The US Capitol Bioterrorism Anthrax Exposures: Clinical Epidemiological and Immunological Characteristics

Denise L. Doolan,1,6,a Daniel A. Freilich,2,4,a Gary T. Brice,1,5a Timothy H. Burgess,3,4,5 Mara P. Berzins,1,7 Robert L. Bull,1,7 Norma L. Graber,1,7 Jason L. Dabbs,3,7 Lori L. Shatney,1,7 David L. Blazes,3,5b Lolita M. Bebris,1,7 Maria F. Malone,1,7 John F. Eisold,5,8 Alfred J. Mateczun,3,5 and Gregory J. Martin 4,5,b

1Infectious Diseases Directorate, 2Combat Casualty Directorate, and 3Biological Defense Research Directorate, Naval Medical Research Center, Silver Spring, 4Infectious Diseases Service, National Naval Medical Center, and 5Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, 6Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, and 7Henry M. Jackson Foundation, Rockville, Maryland; 8Office of the Attending Physician to Congress, United States Capitol, Washington, DC

(See the editorial commentary by Hadler, on pages 163–4.)

Background. Bioterrorism-related anthrax exposures occurred at the US Capitol in 2001. Exposed individuals received antibiotics and anthrax vaccine adsorbed immunization.

Methods. A prospective longitudinal study of 124 subjects—stratified on the basis of spore exposure, nasopharyngeal culture results, and immunization status from inside and outside an epidemiologically defined exposure zone—was performed to describe clinical outcome and immune responses after Bacillus anthracis exposure. Antibody and cell-mediated immune (CMI) responses to protective antigen (PA) and lethal factor were assayed by enzyme-linked immunosorbent assay and fluorescence-activated cell sorting.

Results. Antibody and CMI dose-exposure responses, albeit generally of low magnitude, were seen for unimmunized subjects from inside, within the perimeter, and outside the exposure zone and in nonexposed control subjects. Anti-PA antibody and CMI responses were detected in 94% and 86% of immunized subjects. No associations were seen between symptoms and exposure levels or immune responses.

Conclusions. Anthrax spores primed cellular and possibly antibody immune responses in a dose-dependent manner and may have enhanced vaccine boost and recall responses. Immune responses were detected inside the perimeter and outside the exposure zone, which implies more-extensive spore exposure than was predicted. Despite postexposure prophylaxis with antibiotics, inhalation of B. anthracis spores resulted in stimulation of the immune system and possibly subclinical infection, and the greater the exposure, the more complete the immune response. The significance of low-level exposure should not be underestimated.

Bioterrorism-related anthrax exposures occurred at the US Capitol in 2001 after the opening of a spore-filled envelope. A handheld ELISA was positive for Bacillus anthracis, staff were evacuated, ventilation was secured, and nasopharyngeal cultures (NPCs) were performed to delineate an exposure zone (EZ). Individuals with a definite or high risk of exposure were promptly given 60 days of antibiotics. Of 63 NPCs, 28 were positive: all 13 from persons in the immediate vicinity, 9 of 41 from persons in adjacent areas, and 6 of 9 from first responders. Repeat NPCs at 7 days were negative. None of ~6000 NPCs from outside the EZ were positive.

Received 13 March 2006; accepted 27 June 2006; electronically published 6 December 2006.

Reprints or correspondence: Dr. Denise Doolan, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, MD 20910-7500 (dooland@nmrc.navy.mil).

The Journal of Infectious Diseases 2007; 195:174–84 © 2006 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2007/19502-0005$15.00

Presented in part: 40th Annual Meeting of the Infectious Disease Society of America, Chicago, 27 October 2002 (late breaker abstract, oral presentation); American Society of Tropical Medicine and Hygiene, Denver, 11 November 2002 (late breaker abstract, oral presentation).

Potential conflicts of interest: none reported.

Financial support: US Army Medical Research Material Command (funds allocated to the Naval Medical Research and Development Center, work units N0239802RCCDO01B98 and 61102A.313.F.A0009). The funding source had no impact on design, conduct, and reporting of the study.

