A Short Course of Antibiotic Treatment Is Effective in Preventing Death from Experimental Inhalational Anthrax after Discontinuing Antibiotics

Nicholas J. Vietri,1 Bret K. Purcell,1 Steven A. Tobery,1 Suzanne L. Rasmussen,1 Elizabeth K. Leffel,4,a Nancy A. Twenhafel,2 Bruce E. Ivins,1 Mark D. Kellogg,3,a Wendy M. Webster,1 Mary E. Wright,6 and Arthur M. Friedlander5

Divisions of 1Bacteriology, 2Pathology, and 3Diagnostics Systems, 4Center for Aerobiological Sciences, and 5Headquarters, US Army Medical Research Institute of Infectious Diseases, Frederick, and 6National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

Background. Postexposure prophylaxis of inhalational anthrax requires prolonged antibiotic therapy or antibiotics and vaccination. The duration of treatment for established anthrax is controversial, because retained spores may germinate and cause disease after antibiotics are discontinued. Using rhesus macaques, we determined whether a short course of antibiotic treatment, as opposed to prophylaxis, could effectively treat inhalational anthrax and prevent disease caused by the germination of spores after discontinuation of antibiotics.

Methods. Two groups of 10 rhesus macaques were exposed to an aerosol dose of Bacillus anthracis spores. Animals in group 1 received ciprofloxacin prophylaxis beginning 1–2 h after exposure. Those in group 2 began receiving ciprofloxacin after becoming bacteremic, and treatment was continued for 10 days. When each group 2 animal completed 10 days of therapy, the prophylactic antibiotic was discontinued in the paired group 1 animal.

Results. In group 1 (prophylaxis), no deaths occurred during antibiotic treatment, but only 2 (20%) of 10 animals survived after antibiotics were discontinued. In contrast, in group 2 (treatment), 3 deaths occurred during antibiotic treatment, but all 7 animals (100%) alive after 10 days of therapy survived when antibiotics were discontinued.

Conclusions. In the treatment of inhalational anthrax, the prolonged course of antibiotics required to achieve prophylaxis may not be necessary to prevent anthrax that results from the germination of retained spores after the discontinuation of antibiotics.

Inhalational anthrax, which is caused by the inhalation of spores of the bacterium Bacillus anthracis, was first recognized as an occupational illness among British woolsorters and ragpickers in Germany in the mid-1800s [1]. Naturally occurring inhalational anthrax is presently a very rare disease; from 1900 until the 2001 anthrax attacks in the United States, there were only ~20 reported cases of inhalational anthrax in the United States and 8 cases in Great Britain [2]. Because of the paucity of human cases, researchers have had to rely on animal models to understand the pathogenesis and treatment of anthrax. The animal model that most closely mimics human inhalational anthrax is the rhesus macaque [3]. In both macaques and humans, spores are thought to be ingested by alveolar phagocytic cells after being deposited into the terminal airways. Some spores survive and are transported to regional lymph nodes, where they are thought to germinate into vegetative bacilli [4]. Although most spores probably germinate during the first few days after inhalation, germination is not synchronous. Using data obtained from 50 rhesus macaques, Henderson et al. [5] demonstrated that spores could be detected in the lungs for as long as 100 days, even after low-dose aerosol exposure. Because spores can remain dormant for long periods and antibiotics are active only after spores have

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Present affiliations: National Biodefense Analysis and Countermeasures Center, Frederick, Maryland (E.K.L.); Department of Laboratory Medicine, Children’s Hospital, Boston, Massachusetts (M.D.K.).

Reprints or correspondence: Dr. Vietri or Dr. Friedlander, USAMRILID, 1425 Porter St., Frederick, MD 21702 (nicholas.vietri@amedd.army.mil or arthur.friedlander@amedd.army.mil).

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germinated [6], a prolonged course of prophylactic antibiotics is required after an inhalational exposure to prevent disease resulting from the germination of retained spores after discontinuation of antibiotics. For example, rhesus macaques that received 30 days of antibiotic prophylaxis after a low-dose aerosol challenge with \( B. \) \( \text{anthracis} \) spores were protected while receiving antibiotic therapy. However, 5 of 29 animals died after antibiotics were discontinued [7]. Moreover, recent computer modeling has suggested that >4 months of antibiotic prophylaxis would be required to protect individuals exposed to a high aerosol dose of spores [8].

