Lethal Factor Toxemia and Anti-Protective Antigen Antibody Activity in Naturally Acquired Cutaneous Anthrax

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Cutaneous anthrax outbreaks occurred in Bangladesh from August to October 2009. As part of the epidemiological response and to confirm anthrax diagnoses, serum samples were collected from suspected case patients with observed cutaneous lesions. Anthrax lethal factor (LF), anti-protective antigen (anti-PA) immunoglobulin G (IgG), and anthrax lethal toxin neutralization activity (TNA) levels were determined in acute and convalescent serum of 26 case patients with suspected cutaneous anthrax from the first and largest of these outbreaks. LF (0.005–1.264 ng/mL) was detected in acute serum from 18 of 26 individuals. Anti-PA IgG and TNA were detected in sera from the same 18 individuals and ranged from 10.0 to 679.5 μg/mL and 27 to 593 units, respectively. Seroconversion to serum anti-PA and TNA was found only in case patients with measurable toxemia. This is the first report of quantitative analysis of serum LF in cutaneous anthrax and the first to associate acute stage toxemia with subsequent antitoxin antibody responses.

Anthrax is a zoonotic disease caused by the gram positive bacterium *Bacillus anthracis*. The disease is primarily associated with herbivores and domestic livestock and occurs with regularity in countries where widespread vaccination of animals is not practiced (http://www.promedmail.org). Human anthrax is less common and usually occurs subsequent to handling infected domestic animals including cattle and goats or their products (eg, skin, meat, hides, and bones). There are 3 primary forms of the disease in humans; cutaneous (CA), gastrointestinal (GA), and inhalation (IA),

depending on the route of exposure to *Bacillus anthracis*. The case-fatality rate in humans for untreated anthrax ranges from 20% for CA, to 25%–60% for GA and 86%–89% for IA (http://www.bt.cdc.gov/agent/anthrax/faq/signs.asp) [1]. The case-fatality rate for IA in the 2001 anthrax letter attacks in the United States was 45% even with antimicrobial treatment and aggressive supportive patient care [2, 3].

The pathogenesis of anthrax is primarily attributed to the protein exotoxins, lethal toxin (LTx) and edema toxin (ETx), secreted by *B. anthracis*. LTx has been shown to be sufficient to cause death in a variety of animal models. The pivotal protein of these toxins is the "protective antigen" (PA) that binds to enzymatic components lethal factor (LF) and edema factor (EF) [4] and transports them into host target cells. LF is a zinc-dependent endoproteinase that hydrolyzes and inactivates at least 5 members of the mitogen-activated protein kinase kinase (MAPKK) family of response regulators involved in immune activation [5]. EF is a calmodulin dependent adenylate cyclase that converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [6].

Elevated levels of cAMP cause edema and suppress inflammatory responses. The combined effects of the toxins are local necrosis and edema in cutaneous anthrax, and hemorrhagic mediastinal necrosis, hypoxic insult and pleural edema in inhalation anthrax [7]. A consequence of the combined activities of these toxins is to inhibit innate and acquired immune responses thus facilitating the extensive bacterial proliferation of systemic anthrax [4]. Protection against anthrax is mediated by antitoxin antibody responses whether actively induced or passively administered.

Due to the low frequency of occurrence of natural anthrax in humans, there are only limited data available on the human immune response to B. anthracis infections and in particular for CA [8]. In the present study, sensitive and specific LF quantification, anti-PA immunoglobulin G (IgG) and toxin neutralization assays were used in an evaluation of acute and convalescent sera from 26 suspected human CA cases from an outbreak reported in North-Western Bangladesh between August and October 2009 [9, 10, 11, 12].

LF was present in acute serum from CA cases and after antimicrobial treatment. Anti-PA and TNA were observed only in convalescent sera from individuals with measureable LF. The data indicate that in cases of confirmed cutaneous anthrax, toxins are secreted into the blood inducing an antitoxin immune response. Anti-PA IgG levels in naturally acquired CA were statistically significantly higher than those reported for bioterrorism associated CA.

MATERIALS AND METHODS

Materials
All chemicals and reagents were obtained from Sigma-Aldrich except where indicated.

Case Definitions
Case patients for evaluation occurred from the date of slaughtering the first sick animal on August 18th in the outbreak area until 3 weeks after the last sick animal was slaughtered. Anthrax cases were defined as suspect (possible), probable (presumptive), or confirmed according to the following criteria. Suspect CA was defined as acute onset of a painless skin lesion(s) that developed over 2–6 days from a papular through a vesicular stage to skin ulceration with raised margin and central black eschar. Probable CA was defined by the presence of a documented lesion accompanied by an epidemiological link to either a known exposure or a positive result for LF toxemia. Confirmed CA was defined by the presence of a cutaneous anthrax lesion accompanied by one or more of the following: positive identification of B. anthracis by growth in culture, M'Fadyean (MFad) stain, immunohistochemical (IHC) stain, or evidence of a 4-fold change in anti-PA IgG antibody levels between acute and convalescent sera or paired convalescent sera. For acute sera that were nonreactive by anti-PA ELISA, the assay lower limit of quantification (LLOQ) was used to determine 4-fold change. Serum toxin neutralization activity (TNA) measurements were not included in the case definition. Serum TNA provides an assessment of the functional activity of anti-PA antibodies, and enhances the diagnostic sensitivity and specificity of the anti-PA IgG.