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, the Department of Defense, nor the US Government.

a D.L.D. and D.A.F. contributed equally to the study.

b Present affiliations: Naval Medical Research Center Unit 2, Jakarta, Indonesia (G.T.B.); Naval Medical Research Center Detachment, Lima, Peru (D.L.B. and G.J.M.).
Because monkey data have shown that spores are viable for \(\geq 100\) days [1], exposed individuals were offered a 120-day course of postexposure antibiotics and an abbreviated 0/2/6-week anthrax vaccine adsorbed (AVA) immunization schedule (i.e., \(~6, 8,\) and 12 weeks after exposure). None developed inhalational anthrax.

The immunological characteristics of human inhalational anthrax are incompletely understood [1]. The few documented cases, which predominantly occurred in vaccinated mill workers with exposure to anthrax-infected animal products [2, 3], were not extensively studied; thus, protective immunity concepts have been derived from animal models. Active immunization [4–8] and passive transfer [6, 9–13] studies have demonstrated that \(B.\ anthracis\) protective antigen (PA)–specific antibodies correlate with protection, possibly by blocking PA binding to cells and, thus, preventing the internalization and activation of lethal factor (LF) and edema factor; enhancing phagocytic opsonization; and inhibiting spore germination [9]. This correlation has been consistent in toxin-neutralizing antibody assays but not in ELISA (depending on the model) [4, 14]. Less data are available regarding cell-mediated immunity (CMI) responses. In vitro, LF has been shown to induce monocyte-dependent T cell mitogenic proliferation, and, in vivo, PA and LF fragments fused to T cell epitopes derived from lymphocytic choriomeningitis virus (LCMV) induce LCMV-specific CD4+ T cell responses in mice [15–17]. These studies provided no information about antigen-specific CMI responses.

The US Capitol outbreak provided the opportunity for a prospective longitudinal study of humans exposed to \(B.\ anthracis\). We hypothesized that acute exposure to \(B.\ anthracis\) might result in subclinical (asymptomatic or mild) infection, as suggested in nonbioterrorism settings [2, 3, 18]. Specifically, we hypothesized that exposed individuals who remained asymptomatic while receiving antibiotics would mount PA- and LF-specific CMI responses even in the absence of detectable anti-PA or anti-LF antibodies and that this priming would manifest as a dose-exposure relationship. Subsequently, we hypothesized that immune responses induced by AVA would be enhanced by priming from spore exposure.

Our underlying hypotheses were based on observations regarding anthrax and on extrapolation from other disease models. First, rhesus monkeys inhalationally challenged with \(B.\ anthracis\) spores and treated with antibiotics did not develop anti-PA antibodies [19]. Second, antigen-specific T cell responses can be detected in the absence of antibodies after exposure to pathogens (e.g., HIV) [20–23]. Third, that LF plays a role in protective immunity has been suggested by anthrax DNA vaccination in rabbits [8]. Moreover, environmental sampling confirming the presence of spores outside the EZ [24] provided an opportunity to assess the impact of spore dose on immune responses. As an extension of the outbreak investigation, clinical outcome and antibody and cellular responses to \(B.\ anthracis\) antigens were evaluated in a prospective longitudinal study in stratified study cohorts from inside and outside the epidemiologically defined EZ.

SUBJECTS, MATERIALS, AND METHODS

Clinical study. The study protocol was approved by institutional review boards at the Naval Medical Research Center and National Naval Medical Center (NNMC), in compliance with applicable federal regulations governing the protection of human subjects. Written informed consent was obtained.

In the separate public health response, NPC specimens were collected using Dacron fiber-tipped sterile swabs from individuals within the EZ and an \(~6000\) additional swabs from individuals in the perimeter of and outside the EZ. \(B.\ anthracis\) was identified at NNMC’s microbiology laboratory on the basis of blood agar growth, motility, Gram’s stain, and spore formation and was confirmed by direct fluorescent antibody test, \(\gamma\)-phage lysis, and polymerase chain reaction. Most subjects with definite or presumed exposure received postexposure antibiotics (500 mg of ciprofloxacin or 100 mg of doxycycline, orally, twice daily) [26, 27], and AVA \(\sim 6, 8,\) and 12 weeks later.

