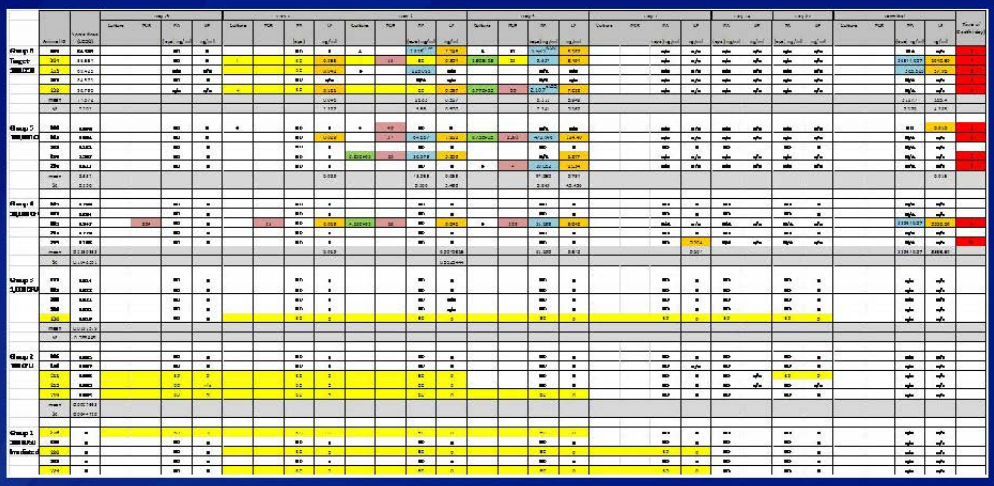


Group 6 – Target Dose 100xLD50 Equivalents



Schematic Alignment of Anti-spore IgM Responses & Other Biomarkers

Acute Exposure Study



Results Summary

- **Anti-anthrose IgM**
  - Day 1
    - 18 of 34 rabbits mounted anti-anthrose IgM responses with >3SD
  - Day 2
    - 14 of 29 >3SD
  - Day 3
    - 15 of 31 >3SD
  - High dose levels had higher frequency of responders
  - Intermediate doses had indeterminate frequencies (inverse trend?)
- **Anti-anthrose IgG response was detected in 2 of 32 rabbits**
  - Rabbit 219 (Group 2) was high from Day -3 pre-exposure and remained elevated
  - Rabbit 230 (Group 3) was elevated at Day 14 and Day 21

Conclusions

- **Exposure Zone Diagnostics**
  - There are no available clinical tests or assays to distinguish *B. anthracis* contaminated individuals at risk of disease
  - Rh. macaque NHP surviving inhalation anthrax mount a specific IgG antibody response against the anthrosyl component of the ATS
  - Specific anti-ATS responses detectable in NHP treated with ciprofloxacin
  - Anti-ATS response is not stimulated by AVA
- **Rabbit acute dose exposure**
  - Significant numbers of animals mounted a qualitative anti-anthrose IgM response
  - 2 animals mounted an anti-anthrose IgG response
  - There was not an obvious exposure-dose / response relationship
- **Differences observed between rh. macaque and rabbit studies**
  - Exposure dose related?
    - (NHP ~ 200LD50 equivalents)
  - Physiological differences
    - NHP – NALT (similar to human)
    - Rabbit – BALT
    - Innate immune response differences between genera

Conclusions

- **Data indicate that *B. anthracis* spores interact with host MALT**
  - Live and killed spores stimulate a response
  - Anti-spore responses detected in the presence of cipro rescue
  - Anti-spore responses are AVA independent
  - Specificity and sensitivity are high in NHP, but not 100%
- **Spore carbohydrate antigens have potential as a biomarker for spore contamination in asymptomatic individuals**

Next Steps

- **Spore Antigens**
  - Evaluation of multiple low dose exposure studies (EPA rabbits)
  - Evaluate IgM responses in NHP
  - Determine the relationship between spore dose and a detectable anti-spore antibody response in NHP
  - Determine spore dose 'antigenicity threshold'
  - Complete aerosol exposure, infection and rescue studies for specific reactivity to the *B. anthracis* carbohydrate antigens
  - Evaluate impact of pre-treatment with antibiotics
  - Evaluate human clinical samples IgM and IgG
  - Evaluate innate and acquired responses to *B. anthracis* spores antigens