Human Cutaneous Anthrax
Cutaneous anthrax cases were defined as described above. Reported exposures consisted of but were not limited to one or more of the following: handled raw meat, contacted sick animal, contacted dead animal, and/or was present close to the slaughtering site. Acute and convalescent sera from 26 suspect CA cases were obtained as part of the Government of Bangladesh, International Centre of Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and Centers for Disease Control and Prevention (CDC) response to the 2009 outbreak. Acute stage serum samples were those collected when cutaneous lesions were first identified; convalescent stage sera were those collected 16–28 days after appearance of the lesions. Antimicrobial treatment with ciprofloxacin commenced prior to or on the date of acute sample collection (Table 1). Swabs of vesicles were obtained for bacterial culture when feasible. In case patients where swabs were not collected, the vesicles were either dry or insufficiently developed. The details of this anthrax outbreak epidemiology, management and informed consent are described elsewhere (Chakraborty et al 2011, submitted).

Serological Methods
Sera were collected using serum separation tubes (SST) (Becton Dickinson). Detailed methods for quantification of LF, anti-PA IgG, and TNA are described elsewhere [10, 11, 12, 13]. LF toxemia was quantified using a validated mass spectrometry (LF-MS) method that reports specific LF endoproteinase activity in ng/mL of serum [10]. The LF-MS assay had precision of 8%–14%, accuracy of 92%–98%, and 100% diagnostic sensitivity and specificity (M. Gallegos, unpublished data).

Anti-PA IgG was measured by enzyme-linked immunosorbent assay (ELISA) and reported in µg/mL [12]. The anti-PA ELISA had a diagnostic sensitivity of 99.8% and diagnostic specificity of 98.4%. As case patients were symptomatic at the time of earliest serum acquisition and actual exposure times were not determined, seroconversion was defined as a ≥4-fold increase in anti-PA IgG concentration in the convalescent serum compared with the acute serum. If an acute serum was not available or was obtained later than 7 days after symptoms onset, seroconversion was defined as a ≥4-fold increase over the assay lower limit of quantification (LLOQ, 3.7 µg/mL).

The TNA assay measured the ability of a test serum to neutralize lethal toxin mediated killing of the macrophage cell line J774A.1 [11]. The primary endpoint used in this study was

Table 1. ***B. anthracis*** **Was Detected by Culture (Cult) of Lesion Swabs, M'Fadyean Staining (MFad) of Lesion Smears, and Immunohistochemistry (IHC) of Tissue Biopsies, Indicated by + (Positive), – (Negative), or NS (No Sample)**

Case ID	Antimicrobials pre-acute (days)	Acute post-onset (days)	Conv. post-onset (days)	BA confirmed by culture, IHC, or MFad	Acute			Convalescent			≥4-fold change in anti-PA	Diagnostic status
					Anti-PA (µg/mL)	TNA (ED$_{50}$)	LF (ng/mL)	Anti-PA (µg/mL)	TNA (ED$_{50}$)	LF (ng/mL)		
pab1	2	8	28	+ (IHC)	<LLOQ	0	0.996	47.4	147	<LOD	Yes	Confirmed
pab2	3	8	28	–	<LLOQ	0	0.943	78.8	438	<LOD	Yes	Confirmed
pab3	0	8	28	+ (IHC)	4.9	0	0.041	44.1	27	NA	Yes	Confirmed
pab4	0	8	28	NS	<LLOQ	0	0.069	88.0	93	<LOD	Yes	Confirmed
pab5	2	8	28	+ (IHC)	<LLOQ	0	1.264	55.9	109	<LOD	Yes	Confirmed
pab7	3	7	27	–	<LLOQ	0	0.310	230.6	593	NA	Yes	Confirmed
pab8	0	7	27	–	<LLOQ	0	0.011	26.8	0	<LOD	Yes	Confirmed
pab9	7	7	27	–	<LLOQ	0	0.161	42.8	189	<LOD	Yes	Confirmed
pab10	5	7	27	–	<LLOQ	0	1.166	76.0	80	<LOD	Yes	Confirmed
pab11	7	7	27	–	<LLOQ	0	0.031	21.1	39	<LOD	Yes	Confirmed
pab12	0	7	27	+ (MFad)	<LLOQ	NS	0.159	55.9	104	<LOD	Yes	Confirmed
pab13	0	6	26	–	<LLOQ	0	0.675	83.5	88	<LOD	Yes	Confirmed
pab14	0	3	23	–	<LLOQ	0	0.040	10.0	40	NA	No	Confirmed*
pab15	0	3	23	–	19.1	0	0.005	85.8	65	<LOD	Yes	Confirmed
pab16	0	1	21	NS	<LLOQ	0	<LOD	<LLOQ	0	<LOD	No	Probable
pab18	0	8	28	+ (Cult/Mfad)	<LLOQ	0	0.486	144.8	277	<LOD	Yes	Confirmed
pab19	0	7	27	–	<LLOQ	0	<LOD	<LLOQ	0	NA	No	Probable
pab20	0	6	25	+/– (MFad)	<LLOQ	0	<LOD	4.0	0	<LOD	No	Probable
pab21	0	3	22	+ (Cult/MFad)	<LLOQ	0	1.105	194.0	83	<LOD	Yes	Confirmed
pab23	0	5	24	NS	<LLOQ	0	<LOD	<LLOQ	0	NA	No	Probable
pab25	0	1	20	NS	<LLOQ	0	<LOD	<LLOQ	0	<LOD	No	Probable
pab27	0	1	20	NS	<LLOQ	0	<LOD	<LLOQ	0	<LOD	No	Probable
pab29	5	5	16	–	<LLOQ	0	<LOD	<LLOQ	0	NA	No	Probable
pab30	5	5	16	NS	<LLOQ	0	<LOD	<LLOQ	0	<LOD	No	Probable
pab31	4	5	16	–	4.0	0	0.035	12.3	45	<LOD	No	Confirmed*
pab32	4	14	25	–	679.5	58	0.086	487.2	241	NA	No	Confirmed*
GMC of positive results		5.0	24.0		22.5[a]		0.153	48.1	107.92			
SE		0.14	0.04		2.3		0.511	0.28	0.23			