Subjects were stratified according to presumed level of exposure (i.e., NPC status and proximity to Senator Daschle’s office, where the envelope was opened). Spore-count data were not collected. Subjects within the EZ were assigned to group 1 (high exposure, positive NPC) or group 2 (high exposure, negative NPC). Subjects in the outer perimeter of the EZ (elsewhere in the Hart Senate Office Building [HSOB]) were assigned to group 3 (intermediate exposure, negative NPC). Subjects in the Capitol complex outside the HSOB were assigned to group 4 (presumed no exposure, negative NPC). Three comparator groups were enrolled from outside the Capitol: individuals with confirmed anthrax (from 2001 US bioterrorism events; group 5), negative control subjects (no known \(B.\ anthracis\) exposure; group 6), and positive control subjects (history of AVA [mean \(\pm\) SE number of immunizations, 4.5 \(\pm\) 0.3; interval until immunization blood draw, 84.5 \(\pm\) 28.8 days]; group 7). One subject each from groups 1 and 2 with a history of prior AVA were analyzed separately (“preimmunized”). Cohort demographics are summarized in table 1; additional details have been published elsewhere [24, 25].

Subjects completed a survey detailing self-recalled symptoms, antibiotic use, and AVA history (\(~6\) weeks after exposure). Heparinized whole blood was collected before and 2 weeks after the first and third immunizations (\(~6, 8,\) and 14 weeks after exposure); comparable samples were obtained from unimmunized and control subjects.

Antibody assays. Antibodies were evaluated by ELISA using recombinant PA or LF proteins (provided by Dr. S. Leppla, National Institutes of Health, Bethesda, MD) as capture anti-
Table 1. Demographic characteristics of enrolled subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>Criteria</th>
<th>Group risk</th>
<th>Rationale</th>
<th>Age, years</th>
<th>Male sex, %</th>
<th>AVA immunization status</th>
<th>Duration of antibiotics, days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>Preimm</td>
</tr>
<tr>
<td>1</td>
<td>EZ+ and NPC+</td>
<td>Definite exposure</td>
<td>Key study group</td>
<td>28</td>
<td>31</td>
<td>57</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>EZ+ and NPC-/H11002</td>
<td>High risk of exposure</td>
<td>Key study group</td>
<td>31</td>
<td>31</td>
<td>52</td>
<td>3.2</td>
</tr>
<tr>
<td>3</td>
<td>Outer perimeter of EZ</td>
<td>Intermediate risk of exposure</td>
<td>Dose response comparator</td>
<td>24</td>
<td>36</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>EZ-, in Capitol outside HSOB</td>
<td>Low risk of exposure</td>
<td>Dose response comparator</td>
<td>20</td>
<td>37</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Confirmed anthrax</td>
<td>Definite exposure and disease</td>
<td>Infected positive controls</td>
<td>2</td>
<td>66</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Nonexposed controls</td>
<td>No risk of exposure</td>
<td>True negative controls</td>
<td>12</td>
<td>36</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Vaccinated controls</td>
<td>NA</td>
<td>Vaccinated positive controls</td>
<td>7</td>
<td>42</td>
<td>86</td>
<td>0</td>
</tr>
</tbody>
</table>

**NOTE.** There were 124 subjects in the study (65% male), with a median age of 34 years. +, positive; −, negative; AVA, anthrax vaccine adsorbed vaccine; EZ, exposure zone; HSOB, Hart Senate Office Building; imm, immunized; NA, not applicable; NPC, nasopharyngeal culture; preimm, immunized previously.
gens (0.5 μg/mL). Serial 2-fold dilutions of plasma were assayed in triplicate. Samples were considered to be positive if the OD_{410} in replicate assays at dilutions ≥1:400 was ≥0.150, in accordance with validation studies in 20 presumed anthrax-naive control subjects.