Panel Charge Questions

- To determine if an effect is due to *B. anthracis* infection/illness:
  - Is there an obvious dose-response?
    - *There is a trend toward anti-ATS responses in the higher exposure doses; control and virulent*
    - *Indications of a trend to inverse dose-response relation?*
  - Is the effect due to findings in one or more rabbits that could be considered outliers?
    - *Sample sizes were too low to be definitive*
  - Is the effect within normal biological variation (i.e. within the range of historical control or reference values)? (What are the most appropriate designs to capture variation?)
    - *Sample sizes were too low to be definitive*
  - Is there a lack of biological plausibility (i.e. lack of direct causal-connection of *B. anthracis* infection and measured responses)?
    - *Connection is plausible, but data are incomplete*

Panel Charge Questions

- To determine if an effect is adverse:
  - Is the response adaptive?
    - *Detection of IgM and IgG indicate an adaptive response*
  - Is the response transient?
    - *IgM responses appear to be transient*
    - *Underlying immune response may be 'boostable'*
  - Is the effect isolated or independent (i.e. changes in other parameters usually associated with the effect of concern are not observed)?
    - *Effect is dependent on spore exposure*

Acknowledgements

- **Nazia Kamal - NCIRD/CDC**
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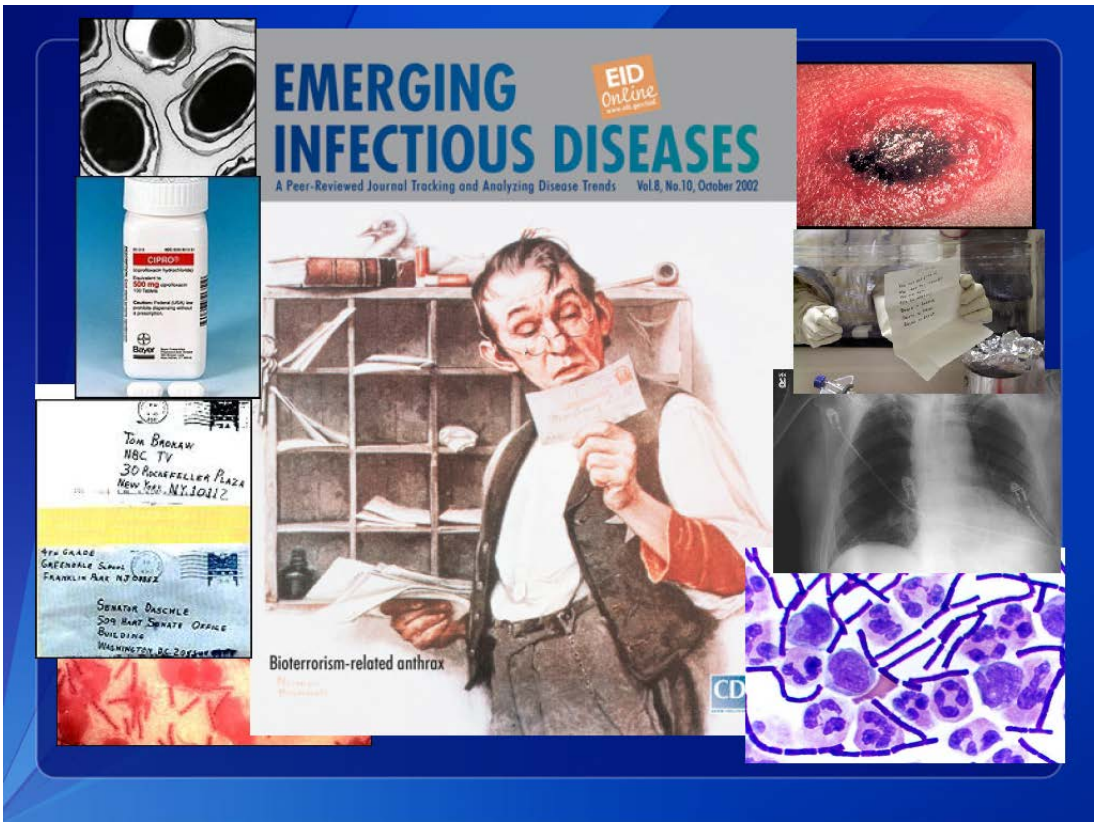
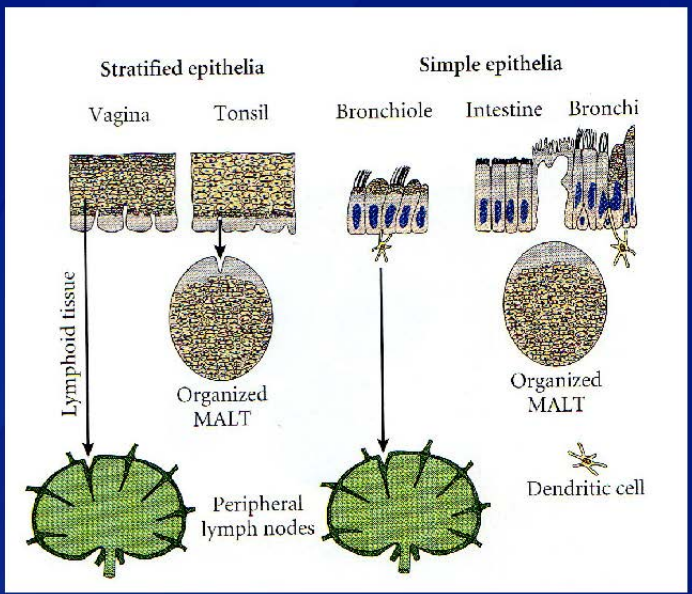
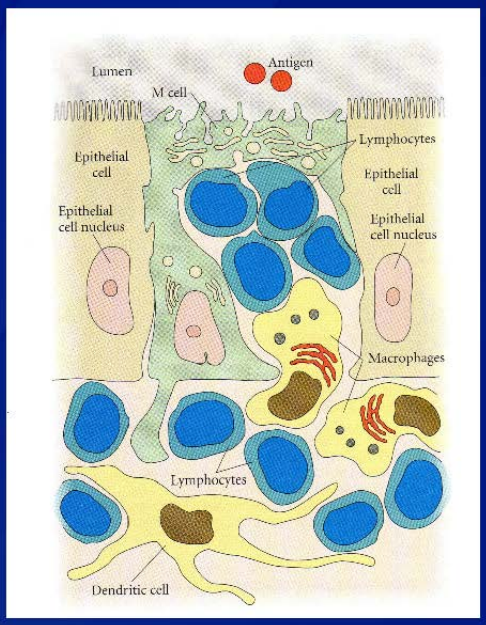
- **Anne Boyer - NCEH/CDC**
- **John R. Barr - NCEH/CDC**
- **Jim L. Pirkle - NCEH/CDC**
- **Alex Hoffmaster - NCEZID/CDC**
- **Russ Carlson - CCRC/UGA**
- **Geert-Jan Boons - CCRC/UGA**
- **Elmar Kannenberg - CCRC/UGA**



