

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE 06 JUN 2008	2. REPORT TYPE N/A	3. DATES COVERED -			
4. TITLE AND SUBTITLE Nosocomial infection of Serratia marcescens may induce a protective effect in monkeys exposed to Bacillus anthracis. Journal of Infection 57:162-164		5a. CONTRACT NUMBER			
		5b. GRANT NUMBER			
		5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S) Leffel, EK Twenhafel, NA Whitehouse, CA		5d. PROJECT NUMBER			
		5e. TASK NUMBER			
		5f. WORK UNIT NUMBER			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD		8. PERFORMING ORGANIZATION REPORT NUMBER TR-07-091			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)			
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)			
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This study was originally designed to collect data on the natural history of inhalational anthrax in a new nonhuman primate model. An uncontrollable event created a new experimental condition which allowed us to retrospectively evaluate the power of the innate immune system to protect from an aerosol exposure of B. anthracis. Five African green monkeys (AGMs) had intravenous catheters implanted. One catheter was accidentally pulled out, leaving four AGMs with catheters and one without. All were exposed, to multiple lethal doses of B. anthracis Ames strain. Blood was collected twice daily to evaluate bacteremia. The AGM with no catheter had blood drawn from a femoral vein and became bacteremic on Day 9; succumbed to inhalational anthrax on Day 10. The other four AGMs had S. marcescens contamination in the catheter; indicated by pure colonies grown from the blood. None of these AGMs showed clinical signs of illness, had B. anthracis or a detectable level of protective antigen in the bloodstream. It appears that the presence of S. marcescens may have induced a "Coley's toxin" effect in this experiment. The innate immune response may have protected the AGMs from a lethal inhalational dose of B. anthracis spores.					
15. SUBJECT TERMS Bacillus anthracis, anthrax, Serratia marcescens, protective effect, Coley's toxin, efficacy, laboratory animals, nonhuman primates					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 3	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			



ELSEVIER



www.elsevierhealth.com/journals/jinf

CASE REPORT

Nosocomial infection of *Serratia marcescens* may induce a protective effect in monkeys exposed to *Bacillus anthracis*

Elizabeth K. Leffel ^{a,*}, Nancy A. Twenhafel ^b, Chris A. Whitehouse ^c

^a Center for Aerobiological Sciences, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

^b Division of Pathology, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

^c Division of Diagnostics Systems, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

Accepted 28 April 2008

Available online 6 June 2008

KEYWORDS

Inhalation anthrax;
Innate immunity;
B. anthracis;
S. marcescens;
African green monkey

Summary This study was originally designed to collect data on the natural history of inhalational anthrax in a new nonhuman primate model. An uncontrollable event created a new experimental condition which allowed us to retrospectively evaluate the power of the innate immune system to protect from an aerosol exposure of *B. anthracis*. Five African green monkeys (AGMs) had intravenous catheters implanted. One catheter was accidentally pulled out, leaving four AGMs with catheters and one without. All were exposed, to multiple lethal doses of *B. anthracis* Ames strain. Blood was collected twice daily to evaluate bacteremia. The AGM with no catheter had blood drawn from a femoral vein and became bacteremic on Day 9; succumbed to inhalational anthrax on Day 10. The other four AGMs had *S. marcescens* contamination in the catheter; indicated by pure colonies grown from the blood. None of these AGMs showed clinical signs of illness, had *B. anthracis* or a detectable level of protective antigen in the bloodstream. It appears that the presence of *S. marcescens* may have induced a “Coley’s toxin” effect in this experiment. The innate immune response may have protected the AGMs from a lethal inhalational dose of *B. anthracis* spores.

Published by Elsevier Ltd on behalf of The British Infection Society.

* Corresponding author. Tel.: +1 301 620 7544; fax: +1 301 682 5268.

E-mail address: leffele@nbacc.net (E.K. Leffel).

Bacillus anthracis is the causative agent of anthrax, a zoonotic disease, which is also of great concern as a potential agent of bioterrorism. Infection in humans occurs as cutaneous, gastrointestinal, or inhalational depending on the route of exposure. Because inhalation anthrax is the form

most likely to occur after a bioterrorism attack, much effort has been made by the U.S. military and others to develop animal models of inhalation anthrax to test drugs and vaccines and to study the pathogenesis of the disease.