**Cellular assays.** Heparinized whole blood (0.5 mL) was stimulated with recombinant PA, LF, or control (*Plasmodium falciparum*) proteins (10 μg/mL) or with PBS in the presence of costimulatory anti-CD28 and -CD49d antibodies (1 μg/mL; BD Biosciences) and cultured for 16 h. Brefeldin A (10 μg/mL; BD Biosciences) was added during the last 4 h. Samples were treated with 2 mmol/L EDTA and 10 mL of 1× FACS lysis solution (BD Biosciences) and were stored at −80°C pending batch processing. Samples were thawed, permeabilized with 2 mL of 1× FACS permeabilizing solution (BD Biosciences), and stained using standard methodologies (available at: http://www.bd biosciences.com) with interferon (IFN)–γ–fluorescein isothiocyanate (FITC), tumor necrosis factor (TNF)–α–phycoerythrin (PE), CD69–peridinin-chlorophyll-protein (PerCP), and CD3–allophycocyanin (APC) (panel 1); HLA-DR–FITC, interleukin (IL)–1α–PE, CD69–PerCP, and CD3–APC (panel 2); or IL-6–FITC monokine induced by IFN-γ (MIG)–PE [28], and CD14–APC (panel 3). Samples were resuspended in 0.3 mL of wash buffer and acquired using FACSCalibur and CellQuest software (version 3; BD Biosciences). Lymphocytes (panels 1 and 2) and monocytes (panel 3) were gated on the basis of forward- and side-scatter characteristics. Samples were considered to be positive if responses to LF and PA antigens were ≥0.01%, there were ≥2-fold background responses to control protein and PBS-treated samples, and there was a true difference in response proportions versus the presumed negative controls in group 4 (95% confidence intervals [CIs]) (normalized by internal control).

**Data analysis and statistics.** The subject response rate (RR) was defined as the proportion of subjects with a positive response. The test RR was defined as the ratio of positive assays to the total number of assays. The magnitude of response was defined by optical-density values for antibodies and the frequency of positive fluorescence-activated cell sorting events for CMI. The significance of differences in proportions was compared using a 2-sided Kruskal-Wallis test (EpiCalc 2000; available at: http://www.brixtonhealth.com/epicalc.html). α <.05 was considered to be significant, in comparison with data from group 4, with presumed low or no exposure. Originally, group 4 was the planned negative control, but unanticipated responses (consistent with environmental sampling data [24]) prompted the enrollment of group 6 as a true negative control.
Cell-mediated immune responses in unimmunized subjects at any time point after exposure to anthrax spores. Whole blood was collected at the time points corresponding to the 2-week time points after immunizations 1 and 3 (in immunized subjects) stimulated with recombinant protective antigen (PA), recombinant lethal factor (LF), or control protein (or PBS) and stained with (1) interferon-γ–fluorescein isothiocyanate (FITC), tumor necrosis factor–α–phycoerythrin (PE), CD69–peridinin-chlorophyll-protein (PerCP), and CD3–allophycocyanin (APC); (2) HLA-DR–FITC, interleukin (IL)–1α–PE, CD69–PerCP, and CD3–APC; or (3) IL-6–FITC, monokine induced by interferon-γ–PE, and CD14–APC and was evaluated by fluorescence-activated cell sorting. Lymphocytes and monocytes were gated on the basis of forward- and side-scatter characteristics. Samples were considered to be positive if the response to LF and PA antigens was \( >0.01\% \); if there was a \( >2\)-fold higher background response, compared with control protein– and PBS-treated samples; and if there was a true difference in response proportions vs. group 4 presumed negative controls (95% confidence interval). Data are the subject response rate (RR) of lymphocytic or monocytic (total), lymphocytic, or monocytic (mono) populations, based on the highest response at any time point.

Table 3. Cell-mediated immune (CMI) responses in all-comers after exposure, unimmunized, and immunized subjects.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases*.  

RESULTS

NPCs. A spore exposure–dose response relationship was apparent for NPC positivity: all 13 subjects in Senator Daschle’s sixth-floor office where the envelope was opened; 7 (32%) of 22 subjects in his adjoining fifth-floor office, connected by a stairway; 2 (11%) of 18 subjects in Senator Feingold’s adjoining sixth-floor office without cross-ventilation; and 0 of ∼6000 subjects in the Capitol outside the HSOB \( (P<.001) \). Six (86%) of 7 first responders had positive NPCs. However, only 47.5% of subjects within the EZ (groups 1 and 2) had positive NPCs, despite high exposure.

