The early humoral immune response to *Bacillus anthracis* toxins in patients infected with cutaneous anthrax

Karen E. Brenneman¹,², Mehmet Doganay³, Arya Akmal¹, Stanley Goldman¹, Darrell R. Galloway¹,², Alfred J. Mateczun¹, Alan S. Cross⁴ & Leslie W. Baillie⁵

¹Biological Defense Research Directorate, Naval Medical Research Center, Rockville, MD, USA; ²Department of Microbiology, The Ohio State University, Columbus, OH, USA; ³Department of Infectious Diseases, Faculty of Medicine, Erciyes University, Kayseri, Turkey; ⁴Department of Medicine, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD, USA; and ⁵Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, MD, USA

Correspondence: Alan S. Cross, Department of Medicine, Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore Street, Baltimore, MD 21201, USA; Tel.: +1 410 706 5328; fax: +1 410 706 6205; e-mail: across@medicine.umaryland.edu

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Keywords

anthrax; lethal factor; edema factor; protective antigen.

Abstract

*Bacillus anthracis*, the causative agent of anthrax, produces a tripartite toxin composed of two enzymatically active subunits, lethal factor (LF) and edema factor (EF), which, when associated with a cell-binding component, protective antigen (PA), form lethal toxin and edema toxin, respectively. In this preliminary study, we characterized the toxin-specific antibody responses observed in 17 individuals infected with cutaneous anthrax. The majority of the toxin-specific antibody responses observed following infection were directed against LF, with immunoglobulin G (IgG) detected as early as 4 days after the onset of symptoms in contrast to the later and lower EF- and PA-specific IgG responses. Unlike the case with infection, the predominant toxin-specific antibody response of those immunized with the US anthrax vaccine absorbed and UK anthrax vaccine precipitated licensed anthrax vaccines was directed against PA. We observed that the LF-specific human antibodies were, like anti-PA antibodies, able to neutralize toxin activity, suggesting the possibility that they may contribute to protection. We conclude that an antibody response to LF might be a more sensitive diagnostic marker of anthrax than to PA. The ability of human LF-specific antibodies to neutralize toxin activity supports the possible inclusion of LF in future anthrax vaccines.

Introduction

Anthrax is a zoonotic disease caused by *Bacillus anthracis*, a Gram-positive spore-forming microorganism whose manifestations in humans depend on the route of infection. The cutaneous form of the disease accounts for more than 95% of reported cases (Shafazand et al., 1999) and, with treatment, does not usually pose a threat to human life (Little & Ivins, 1999). The gastrointestinal and inhalational forms of the disease, although not as common, are much more severe (Little & Ivins, 1999). The ability of the organism to form environmentally resistant spores, be dispersed as aerosols and cause lethal infection following inhalation has resulted in its development and use as a biological weapon.

Following infection, spores are phagocytosed by macrophages and transported to the draining lymph nodes, where they germinate into vegetative bacilli and escape from the macrophage (Lincoln et al., 1965; Dixon et al., 2000; Guidi-Rontani et al., 2001). In cutaneous anthrax, this results in a localized infection; in inhalational anthrax, the bacilli multiply in the lymphatic system and spread to the blood, resulting in massive bacteremia and toxemia (Fish & Lincoln, 1968).

Within 3 h of spore germination, the expression of the toxin proteins begins (Guidi-Rontani et al., 1999). The extracellular tripartite toxin of anthrax is composed of two enzymatically active subunits, lethal factor (LF) and edema factor (EF), and a cell-binding and translocation component, protective antigen (PA). Both lethal (PA+LF) and edema (PA+EF) toxins are able to suppress key parts of the innate immune response to the developing infection (O’Brien et al., 1985; Wright & Mandell, 1986; Duesbery...
Bacillus anthracis, the causative agent of anthrax, produces a tripartite toxin composed of two enzymatically active subunits, lethal factor (LF) and edema factor (EF), which, when associated with a cell-binding component, protective antigen (PA), form lethal toxin and edema toxin, respectively. In this preliminary study, we characterized the toxin-specific antibody responses observed in 17 individuals infected with cutaneous anthrax. The majority of the toxin-specific antibody responses observed following infection were directed against LF, with immunoglobulin G (IgG) detected as early as 4 days after the onset of symptoms in contrast to the later and lower EF- and FA-specific IgG responses. Unlike the case with infection, the predominant toxin-specific antibody response of those immunized with the US anthrax vaccine absorbed and UK anthrax vaccine precipitated licensed anthrax vaccines was directed against PA. We observed that the LF-specific human antibodies were, like anti-PA antibodies, able to neutralize toxin activity, suggesting the possibility that they may contribute to protection. We conclude that an antibody response to LF might be a more sensitive diagnostic marker of anthrax than to PA. The ability of human LF-specific antibodies to neutralize toxin activity supports the possible inclusion of LF in future anthrax vaccines.
et al., 1998; Pellizzari et al., 1999; Erwin et al., 2001; Kalns et al., 2002; Popow et al., 2002; Moayeri et al., 2003). Later in the disease process, high levels of lethal toxin (LT) induce the cytokine independent shock-like death associated with anthrax (Moayeri et al., 2003).