The persistence of retained spores, which may germinate after the discontinuation of antibiotics and cause disease, has also generated substantial controversy with regard to treatment of inhalational anthrax. For example, guidelines from both the US Centers for Disease Control and Prevention and the European Commission’s Task Force on Biological and Chemical Agent Threats recommend 60 days of antibiotic treatment for inhalational anthrax [9, 10]. However, as reported previously and as expected, a serological response develops in individuals who are treated and recover from established clinical anthrax [11–13]. Furthermore, repeated cutaneous \( B. \) \( \text{anthracis} \) infections have rarely been reported and tend to be much milder [14]. This suggests that the initial \( B. \) \( \text{anthracis} \) infection induces some degree of immunity that protects against the development of subsequent disease. In addition, among nonhuman primates exposed to high doses of spores and given antibiotic prophylaxis, those that survived and that seroconverted after discontinuation of antibiotics were immune to rechallenge [15], suggesting that development of an antibody response indicates immunity. Earlier work demonstrated survival in nonhuman primates treated for 21 days after the onset of bacteremia, but these animals also received vaccination and/or antiserum [16].

To clarify the critical question of whether the treatment of established inhalational anthrax requires a prolonged course of antibiotic therapy, we used the rhesus macaque to model the outcome in humans. We designed an experiment to test the hypothesis that a short course of antibiotic therapy (10 days) for established inhalational anthrax, compared with a short course of postexposure prophylaxis, will protect surviving animals from anthrax caused by germination of retained spores after the discontinuation of antibiotics.

**METHODS**

**Bacterial strain preparation and aerosol exposure.** \( B. \) \( \text{anthracis} \) spores (Ames strain, US Army Medical Research Institute of Infectious Diseases) were produced and rhesus macaques exposed to a spore aerosol as described elsewhere [17]. The animals were exposed to a mean inhaled dose of 442 LD\(_{50}\) (range, 170–694 LD\(_{50}\)); 1 LD\(_{50}\) for aerosol exposure corresponds to 5.5 \( \times \) 10\(^4\) spores [17].

**Experimental groups.** Twenty adult rhesus macaques (\( \text{Macaca mulatta} \)) with a mean weight of 7.05 kg (range, 4.9–10.3 kg) were randomly distributed by sex into 2 groups: a prophylaxis group (group 1) and a treatment group (group 2). Each animal in group 1 was randomly paired with an animal in group 2. One week before challenge, all animals underwent placement of a Hickman 7F dual-lumen central venous catheter (Bard Access System) into the internal jugular vein while under general anesthesia. The catheters were used for obtaining blood samples and administering antibiotics, and the animals were prevented from manipulating them by a jacket-and-tether system.

Animals in the prophylaxis group received antibiotics beginning 1–2 h after aerosol exposure, whereas antibiotic therapy for animals in the treatment group began once an animal had 2 consecutive blood cultures positive for \( B. \) \( \text{anthracis} \). To ensure that spore burdens remained equivalent in the 2 groups of animals after antibiotics were discontinued, the duration of antibiotic therapy in the prophylaxis group was linked to that in the treatment group. Once the animal in the treatment group had completed or was scheduled to complete 10 days of antibiotic therapy, antibiotics were discontinued in both that animal and its paired match in the prophylaxis group. Animals in both groups received an initial loading dose of 200 mg of ciprofloxacin intravenously, followed by 100 mg given intravenously every 12 h. Blood was collected from each animal every 12 h for 7 days beginning 1 day after aerosol challenge, and it was quantitatively cultured on sheep blood agar plates.

**Clinical, microbiological, and pathological studies.** Necropsy was performed in all animals that died or were euthanized. Moribund animals were anesthetized before being euthanized. Samples of blood, spleen, lungs, brain, and lymph nodes from all animals that died were cultured for bacteria. Tissues were stained with hematoxylin-eosin and Lillie-Twort (Gram) stain, as described elsewhere [15]. Immunohistochemistry was performed on tissue sections by means of a monoclonal mouse anti–\( B. \) \( \text{anthracis} \) capsular antibody [18]. Serum anti-IgG antibodies to the protective antigen (PA) component of anthrax toxin were measured on study days 14, 21, 28, 35, and 42 by ELISA [19]. Plasma samples for peak ciprofloxacin levels (obtained 1 h after the antibiotic dose on days 1, 4, and 8 of therapy) and trough levels (obtained just before the antibiotic dose on days 2, 5, and 9 of therapy) were analyzed as described elsewhere [15]. Immunomagnetic electrochemiluminescence (ECL) detection of \( B. \) \( \text{anthracis} \) PA was performed on selected plasma samples, as described elsewhere [20].