AVA, antibiotics, and symptoms. Of 59 subjects for whom AVA was recommended (groups 1 and 2), 51 (86%) chose immunization and 4 (7%) chose only antibiotics. All subjects in groups 1 and 2 were prescribed antibiotics for 60–120 days (adherence was unknown); the duration was <60 days for most subjects in group 3. No individuals in group 4 received \( >14 \) days of prophylaxis (table 1). There were no significant differences in symptoms in subjects with or without spore exposure and with or without immune responses (antibodies or CMI; data not shown), except for headache, which was more common in exposed subjects but was correlated with the duration of antibiotic use \( (P = .03) \) \[24\].  

Antibodies. In unimmunized subjects, there was a trend toward a higher anti-PA total immunoglobulin RR in EZ groups 1 and 2 than in group 3 and compared with the presumed unexposed groups 4 and 6 (figure 1). Anti-PA IgM and anti-LF antibodies were detected only in groups 1 and 2. Anti-PA
Figure 3. Individual cell-mediated immune marker responses in unimmunized subjects at any time point after exposure to anthrax spores. Samples were collected, processed, and analyzed as described in the legend to figure 2. Data are the subject response rate (RR) for identified markers, based on the highest response at any time point. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

IgG was detected in groups 1 and 2 and in 2 (9%) of 23 subjects in group 3. For anti-PA antibodies (but not LF), there were trends toward increasing subject RRs and magnitude over time in groups 1 and 2 (P> .05) (data not shown). Of subjects who had positive responses at 8 weeks, 11 (79%) of 14 remained positive at 14 weeks for anti-PA total immunoglobulin, 2 (67%) of 3 remained positive at 14 weeks for IgG, 3 of 3 remained positive at 14 weeks for IgM, and 0 of 1 remained positive at 14 weeks for anti-LF total immunoglobulin.

AVA induced anti-PA IgG and/or IgM antibodies in ≥94% of subjects. Anti-LF antibodies were detected in subjects in immunized groups 1, 2, and 3 but not in subjects in nonexposed, immunized group 7 (figure 1; group 3 not shown). In the 2 exposed, previously immunized individuals, anti-PA titers after 1 dose of AVA were comparable to those achieved in vaccine-naive subjects after 3–6 doses, and titers were subsequently boosted to high levels (total immunoglobulin and IgG, ≥204,800). Titers of anti-LF antibodies were higher than those in other immunized subjects.

In 2 subjects in group 5 who had had recent inhalational anthrax, anti-PA total immunoglobulin and IgG levels were comparable to those in immunized subjects (total, 204,800 and

Figure 4. Ranked cell-mediated immune responses in unimmunized subjects at any time point after exposure to anthrax spores. Samples were collected 2 weeks after immunizations 1 and 3 and were processed and analyzed as described in the legend to figure 2. Data are the ranked subject response rate (RR) of either lymphocyte or monocyte populations. Responses were categorized as high or low, relative to the group mean (P< .05).
Figure 5. Cell-mediated immune subject and test responses in anthrax vaccine adsorbed–immunized subjects at any time point after exposure to anthrax spores. Samples were collected 2 weeks after immunizations 1 and 3 and were processed and analyzed as described in the legend to figure 2. Data are the subject response rate (RR) (A) and test RR (B) of lymphocytic or monocytic (total), lymphocytic (lymph), or monocytic (mono) populations, based on the highest response at any time point. LF, lethal factor; PA, protective antigen.

25,600; IgG, 204,800 and 12,800). However, anti-LF antibody responses were higher than those in other subjects (25,600 vs. 200–3200).