Animal studies suggest that as the concentration of toxin increases the likelihood of successfully treating an infected individual decreases until it reaches a level at which antibiotics are no longer effective (Albrecht et al., 2007; Baillie, 2009). Given that the early detection of toxin is a key diagnostic marker, it surprising how little is known of the time course of toxin production in humans and thus we sought to characterize the early immune responses to individuals infected with anthrax (Baillie, 2009).

To achieve this aim, clinical serum samples previously obtained at various time points postinfection from individuals who had contracted cutaneous anthrax were examined for the presence of toxin-specific immunoglobulin M (IgM) and IgG antibodies. In addition, we compared these antibody responses with those seen following immunization with the US anthrax vaccine absorbed (AVA) and UK anthrax vaccine precipitated (AVP) licensed human anthrax vaccines. Finally, the protective function of the toxin-specific antibody responses stimulated following infection were assessed using an assay that measures toxin neutralization, a recently demonstrated correlate of protection (Reuveny et al., 2001; Little et al., 2004).

Materials and methods

Expression and purification of toxin components

The PA, LF and EF genes were cloned into the Escherichia coli expression vector pQE-30 (Qiagen) and confirmed by sequencing (Read et al., 2003). Proteins were expressed from either the M15 (PA) or the SG13009 (LF and EF) strain of E. coli. Host strains were grown in Luria–Bertani medium to an OD_{600nm} of 0.55–0.65 and induced with 1 mM isopropyl-β-D-thiogalactoside either 4 h at 37 °C (PA) or 20 h at 25 °C (LF and EF). Cells were pelleted and lysed by French press at 16000 p.s.i.; lysates were cleared at 45000 g for 15 min. Recombinant proteins were purified by cobalt affinity chromatography. Cleared lysate was batch bound to TALON resin (Clontech) and then washed with 10 CV 300 mM NaCl, 50 mM Na_2HPO_4 and 20 mM imidazole, pH 7.0. Proteins were eluted in 5 CV 300 mM NaCl, 50 mM Na_2HPO_4 and 150 mM imidazole, pH 7.0. Fractions containing protein [determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] were pooled and dialyzed into 10 mM HEPEs and 50 mM NaCl, pH 7.5. Proteins purified using this procedure were approximately 90% pure as assessed by SDS-PAGE with Coomassie staining.

Serum samples

Serum samples were obtained from volunteers who had received at least a priming series of the AVA (six Maryland-based volunteers) or AVP (four UK-based volunteers visiting Maryland) vaccines. Control samples were obtained from six nonimmunized, noninfected Maryland-based individuals. All samples were obtained under a protocol approved by the University of Maryland and the Naval Medical Research Center’s Institutional Review Boards, as well as by the Ethics Committee at Erciyes University. Informed consent was obtained from all individuals. Clinical samples were obtained from 17 cutaneous anthrax patients attending the infectious diseases clinic at Erciyes University in Turkey (Table 1). Serum samples were not collected prospectively from patients under a set protocol, but were instead collected when patients presented to the outpatient clinics for up to 21 days after the initial visit. Anthrax was diagnosed by exposure history, clinical presentation consistent with anthrax, Gram stain and positive culture from the lesion.

Antitoxin IgG and IgM enzyme-linked immunosorbent assay (ELISA)

Antitoxin IgG and IgM levels were measured using an ELISA as described previously, with minor variations (Hepburn et al., 2007). Data values were compared with a standard curve of purified human IgG or IgM (Sigma). Data in the linear portion of the ELISA graph and within the range of the standard curve were used to calculate the quantitative titer (μg mL^{-1}) for the serum sample. For each antigen, four to six naïve serum samples were assayed and their titer were averaged (geometric mean) and the 95% confidence interval of the distribution was calculated. Experimental data were scored as a positive result only if the calculated titer exceeded the upper limit of the confidence interval of the naïve control samples.