This study was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhering to principles stated in the Guide for the Care and Use of Laboratory Animals [21]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animals Care International.

**Statistical analysis.** The difference in mortality between the prophylaxis and treatment groups was determined using a 2 × 3 two-tailed or one-tailed Fisher’s exact test. Differences in ciprofloxacin levels between prophylaxis and treatment groups were
assessed for statistical significance by Student’s t test. Analyses were conducted using SAS (version 9.1; SAS Institute) or GraphPad (version 5.01) software.

RESULTS

As expected, all 10 animals in the prophylaxis group whose antibiotic regimen started within 1–2 h after spore exposure remained clinically well, with no deaths during antibiotic therapy (figure 1). The duration of antibiotics for animals in the prophylaxis group was 12–16 days (mean ± SD, 13.4 ± 1.35 days). In contrast to the lack of deaths in animals whose antibiotic prophylaxis began soon after aerosol exposure, animals in the treatment group, which began receiving antibiotics after bacteremia developed, had a worse outcome. Three (30%) of 10 animals in the treatment group died while receiving the 10-day course of antibiotic therapy (figure 1). There was no difference in aerosol dose between the 3 animals that died and the 7 that survived (data not shown). The 3 animals died 1, 4, and 8 days after the start of antibiotic treatment. Postmortem blood culture results were negative, but B. anthracis grew in lung tissue from all 3 animals. At necropsy, all 3 animals had evidence of anthrax manifested by necrotizing lymphadenitis of the tracheobronchial lymph nodes and hemorrhagic mediastinitis. Gram-positive bacilli, confirmed to be B. anthracis by immunohistochemical staining, were observed in the meninges of 2 of the 3 animals, and antemortem plasma samples from 2 of the 3 were positive for PA assayed by ECL. In all 7 animals that survived during treatment, the blood cultures became negative within 12 h after the start of antibiotic treatment. However, PA could be detected by ECL for an average of 6 days (mean ± SD, 6.43 ± 0.79 days) after antibiotic treatment was begun.

After cessation of the antibiotic regimen, there was a marked difference in survival between animals in the prophylaxis and treatment groups. In the prophylaxis group, only 20% survived; 8 of 10 animals died after antibiotics were discontinued. These 8 deaths occurred 4–9 days (mean ± SD, 7.25 ± 2.05 days) after cessation of antibiotics. Five of the 8 animals in the prophylaxis group that died were found dead, and the remaining 3 were euthanized. Antemortem blood cultures of these 3 euthanized animals demonstrated 5.2 × 10⁵, 9.3 × 10⁵, and 5.9 × 10⁴ cfu of B. anthracis per milliliter. All 8 animals that died in the prophylaxis group had cultures of blood, spleen, brain, lungs, and lymph nodes that were positive for B. anthracis. Necrohemorrhagic mediastinal lymphadenitis was present in all 8 animals, and 3 had meningitis.

In marked contrast to the 80% mortality rate in the prophylaxis group after cessation of antibiotics, 100% of animals (7/7) in the treatment group survived after completing the 10-day course of antibiotic therapy. Those 7 surviving animals remained free of signs.

Figure 1. Survival of rhesus macaques during and after completion of antibiotic prophylaxis or treatment for inhalational anthrax. The duration of antibiotic prophylaxis (blue bars) or treatment (green bars) is indicated for individual animals. The outcome after discontinuation of antibiotics is also indicated for the same animals in the prophylaxis (blue circles) and treatment (green triangles) groups.
of illness for as long as 120 days after exposure (figure 2). All 7 seroconverted and had detectable levels of anti-PA IgG by day 14 after aerosol exposure (data not shown). In contrast, none of the 10 animals in the prophylaxis group had similar evidence of a rapid development of immunity to \textit{B. anthracis} by day 14, although the 2 survivors in this group seroconverted by day 28. The difference in outcome between the prophylaxis and treatment groups in our study was not due to a difference in ciprofloxacin pharmacokinetics, because there was no significant difference in either the peak ($P = .189$) or trough ($P = .253$) ciprofloxacin levels between the 2 groups (data not shown).