**CMI response.** There was also a dose relationship between presumed spore exposure and total PA or LF CMI responses for subject and test RRs, for all subjects (immunized and unimmunized) and for unimmunized subjects analyzed separately (figure 2 and table 3). For example, overall monocytic subject RRs for groups 1, 2, 3, 4, and 6 were 73.9%, 72.0%, 37.5%, 30.0%, and 9.1% (P < .001), and the respective test RRs were 40.1%, 31.5%, 18.2%, 9.2%, and 1.5% (P < .001). Among groups 1 and 2, differences were not significant for either subject or test RRs (P > .05). Unexpectedly, monocytic responses were detected in a higher proportion of subjects in groups 3 and 4 than in nonexposed control subjects in group 6 (subject RR, P = .22; test RR, P = .001). The group 3 test RR was higher than that in group 4, but there was no difference in subject RR (subject RR, P = .6; test RR, P = .04). Total and monocytic (but not lymphocytic) CMI subject RRs increased over time (significantly in groups 1, 2, and 4; trends in group 3) (data not shown). The number of subjects with positive responses at 8 weeks who remained positive at 14 weeks was 2 (40%) of 5 for total PA/LF, 0 of 4 for lymphocytic, and 0 of 1 for monocytic responses. Data showed that antibody responses (at least anti-PA responses) may have been sustained over time, whereas CMI responses were not.
Dose-exposure relationships were particularly evident for certain markers. CD14/IL-6, CD3/CD69, and CD14/MIG appeared to best predict spore exposure ($P<.05$); no associations were seen for CD3/IFN-γ, CD3/TNF-α, or CD14/TNF-α (figure 3).

Although generally low, magnitudes of responses were higher in groups 1–3 subjects than in control subjects in group 6 (table 3). Ranking of subject RRs in unimmunized subjects revealed a dose-exposure response, with high responses only in groups 1, 2, and 3 (figure 4 and table 3).

A trend toward a relationship between spore exposure dose and CMI response was also seen in immunized subjects. For example, in groups 1, 2, and 7, LF monocytic subject RRs were 57.1%, 45.5%, and 0% ($P = .07$) (figure 5A); test RRs were 24.2%, 16.9%, and 0% ($P = .09$) (figure 5B); and respective magnitudes of response were 0.38%, 0.31%, and 0.03% ($P < .001$) (table 3). PA/LF monocytic test RRs were 43.2%, 33.6%, and 11.4% ($P = .004$), and magnitudes of responses were 1.51%, 0.67%, and 0.07% ($P < .001$).

Overall, in groups 1 and 2, PA/LF total, lymphocytic, and monocytic subject RRs were 84.4%, 51.1%, and 77.3%, respectively. There were no significant differences in any or high CMI subject RRs among subjects when results were stratified by antibody response (none, low, intermediate, or high) (data not shown).

An inverse relationship between antibiotic use and CMI (but not antibodies) was demonstrated, particularly for monocytic responses ($P = .02$) (table 4). CMI responses were considerably higher in preimmunized subjects ($n = 2$) and in those who had recovered from inhalational anthrax ($n = 1$) than in those without prior exposure. Although high PA-specific responses were noted in both groups, high LF-specific responses were detected only in subjects who had recovered from inhalational anthrax (group 5) (figure 6).

**DISCUSSION**

The present study is the first comprehensive longitudinal study of the epidemiological and immunological characteristics of *B. anthracis* exposure in humans, in a bioterrorism-related outbreak, and with postexposure prophylaxis with antibiotics and AVA. In particular, to the best of our knowledge, the delineation of CMI responses to *B. anthracis* has not been previously reported.

Confirming our hypothesis, the data demonstrate that spore exposure primed immune responses in an apparently dose-dependent manner. Responses were detected in exposed subjects with positive and negative NPCs and in individuals from the EZ perimeter. That we did not detect differences in immune response profiles in groups 1 (positive NPC) and 2 (negative NPC) suggests similar spore exposure within the EZ. Unexpectedly, immune responses were detected in subjects from outside the HSOB EZ but within the Capitol complex (presumed insignificant or no spore exposure). Possible explanations include (1) Capitol contamination, which is consistent with published environmental sampling data [24] and is supported by our data; (2) cross-contamination, in that individuals may have been exposed via contact with subjects in groups 1 and 2 who left the HSOB without decontamination; and (3) prior exposure, in that some individuals were exposed to the spore-laden envelope during the 3 days before it was opened.