LT neutralization assay

The toxin neutralization assay was performed on the mouse monocytic cell line J774A.1 (ATCC) as described previously, with cell viability determined by the addition of DMEM containing sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (Roche) for 16 h. The assay was read at 480 nm. The dilution series data (A_{480nm} vs. toxin concentration) were modeled with four-parameter logistic curves of the form:

\[ OD_{480nm}(y) = \beta_1 + (\beta_2 - \beta_1)/[1 + \exp(\beta_3(\beta_4 - x))] \]

The data were fit using MATLAB software via a nonlinear least-squares analysis, yielding the parameters (\beta_1, \beta_2, \beta_3, \beta_4).
### Table 1. Details for the patients with cutaneous anthrax

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Incubation period (days)</th>
<th>Contact with contaminated animal materials</th>
<th>Site of the lesion</th>
<th>Gram stain for B. anthracis</th>
<th>Positive culture for B. anthracis</th>
<th>Previous antibiotic use</th>
<th>Antibiotic initiation day of disease</th>
<th>Given antibiotic</th>
<th>Duration of therapy (days)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>+</td>
<td>Right eye lids</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Third</td>
<td>Pen G</td>
<td>15</td>
<td>Left deep tissue scar, recovered</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>+</td>
<td>Right arm</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Fourth</td>
<td>Pen G + Cipro</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>+</td>
<td>Right arm</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Second</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>+</td>
<td>Right hand</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Fourth</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>+</td>
<td>Left hand</td>
<td>–</td>
<td>–</td>
<td>No</td>
<td>Sixth</td>
<td>Pen G</td>
<td>7</td>
<td>Recovered</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>+</td>
<td>Left forearm</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Fifth</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>+</td>
<td>Right hand fingers</td>
<td>+</td>
<td>+</td>
<td>No</td>
<td>Fifth</td>
<td>Pen G</td>
<td>7</td>
<td>Recovered</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>+</td>
<td>Left hand finger</td>
<td>+</td>
<td>–</td>
<td>Yes</td>
<td>Fourth</td>
<td>Pen G</td>
<td>15</td>
<td>Recovered</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>+</td>
<td>Both hands</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Third</td>
<td>Pen G</td>
<td>14</td>
<td>Recovered</td>
</tr>
<tr>
<td>10*</td>
<td>2</td>
<td>+</td>
<td>Anterior neck</td>
<td>+</td>
<td>+</td>
<td>No</td>
<td>Second</td>
<td>Pen G</td>
<td>14</td>
<td>Left deep tissue scar, recovered</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>+</td>
<td>The eye lid</td>
<td>+</td>
<td>+</td>
<td>No</td>
<td>Third</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>+</td>
<td>Left eye lids</td>
<td>+</td>
<td>–</td>
<td>Yes</td>
<td>Third</td>
<td>Pen G</td>
<td>14</td>
<td>Recovered</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>+</td>
<td>Right wrist</td>
<td>+</td>
<td>–</td>
<td>Yes</td>
<td>Fifth</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>14</td>
<td>?</td>
<td>+</td>
<td>Left elbow</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Tenth</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>+</td>
<td>Right face</td>
<td>+</td>
<td>–</td>
<td>Yes</td>
<td>Fifth</td>
<td>Pen G</td>
<td>10</td>
<td>Recovered</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>+</td>
<td>Anterior neck</td>
<td>+</td>
<td>–</td>
<td>No</td>
<td>Seventh</td>
<td>Pen G</td>
<td>7</td>
<td>Recovered</td>
</tr>
<tr>
<td>17</td>
<td>7</td>
<td>+</td>
<td>Right arm and left wrist</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
<td>Fourth</td>
<td>Pen G</td>
<td>10</td>
<td>Left deep tissue scar, recovered</td>
</tr>
</tbody>
</table>

*The patient gave a history of contact with an ill animal or contaminated animal materials (such as slaughtering of ill animals, skinning, chopping meat, carrying raw skin or splashing blood from dying animal during slaughtering). One case gave a history of fly bite.