The course of the study included 2 distinct periods when the 2 groups differed: the first during antibiotic treatment, when survival was lower in the treatment group, and the second after antibiotics were discontinued, when survival was higher in the treatment group. A statistical analysis of the outcomes in all 20 animals demonstrated that outcomes were significantly different between the prophylaxis and treatment groups ($P < .001$, 2 $\times$ 3 two-tailed Fisher’s exact test). The overall survival rate in the treatment group was significantly higher than that in the prophylaxis group ($P = .035$, one-tailed Fisher’s exact test). If survival after discontinuing antibiotics is analyzed separately, then the difference between the treatment and prophylaxis groups (7/7 vs. 2/10 survivors) is significant ($P = .002$, two-tailed Fisher’s exact test).

**DISCUSSION**

The 2001 anthrax attacks in the United States resulted in 11 cases of inhalational anthrax, 5 of which were fatal. This event quickly highlighted the medical community’s unfamiliarity with inhalational anthrax and the uncertainty regarding recommendations for prophylaxis and treatment of this disease. For example, 2 months after the attacks, when many of the 10,000 persons with suspected or confirmed exposure to \textit{B. anthracis} spores were concluding their 60-day course of antibiotic prophylaxis, the US Department of Health and Human Services released additional options for individuals who wished to take further precautions against inhalational anthrax. These included (1) 40 additional days of antibiotic therapy to protect against the theoretical possibility that spores might cause infections up to 100 days after exposure and (2) 40 additional days of antibiotic therapy plus the anthrax vaccine to provide immunity to infection over a longer period of time [22].

The rhesus macaque treatment model of inhalational anthrax used in the present study was developed to answer a critical clinical question about therapy for inhalational anthrax. Specifically, we sought to determine whether animals treated with a short course of antibiotics for established inhalational anthrax would develop immunity from the infection and resist developing subsequent disease from retained spores that may have germinated after antibiotic therapy was discontinued. Because of the rarity of inhalational anthrax in humans, the current guidelines recommending a 60-day course of antibiotic therapy for both prophylaxis and treatment are based on data extrapolated from studies involving rhesus macaques [5, 7] as well as observations of individuals during the 1979 Sverdlovsk anthrax outbreak in the former Soviet Union, for which details of patient management are unfortunately lacking [23].

![Survival of rhesus macaques after completion of antibiotic prophylaxis (blue circles) or treatment (green triangles) for inhalational anthrax. Antibiotics were given for 10 days in the treatment group and for a mean of 13.4 days (range, 12–16 days) in the prophylaxis group. For the treatment group, $n = 7$ because 3 animals died while receiving antibiotic treatment.](image-url)
Results from the present study suggest that, although a prolonged course of antibiotics may be necessary for prophylaxis, it is not required to treat established disease from inhalational anthrax and prevent disease due to germination of retained spores after discontinuation of antibiotics. Although the 10 animals in this study treated with a 10-day course of antibiotics beginning after the onset of bacteremia had high initial mortality, as occurred in the human cases in 2001 [24], if they survived they were not at risk of disease once antibiotics were discontinued. The 100% protection from late disease was associated with the early development of anti-PA IgG by day 14 after challenge, indicating a robust immune response. In marked contrast, animals in the prophylaxis group, which were given prophylactic antibiotics soon after aerosol exposure and before the development of bacteremia, experienced high mortality (80%) after the short course of antibiotics was discontinued. This finding is consistent with those of older studies in which rhesus macaques given either a 5- or 10-day course of postexposure antibiotic prophylaxis died of inhalational anthrax after discontinuation of antibiotics [5]. The high mortality in the prophylaxis group after antibiotics were discontinued also supports the use of postexposure vaccination in addition to antibiotics to induce a protective immune response [15].