In 1841, Dr. William B. Coley, a surgeon, observed that in a patient with an inoperable sarcoma, who subsequently contracted erysipelas (caused by *Streptococcus pyogenes*), complete regression of the tumor occurred.¹ Over the next 45 years, Coley treated nearly a thousand other patients, who had a variety of malignant tumors, with a mixture of *S. pyogenes* and *Serratia marcescens*. It was the combined injection of these two bacteria that would eventually be referred to as "Coley's toxin".² The exact mechanisms of tumor regression induced by treatment with Coley's toxin remain unknown; however, modern research supports the concept that infection-stimulated tumor regression generally results from a non-specific innate immune response.³

Here, we report the survival of four naïve (unvaccinated) African green monkeys (*Chlorocebus aethiops*) (AGMs), after exposure to multiple lethal doses of aerosolized *B. anthracis*. All developed a systemic nosocomial infection with *S. marcescens*, and we present preliminary results which suggest a mechanism of action similar to that of Coley's toxin (i.e., induction of the innate immune response). Reports of unlikely survival, in patients with poor prognosis from other diseases, after 'treatment' with Coley's toxin or similar therapy, suggest that stimulation of the innate immune system could be sufficient to protect AGMs from otherwise fatal inhalation anthrax.⁴⁻⁶

The study described herein was designed to collect data on the natural history of inhalation anthrax in a new nonhuman primate model using AGMs. Five AGMs had central venous catheters (CVC) surgically implanted to facilitate frequent blood collection (approximately every 12 h from Day 0 to Day 3; daily on Day 4-12, 15, 28, and 35). All were exposed to approximately 1200 LD₅₀ (lethal dose in 50% population) of *B. anthracis* (Ames strain) spores via an automated bioaerosol exposure system.⁷ While the AGMs were in the exposure chamber, air samples from the chamber were collected in media and subsequently cultured so that the doses could be calculated. Therefore, we are confident that all AGMs were indeed exposed to extreme lethal doses and should have succumbed to disease; it has not been the author's experience that an untreated nonhuman primate survives such a dose.

During the aerosol exposure, the CVC from one AGM, #V509, was inadvertently removed, leaving four of five AGMs with catheters. Blood was collected to evaluate bacteremia and the presence of protective antigen (PA). It was at this point the natural history study, as designed, actually became a different experiment. We made an interesting observation in this small group of AGMs.

The AGM without a CVC had blood drawn from a femoral vein and became bacteremic (with only *B. anthracis*) on Day 9 postexposure. The animal succumbed to inhalation anthrax on Day 10. V509 had multiple gross and histologic lesions consistent with inhalational anthrax (necrotizing splenitis, necrotizing hepatitis, lymphoid depletion of tracheobronchial and mediastinal lymph nodes, pulmonary and mediastinal edema with many gram-positive bacilli). Immunohistochemistry for *B. anthracis* capsular antigen in V509 identified positive bacilli in the lungs, spleen, tracheobronchial and

mediastinal lymph nodes. The four other AGMs with CVC had *S. marcescens* contamination in the catheter that was discovered from laboratory tests designed to identify bacteremia due to *B. anthracis*. Once the contamination was identified, samples were collected from the CVC and the femoral vein to confirm the systemic infection. *S. marcescens* was identified morphologically and then confirmed by both Vitek and 16S rRNA PCR sequencing. None of these AGMs showed clinical signs of illness or had *B. anthracis* bacteremia. Using an electrochemiluminescence assay, PA (secreted by *B. anthracis*) was not detected in the blood of any of the four surviving AGMs. These tests were completed on samples obtained at the same time-points as ones for the blood culture, so the results are consistent with negative bacteremia. All four AGMs were still positive for *S. marcescens* in blood culture on Day 35 and were euthanized on Day 42 postexposure. At necropsy, complete gross and histologic examination revealed no lesions consistent with inhalational anthrax. Sections of lung, spleen, tracheobronchial lymph node, and mediastinal lymph node were negative for gram-positive bacilli using Lily-twort Gram stain. Immunohistochemistry for *B. anthracis* capsular antigen were negative on sections of lung, spleen, tracheobronchial lymph node and mediastinal lymph node. Histologic diagnoses of the four AGMs included subacute to chronic mild pleuropneumonia of unknown etiology with generalized marked lymphoid hyperplasia of many lymphoid tissues. A non-specific change of lymphoid hyperplasia was consistently present in the AGMs with CVC and tissues included the white pulp of the spleen, multiple lymph nodes (axillary, tracheobronchial, and mesenteric), bronchial associated lymphoid tissue (BALT), and gut-associated lymphoid tissue (GALT).