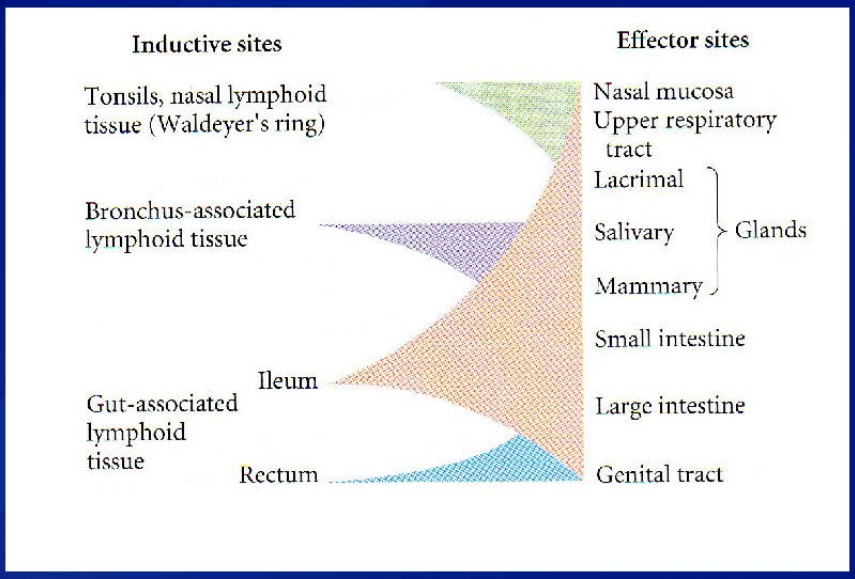
Antigen Sampling at Mucosal Surfaces



Antigen Transporting 'M' Cells



Human MALT Inductive & Effector Sites



Options for Follow-on Studies

Sarah Taft, EPA National Homeland Security Research Center

FUTURE?.