*The swabs for Gram stain and culture were taken from the vesicles of fluid or under the crust if developed.

*Time from first symptoms to the diagnosis and initiation of antibiotic treatment.

*In this case, anthrax sepsis was developed from the cutaneous lesion.

Pen G, penicillin G; Cipro, ciprofloxacin.
β₄) of the best fit. The inflection point of the fit (β₄ parameter) corresponds to the serum dilution that ensures the survival of 50% of the cells in the assay (ED₅₀) (Quinn et al., 2004).

**Results**

**Clinical characteristics**

Cutaneous anthrax in the patients was diagnosed by the history of contact with ill animals and animal products, typical cutaneous lesions and demonstration of Gram-positive bacilli (eight cases) and/or -positive cultures (three cases) from the lesion (Table 1). The upper body was the usual site of the cutaneous anthrax lesion. The finding that cultures were positive only in three cases was presumed to be due to the fact that many patients had taken an antibiotic before their initial visit. With cultures of both the lesion and the blood being positive, one patient was diagnosed with anthrax sepsis originating from the cutaneous lesion. The incubation period varied between 1 and 15 days. Seventeen patients gave a history of contact with an ill animal or contaminated materials of animals that died. The time from first symptom to the initiation of antibiotics ranged from 2 to 10 days, with 16 subjects receiving antibiotics, predominantly penicillin, within the first 7 days, while 10 patients received antibiotics within 4 days. Typically, the patients were treated for 7–15 days, and all recovered. Three subjects (patients 1, 10 and 17), however, were left with deep tissue scars.

**Antitoxin IgM responses**

In order to examine the early immune response to infection, the serum IgM levels against PA, LF and EF were measured and compared with the anthitoxin IgM titer of six naïve volunteers. Of the 17 infected individuals, six (35%) had an IgM response to at least one of the toxin components that was higher than that of the naïve controls (Table 2). There was no statistical difference in the level of toxin-specific IgM between the remaining 11 patients and the naïve controls. An anti-LF-specific IgM was detected in five individuals within the first and second weeks following symptoms, with the earliest response detected at day 4. In contrast, an anti-PA IgM was detected in four individuals, but not until the second and third weeks following symptoms, with the earliest titers appearing at day 12. Finally, anti-EF titers were detected in only two patients with titers occurring in the second week after symptoms.

**Antitoxin IgG responses**

The serum IgG titers against the anthrax toxin proteins in 17 infected individuals were measured and compared with the geometric mean IgG titer (GMT) of six naïve volunteers. In contrast to the IgM responses, 11 of the 17 patients (65%) demonstrated a measurable IgG titer to at least one of the toxin components on the days assayed (Table 3). While each positive patient generated an anti-LF IgG response with titers ranging from 13.6 to 817 µg mL⁻¹, only four individuals showed an anti-EF IgG response (patients 5, 6, 8 and 16) and only three patients (patients 2, 6 and 7) had IgG specific for PA. The anti-LF IgG response in all infected individuals exceeded that directed against PA and EF. During the second week of infection, the GMT of all responders to LF (69.3 µg mL⁻¹) was almost twice the anti-EF titer (37.4 µg mL⁻¹) and three times the anti-PA titer (22.6 µg mL⁻¹).

LF-specific IgG responses were detected as early as 4 days after onset of symptoms while anti-EF IgG responses were not seen until day 6 and anti-PA IgG responses first being detected at day 13. These preliminary results would appear to indicate that of the three toxins components, the antibody response to LF represents the most appropriate early diagnostic indicator.

To further characterize the protective nature of the antibody response induced by infection, we compared the spectrum of the resulting toxin-specific IgG responses to those seen following immunization of healthy volunteers with the US AVA and the UK AVP licensed human anthrax vaccines (Pittman et al., 2002; Baillie et al., 2003, 2004). As can be seen from Fig. 1a and b, all of the vaccinees mounted a PA-specific IgG response, the level of which varied depending on the vaccine used and the immunization schedule of each individual. In contrast to the responses observed following infection, little if any LF-specific IgG was