Our finding of immunological priming was consistent with subclinical and possibly antibiotic-aborted infection after the germination of viable spores (a similar conclusion has been

### Table 4. Effect of postexposure antibiotic prophylaxis on cell-mediated immune responses in unimmunized subjects

<table>
<thead>
<tr>
<th>Group, antibiotic treatment</th>
<th>Subjects, no.</th>
<th>Test response rate, response/total (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Lymphocytic</td>
<td>Monocytic</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Received</td>
<td>19</td>
<td>21/261 (8)</td>
<td>.09</td>
<td>4/152 (3)</td>
<td>.52</td>
</tr>
<tr>
<td>Not received</td>
<td>3</td>
<td>7/42 (17)</td>
<td>1/24 (4)</td>
<td>6/18 (33)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Received</td>
<td>9</td>
<td>7/238 (3)</td>
<td>.07</td>
<td>1/136 (1)</td>
<td>.28</td>
</tr>
<tr>
<td>Not received</td>
<td>11</td>
<td>4/42 (10)</td>
<td>1/24 (4)</td>
<td>3/18 (17)</td>
<td></td>
</tr>
<tr>
<td>3 and 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Received</td>
<td>28</td>
<td>28/499 (6)</td>
<td>.02</td>
<td>5/288 (2)</td>
<td>.06</td>
</tr>
<tr>
<td>Not received</td>
<td>14</td>
<td>11/84 (13)</td>
<td>2/48 (4)</td>
<td>9/36 (25)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Whole blood was collected from unimmunized (groups 3 and 4) subjects at time points corresponding to the 2-week postimmunization 1 and 3 time points (in immunized subjects). Samples were processed and analyzed as described in the legend to figure 2 and were stratified according to antibiotic usage. Data are the test response rate of lymphocytic or monocytic (total), lymphocytic, or monocytic populations.
In general agreement with results obtained by US Centers for Disease Control investigators [24, 25], anti-PA antibodies in unimmunized subjects were low to negligible 8 weeks after exposure. Over time, low titers of anti-PA IgG and IgM and anti-LF antibodies were detected in a minority of exposed volunteers but in no one who had been outside the EZ. Higher titers were generally seen only in groups 1 and 2. These data suggest possible seroconversion and an exposure-dose trend.

Consistent with our hypothesis regarding HIV, PA/LF-specific CMI responses were detected in the absence of detectable antibodies, with apparent dose-exposure relationships and increased responses over time. As a predictor of high exposure, the overall sensitivity and negative predictive value of CMI assays were 67% and 82% (vs. 38% and 44%, respectively, for anti-PA antibodies). High-magnitude responses occurred only in subjects with the most exposure. Monocytic responses were detected in all groups, but lymphocytic responses were detected predominantly in highly exposed subjects, which suggests that monocytic responses were more sensitive markers of exposure. Of the specific CMI markers tested, IL-1α, IL-6, MIG [28], and CD69 were most sensitive. In general, monocytic responses are associated with antigen presentation and immunological priming, whereas lymphocytic responses are associated with antigen-specific effector-cell function, although there is overlap and redundancy. For example, major histocompatibility complex-restricted and antigen-specific monocytic responses do occur [28], and TNF-α can be secreted by both monocytic and lymphocytic cells. Our data demonstrate that anthrax spores primed CMI responses (especially monocytic) in a dose-dependent manner analogous to and complementing antibody response trends.

That postexposure antimicrobial prophylaxis decreased CMI responses suggests that B. anthracis spore germination was effectively impeded. This is consistent with studies in monkeys in which animals aerosol challenged with B. anthracis spores and then administered antibiotics within 24 h did not develop clinical disease [19]. That none of our subjects developed clinical disease after the cessation of antibiotics suggests that insufficient numbers of spores survived to induce clinical disease, despite the known latency of B. anthracis spores [17, 29, 30] and documented small spore particle size (which has a higher propensity to cause lower-respiratory-tract infection) in this bioterrorism event [31–33]. This is analogous to our observation that low-level exposure induced immunological responses in the absence of clinical disease. Moreover, because of the apparent confounding effect of antibiotics, together with a relatively prolonged incubation period [30], the CMI responses that we report may be an underestimation of the sensitivity of CMI assays to predict B. anthracis spore exposure. In any event, the data suggest that postexposure prophylaxis with antibiotics can be expected to decrease the incidence of subclinical and clinical infection caused by B. anthracis.