EPA United States Environmental Protection Agency

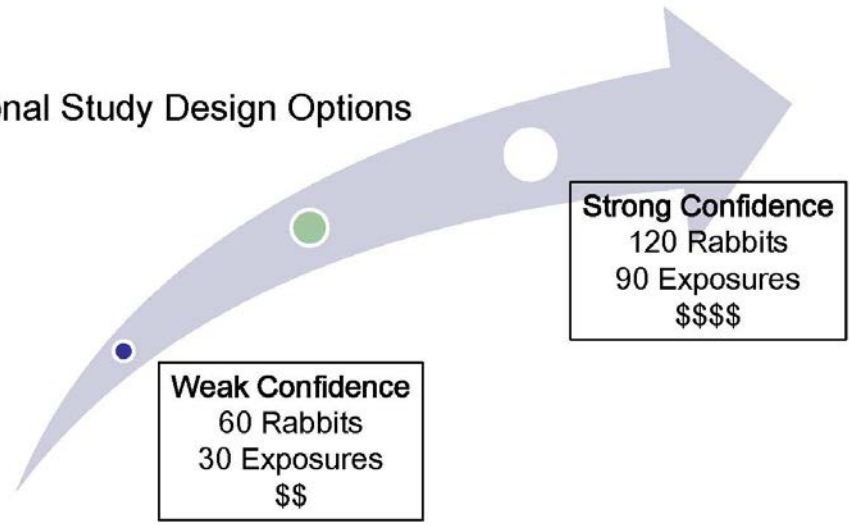
No Standard Protocols for Microbial Health Effects Testing for Multiple Inhalation Exposures

- Code of Federal Regulations, Title 40 Protection of the Environment, Part 798 Health Effects Testing Guidelines (for chemicals)
  - 798.245 Inhalation Toxicity, Subchronic Testing Requirements:
    - At least 8 young adult animals per group (4 males and 4 females)
      - Untreated and Vehicle control groups (Vehicle only given at highest dose level)
      - At least 3 low dose groups
    - Groups exposed 7 days/week for 90 days (but exposure on 5 days/week is minimum acceptable exposure period)
    - Animals observed 90 days post last exposure
      - Daily clinical observations
      - Weekly body weights
      - Blood characterizations:
        - » *Hematology*
        - » *Clinical Chemistry*
      - Full gross pathology and histopathology

FUTURE?.

EPA United States Environmental Protection Agency

Additional Study Design Options



FUTURE?.

Group	Challenge Inhaled Dose (CFU)	No. of Rabbits	No. of Consecutive Challenge Days	Days of Obs post-last challenge day	Blood Draws	Assays on each blood draw	Pathology
1	NA	**20**	**NA**	90	Day -7, 0, and once per week throughout the study	Qual Bact, Circ PA ELISA, IgG ELISA, TNA, Hematology, and Clin Chem	Complete Mol Tox tissue list with histo
2	10^3 Killed	**20**	**90**	90			
3	10	**20**	**90**	90			
4	50	**20**	**90**	90			
5	100	**20**	**90**	90			
6	1000	**20**	**90**	90			

HYBRID

Group	Challenge Inhaled Dose (CFU)	No. of Rabbits	No. of Consecutive Challenge Days	Days of Obs post-last challenge day	Blood Draws	Assays on each blood draw	Pathology
1	NA	**10**	**NA**	90	Day -7, 0, and once per week throughout the study	Qual Bact, Circ PA ELISA, IgG ELISA, TNA, Hematology, and Clin Chem	Complete Mol Tox tissue list with histo
2	10^3 Killed	**10**	**30**	90			
3	10	**10**	**30**	90			
4	50	**10**	**30**	90			
5	100	**10**	**30**	90			
6	1000	**10**	**30**	90			

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