**Table 2. Comparison of serum antitoxin IgM levels in cutaneous anthrax patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days after onset of symptoms</th>
<th>Anti-PA IgM</th>
<th>Anti-LF IgM</th>
<th>Anti-EF IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>7.1 (1.6) 8.9 (0.2) 2.7 (0.1)</td>
<td>&lt; NB &lt; NB 10.2 (1.0) 4.7 (2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.1 (2.0) 8.3 (0.1) 3.4 (0.3)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9.3 (2.0) 6.6 (0.8) 3.4 (0.3)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.2 (2.0) 11.3 (1.1) 7.1 (1.6)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.2 (2.0) 8.9 (0.2) 2.7 (0.1)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10*</td>
<td>2.2 (2.0) 8.3 (0.1) 3.4 (0.3)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2.2 (2.0) 8.9 (0.2) 2.7 (0.1)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2.2 (2.0) 8.9 (0.2) 2.7 (0.1)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>2.2 (2.0) 8.9 (0.2) 2.7 (0.1)</td>
<td>&lt; NB &lt; NB &lt; NB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are in µg mL⁻¹ and represent the mean titer (± SD) as determined by ELISA.

*Patient developed sepsicaemia from cutaneous lesion.

< NB, less than the naïve baseline (titer indistinguishable from naïve controls).
Table 3. Serum antitoxin IgG levels in cutaneous anthrax patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days after onset of symptoms</th>
<th>Anti-PA IgG</th>
<th>Anti-LF IgG</th>
<th>Anti-EF IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td></td>
<td>11.6 (5.4)</td>
<td>5.6 (4.1)</td>
<td>6.5 (6.0)</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>&lt; NB</td>
<td>&lt; NB</td>
<td>&lt; NB</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&lt; NB</td>
<td>147.4 (19.0)</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>&lt; NB</td>
<td>&lt; NB</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>&lt; NB</td>
<td>13.6 (3.2)</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>&lt; NB</td>
<td>15.7 (2.1)</td>
<td>14.7 (1.5)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>214 (6.4)</td>
<td>117.3 (26.8)</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>&lt; NB</td>
<td>141.0 (19.3)</td>
<td>27.1 (2.7)</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>26.1 (2.2)</td>
<td>430.8 (91.0)</td>
<td>24.3 (1.6)</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>&lt; NB</td>
<td>&lt; NB</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>&lt; NB</td>
<td>35.6 (13.6)</td>
<td>60.5 (7.9)</td>
</tr>
<tr>
<td>10*</td>
<td>7</td>
<td>&lt; NB</td>
<td>28.9 (9.6)</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>16</td>
<td>14</td>
<td>&lt; NB</td>
<td>31.0 (8.2)</td>
<td>&lt; NB</td>
</tr>
<tr>
<td>21</td>
<td>&lt; NB</td>
<td>214.3 (24.4)</td>
<td>&lt; NB</td>
<td></td>
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<tr>
<td>17</td>
<td>&lt; NB</td>
<td>817.6 (202.8)</td>
<td>26.2 (7.6)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>&lt; NB</td>
<td>60.2 (10.9)</td>
<td>&lt; NB</td>
<td></td>
</tr>
</tbody>
</table>

Data are in μg ml⁻¹ and represent the mean titer (± SD) as determined by ELISA.  
*Patient developed septicemia from cutaneous lesion.  
< NB, less than the naive baseline (titer indistinguishable from naive controls).

detected and then only in recipients of the UK vaccine. While we were unable to draw any conclusions as to the relative efficacy of each vaccine due to the small sample size, we could conclude that PA comprised the major immunogen within the vaccine, particularly those immunized with the AVA vaccine (Fig. 1a).

Neutralization of LT

The ability of the AVA and AVP vaccines to stimulate a protective immune response has been demonstrated across a number of animal studies and is thought to be due to the production of PA-specific antibodies capable of neutralizing anthrax toxin activity (Baillie, 2009). Indeed, toxin-neutralizing PA-specific IgG antibodies have been identified as a correlate of protection (Reuveny et al., 2001; Little et al., 2004).

To determine whether serum from infected individuals also contained toxin-neutralizing antibodies, we assayed the activity of samples collected from 10 infected individuals at the time point that had the highest antitoxin IgG titer (Fig. 2). Neutralizing activity was detected in all samples, with ED₅₀ values ranging from 103.8 (patient #8) to 7983 (patient #5). The presence of LF-specific antibodies suggested a possible correlation between LF antibody titer and toxin neutralization. However, linear regression analysis of the ED₅₀ values vs. anti-LF IgG produced only a weakly positive correlation (R² = 0.323) possibly due to the small number of samples included in the analysis (Fig. 3). No correlate was observed between the ED₅₀ values and anti-PA IgG, anti-PA IgM or anti-LF IgM titers (data not shown).