Unfortunately, only a limited amount of stored serum was available for cytokine evaluation. Because cytokine analysis was not part of the original study design, a comprehensive data set is not available. However, we found in the AGM that succumbed to anthrax, concentrations of both interferon-gamma (IFN- γ) and interleukin-2 (IL-2) were below detectable levels. In the four AGMs with the subclinical infection of *S. marcescens*, which survived inhalation anthrax, concentrations of serum IFN- γ ranged from 119 to 1170 pg/ml and IL-2 from 2346 to 6951 pg/ml. This suggests that there was stimulation of the innate immune system in the AGMs infected with *S. marcescens*, but not in the one AGM that died of inhalation anthrax and was not infected with *S. marcescens* (Table 1).

We acknowledge that definitive conclusions cannot be drawn from a study with such small numbers of AGMs, but propose that the subclinical infection of *S. marcescens* protected these animals from a very high aerosol dose of *B. anthracis*. The contamination in the catheter appears to have induced an innate immune response in the host, which was significant enough to protect AGMs from a lethal aerosol dose of *B. anthracis* spores. The similar lung pathology found in the four "protected" AGMs was not seen in the one that died from inhalation anthrax. We cannot conclusively determine that these changes were the result of *B. anthracis* infection, but it is strongly suggestive that the disease was attenuated by a host response. Additional studies are needed to investigate this interesting serendipitous finding and perhaps conclusively show that a "Coley's toxin" effect can induce protection against lethal inhalation anthrax. The

Table 1 Summary of study results

ID#	<i>B. anthracis</i> exposure dose (# LD ₅₀)	Catheter	<i>B. anthracis</i> bacteremia	<i>S. marcescens</i> bacteremia	Outcome	Cytokine results (pg/ml) (Day postexposure)
V258	1200	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 3	Survived	IFN- γ = 247 (21) IL-2 = ND
V299	1100	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 0	Survived	IFN- γ = 119 (21) IL-2 = 6951 (8)
V500	1400	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 1	Survived	IFN- γ = 518 (11) IFN- γ = 1170 (35) IL-2 = 3605 (8)
V506	1800	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 1	Survived	IFN- γ = 211 (11) IL-2 = 2346 (12)
V509	760	No	Yes (Day 9)	No	Died ^a	IFN- γ = ND IL-2 = <limit of detection

ND = not detected; cfu = colony forming units.

^a Succumbed to inhalation anthrax on Day 10 postexposure.

observations reported herein suggest that therapeutic countermeasures that stimulate the host's innate immune response may protect from an otherwise fatal infectious disease.

Acknowledgment

We thank Dr. Michael Bray (National Institutes of Health) for critically reviewing the manuscript and Dr. Art Friedlander (USAMRIID) for useful discussions regarding this work.

Laboratory Animal Use: Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Disclaimer: Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

References

1. Bickels J, Kollender Y, Merinsky O, Meller I. Coley's toxin: historical perspective. *Isr Med Assoc J* 2002;4(6):471–2.
2. Starnes CO. Coley's toxins in perspective. *Nature* 1992; 357(6373):11–2.
3. Hopton Cann SA, van Netten JP, van Netten C. Dr. William Coley and tumor regression: a place in history or in the future. *Postgrad Med J* 2003;79(938):672–80.
4. Zacharski LR, Sukhatme VP. Coley's toxin revisited: immunotherapy or plasminogen activator therapy of cancer? *J Thromb Haemost* 2005;3(3):424–7.
5. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. *Pharmacol Ther* 1994;64(3):529–64.
6. Keen AR, Frelick RW. Response of tumors to thermodynamic stimulation of the immune system. *Del Med J* 1990;62(8): 1155–6, 1159–64.
7. Hartings JM, Roy CJ. The automated bioaerosol exposure system: preclinical platform development and a respiratory dosimetry application with nonhuman primates. *J Pharmacol Toxicol Methods* 2004;49(1):39–55.