Postexposure AVA administered on an abbreviated 3-dose schedule was highly immunogenic, given that anti-PA IgG antibodies were detected in ≥94% and CMI responses in >86% of vaccinees. In other studies, 99%–100% seroconversion was seen with a preexposure prophylactic 3-dose schedule of AVA [34, 35]. Possible contributing factors to our lower seroconversion rate may include “real world” postexposure prophylaxis in the field during a bioterrorism exposure rather than in controlled clinical trials [34, 35], stress-induced immunosuppression [36], potential immunosuppression due to subclinical infection [37], and more-conservative criteria for antibody positivity (1:400 vs. 1:100) [35]. In previously immunized volunteers, anti-PA and anti-LF antibody titers and PA CMI responses were high even after a single vaccination, which suggests that a single boost may protect nonnaive immunized individuals in the face of an unanticipated exposure. That LF monocytic responses were detected only in postexposure vaccinees and not in previously immunized subjects without spore exposure adds to the evidence that responses against LF may be important mediators of protective immunity [8]. Overall, we demonstrated boosting of primary and recall immune responses in subjects immunized after exposure, although the relative contribution of spore exposure versus an acute immunization schedule cannot be determined. That priming was seen in unimmunized subjects suggests that spore exposure contributed, at least in part, to the boosting of AVA immune responses. This is consistent with data in other pathogen models demonstrating that responses to experimental immunization are boosted by prior pathogen exposure [38, 39].

In subjects with recent inhalational anthrax, anti-PA antibody and CMI responses were similar to those in subjects after AVA and in previously immunized subjects, respectively. Interestingly, these were the only subjects in whom high anti-LF antibody and CMI responses were detected. These data suggest...
that clinical infection results in brisk PA antibody and CMI responses and high LF responses. This is not surprising in light of the potential involvement of LF in the protective response against *B. anthracis* toxin challenge [8] and the low concentration of LF in AVA.

Our study was limited by restrictions of a real-world bioterrorism “outbreak”: baseline samples and measurements were unavailable, and key group stratification and sample sizes were predetermined (which possibly resulted in type II errors). In particular, despite active recruitment effort, only 2 subjects in group 5 elected to participate. Moreover, the EZ was defined on the basis of proximity to the opening of the spore-laden envelope and NPC status rather than true environmental spore sampling, which potentially limited conclusions about the dose-exposure response.

In summary, our data demonstrate that exposure to *B. anthracis* spores primed antibody and cellular responses in a dose-dependent and antigen-specific manner in immunized and unimmunized subjects, enhanced AVA-boost responses, and boosted recall responses in previously immunized subjects. Moreover, even in unimmunized subjects, antibody and cellular responses increased over time. Importantly, an abbreviated postexposure immunization schedule appeared to be highly immunogenic. That immune responses were detected inside, within the perimeter, and outside the defined EZ demonstrates that spore exposure was more extensive than predicted. Our data suggest a pathogen exposure–host response spectrum (at least with postexposure antibiotic prophylaxis) in which low-level exposure induces asymptomatic CMI in the absence of antibodies and intermediate exposure induces CMI and antibodies. It appears that, in young, healthy individuals, despite the induction of immune responses, infection remains subclinical because of the suppressing and/or aborting of spore germination by antibodies. We do not know whether higher exposure could result in clinical disease even with antibiotics or what happens in immunocompromised or immunologically immature populations (e.g., elderly persons, patients with chronic disease, and children) [40]. From a practical and clinical point of view, that no individuals in the Capitol developed clinical anthrax confirms the accuracy of the epidemiologically defined EZ. However, NPC status was a poor predictor of being in the EZ. Our study puts into perspective, validates, and links environmental sampling and clinical outcome. We conclude that the threat of exposure may extend beyond epidemiologically defined EZs, that NPCs distinguish large differences but immune assays detect subtle differences in spore exposure, that the significance of low-level spore exposure should not be underestimated, and that prompt postexposure prophylaxis with antibiotics and abbreviated AVA immunization appears to be highly effective.

**Acknowledgments**

We thank the volunteers, without whom the study would not have been possible; and Dr. Sybil Tasker, Stephanie Gray, Nora Philbin, HM1 Verdelle Nelson, and Ariel Freilich.

**References**