Discussion

Anthrax is primarily a disease of animals that occasionally infects humans and as a consequence opportunities to study the pathology of the disease in humans are rare (Baillie, 2009). The early antibody responses of a small number of individuals suffering from cutaneous anthrax were analyzed for their ability to recognize the individual components of the tripartite toxin produced by B. anthracis. In the context of infection, one would expect the initial antibody response to comprise IgM class antibodies and while this was true for a small number of individuals, the majority showed a mixed...
IgM/IgG toxin-specific antibody response at the first sampling point. In general, the IgM responses were considerably lower than the corresponding IgG levels for all patients, a pattern similar to that observed in AVA-immunized primates (Ivins et al., 1998). Together, these data imply that class switching from IgM to IgG occurs early in the anthrax infection.

We observed differences in the ability of infected individuals to recognize toxin components. While 11 (65%) patients produced an LF-specific IgG response, only four (24%) mounted an IgG response to EF while only three (18%) demonstrated an IgG-specific immune response to PA. In those patients who showed the presence of anti-LF and anti-PA IgG antibodies (patients 2, 6 and 7), the LF titers exceeded the PA response by a factor of at least 3. Because both proteins are produced early in infection (Guidi-Rontani et al., 1999), this disparity in response may reflect fundamental differences in how each protein is processed by the immune system. For example, LF is known to elicit higher IgG antibody titers than PA when administered to experimental animals (Price et al., 2001; Hermanson et al., 2004; Flick-Smith et al., 2005).

Indeed, the overall disparity between anti-PA and anti-LF IgG responses observed in this study is likely to be the result of a combination of factors such as the virulence of the infecting strain, the route of infection and the health and genetic background of the individual. The initiation of antimicrobial therapy is also likely to have affected the level of toxin expression and may explain the absence of detectable response to any anthrax toxin component in six infected individuals (Stepanov et al., 1996; Athamna et al., 2004).

In an anthrax outbreak, it is crucial to identify infected individuals as quickly as possible to enable the initiation of timely treatment. The central role of the toxins in the progression of the infection has led to the proposal to use the antibody response to PA as a diagnostic marker of exposure to anthrax (Quinn et al., 2002, 2004). This recommendation was based on the analysis of the PA-specific antibody response of patients with bioterrorism-related infections (Quinn et al., 2004). While individuals with inhalation anthrax demonstrated an IgG response to PA as early as 11 days following the onset of symptoms, those with the cutaneous infections did not develop anti-PA titers until 21–34 days after the onset of symptoms and those titers did not peak until days 30–60 (Quinn et al., 2004), confirming our findings that the anti-PA immune response develops slowly. Unlike our study, Quinn and colleagues did not screen their samples for the presence of an anti-LF immune response. If they had, they may have also found that the LF-specific antibody response preceded that of the PA response. We found that the LF-specific IgG was detected as early as day 4 after the onset of symptoms, and that all patients had developed anti-LF titers by day 15. In contrast, PA-specific IgG was first detected 13 days after the onset of symptoms, and had appeared in only three patients by day 21. The early production and prevalence of anti-LF antibodies after the development of symptoms suggest that the overall kinetics of the immune response is a rapid anti-LF response, followed by a slower, longer anti-PA response. The speed, strength and prevalence of the anti-LF response in infected patients clearly demonstrate that the presence of an anti-LF response might be a better diagnostic marker of infection than an anti-PA response.

We also sought to compare the response following immunization with the US AVA and UK AVP licensed anthrax vaccines. While both are subunit vaccines, they...
Concerns over the ability to circumvent the current licensed vaccines by altering the antigenic structure of PA have spurred researchers to focus on additional vaccine targets (Schneerson et al., 2003; Hoffmaster et al., 2004). The immunogenicity of LF and its ability to stimulate the production of toxin-neutralizing antibodies makes a biologically inactive form of LF a strong candidate for inclusion in any future anthrax vaccine. We conclude from this preliminary study that an antibody response to LF might be a more sensitive diagnostic marker of anthrax than one to PA. In addition, the ability of human LF-specific antibodies to neutralize toxin activity supports the possible inclusion of LF in future anthrax vaccines.

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Authors’ contribution
K.E.B. and M.D. contributed equally to this work.

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