Two animals in the prophylaxis group survived and had evidence of an anti-PA IgG response, although their response was delayed compared with that in animals in the treatment group, first appearing at day 28 after challenge. This finding suggests that the 2 surviving animals were infected and that, although they remained clinically well, some proliferation of bacilli occurred, eliciting an immune response. The development of an immune response after prophylaxis contrasts with the findings of a previous study in which rhesus macaques that had received 30 days of antibiotic prophylaxis after spore exposure had no evidence of an anti-PA IgG response and thus were susceptible to disease caused by germination of retained spores [7]. In that study, however, the challenge dose was only 8 LD$_{50}$ and antibiotics were given for 30 days; in the present study, animals were exposed to a larger dose of 442 LD$_{50}$ and were given antibiotic prophylaxis only for an average of 13.4 days. In a more recent study in which rhesus macaques were given a very high aerosol dose of $B.\ anthracis$ spores (1646 LD$_{50}$) and antibiotic prophylaxis for 14 days, a few animals survived long term, generated an anti-PA IgG immune response, and were resistant on rechallenge [15]. Thus, aerosol exposure to high doses of $B.\ anthracis$ spores shortly before the beginning of antibiotic prophylaxis may elicit an immune response. This could be due to a priming immunization resulting from proliferation of bacteria after the initial exposure, which did not generate a detectable antibody response by day 14 in the present experiment but enabled a few animals to generate a protective anamnestic response to the residual spores that germinated after antibiotics were discontinued. This is similar to reported findings in guinea pigs, in which an anamnestic response to spore challenge may rarely result in protective immunity [25]. The outcome after discontinuation of prophylactic antibiotics likely depends on the residual spore burden at the time when antibiotics are discontinued and the rapidity of the immune response.

This experimental animal treatment model was designed to approximate what might happen in an individual presenting with inhalational anthrax. In the 2001 cases, all patients, except for those who received prior antibiotics, were bacteremic on admission [26, 27]. Therefore, we delayed antibiotic therapy in the treatment group until after the development of sustained anthrax bacteremia, defined by 2 consecutive blood cultures positive for $B.\ anthracis$ and obtained at 12-h intervals. As would be standard in a hospital setting, intravenous rather than oral antibiotics were given.

In agreement with what has been reported from the most recent human case of inhalational anthrax in the United States [28], once the bacteremic animals in our study were given a dose of antibiotics, the bacteremia resolved quickly (<12 h), but PA persisted in the blood for an average of 6 days after the antibiotic treatment was started. The rapid sterilization of the blood from patients with inhalational anthrax after a single dose of antibiotics may present a diagnostic challenge to clinicians involved in the care of patients with inhalational anthrax, because patients may have received an antibiotic before presenting to the hospital. In such a case, knowledge that anthrax toxin persists in the blood and the ability to detect it may be critical in making the correct diagnosis.

It has been suggested that individuals who have been treated for inhalational anthrax and recover might not need prolonged antibiotic therapy [29]. In a symptomatic individual, extensive bacterial replication will probably have occurred before the beginning of antibiotic treatment. Thus, as long as the active infection is controlled by antibiotics and the patient recovers, the immune response generated should protect against disease resulting from spore germination, which might occur weeks or months after cessation of antibiotic therapy. The development of such an immune response after aerosol exposure to $B.\ anthracis$ was observed during the 2001 anthrax attacks in the United States. Anti-PA IgG responses developed in all 6 patients who were treated for inhalational anthrax and survived. These antibodies were first detected in serum samples 15–28 days after likely exposure and persisted for 8–16 months [13].

The difference in survival between the prophylaxis and treatment groups after discontinuation of antibiotics is similar to the reported difference in disease incidence between postexposure prophylaxis and treatment of experimental Q fever in humans, which has a prolonged incubation period of ~14 days [30]. In that study, disease developed after the antibiotic was discontinued in 4 of 5 volunteers given oxytetracycline prophylaxis for 5–6 days, beginning within 24 h after exposure to $Coxiella\ burnetii$. In contrast, the same 5–6-day regimen of oxytetracycline
given after onset of symptoms resulted in cure, with no relapse in all 29 individuals. This finding suggests that antibiotics given soon after exposure suppressed replication so that immunity did not develop and disease occurred after discontinuation of antibiotics. When antibiotics were given for the same duration to treat established disease, sufficient replication may have occurred so that an immune response developed and no disease occurred when the antibiotic was stopped.

To summarize, rhesus macaques given a short course of antibiotics for prophylaxis of inhalational anthrax were protected while receiving antibiotics but experienced high mortality once the antibiotic treatment was discontinued. In contrast, animals treated for inhalational anthrax after the onset of bacteremia developed a protective immune response and were not at risk for development of disease after cessation of antibiotics. Patients who are treated for symptomatic inhalational anthrax and recover should be checked for seroconversion to the PA of *B. anthracis* toxin. The presence of anti-PA antibodies in such patients may be useful in determining when antibiotics can be safely discontinued, eliminating the need for a prolonged course of antibiotic therapy.

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