Confirmed indicates the presence of cutaneous lesion with one of the following: culture, IHC, MFad, or ≥4-fold change in anti-PA titer over the lower limit of quantification (3.7 µg/mL) or ≥4-fold change in anti-PA titer between paired acute and convalescent serum samples. Confirmed* refers to case patients that exhibit elevated convalescent anti-PA that do not meet the ≥4-fold change from the acute sample but that exhibit a ≥4 fold change in TNA. Probable refers to case patients that exhibited cutaneous anthrax-like lesions and an epi-link to exposure.

Abbreviations: Anti-PA, anti-protective antigen; BA, *B. anthracis*; GMC, geometric mean concentration; LF, lethal factor; LLOQ, lower limit of quantification; LOD, limit of detection; NA, insufficient sample volume available for all measurements; SE, standard error of the mean; TNA, toxin neutralization activity.

[a] The acute stage anti-PA GMC was higher than expected because the immune response to infection for pab32 had passed its peak level and exposure may have been earlier than assessed from the development of the cutaneous lesion.

the reciprocal of a serum sample dilution that resulted in 50% neutralization of anthrax lethal toxin cytotoxicity (ED50). The TNA assay had a LLOQ ED50 titer of 36 with 97% diagnostic sensitivity and 100% diagnostic specificity [8].

M'Fadyean Stain, Immunohistochemistry, and Culture Isolation

Culture analysis, M'Fadyean staining (MFad) of capsule, and immunohistochemistry (IHC) staining of cell wall and capsule antigens for detection of *B. anthracis* in lesion swabs, vesicle smears, and skin biopsies were performed as described elsewhere [14, 15, 16].

RESULTS

Serum samples from 26 suspected CA cases were evaluated for LF toxemia, seroconversion to anti-PA IgG and serum toxin neutralization activity (TNA) and compared with culture and direct observation diagnostic tests for anthrax. Acute stage samples were collected from 1 to 14 days after symptom onset
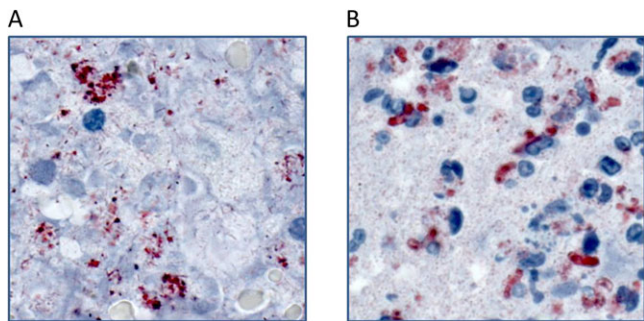
Figure 1. IHC staining of abundant granular and bacilliform antigens in skin biopsy samples with anti–*B. anthracis* capsule antibody (*A*) and anti–*B. anthracis* cell wall antibody (*B*). Immunoalkaline phosphatase with napthol fast red substrate and hematoxylin counterstain. Original magnifications: 400X.

Figure 2. M'Fadyean (MFad) stain for capsulated *B. anthracis* in a cutaneous lesion smear (pab21). The pink-stained poly-D-glutamic acid (PGA) capsule can be seen surrounding the purple stained vegetative bacilli.

and convalescent stage samples from 16 to 28 days. Antimicrobial use was reported in 11 individuals from 2 to 7 days prior to collection of the first available (acute) sample. In the remaining 15 case patients, antimicrobial treatment commenced with the acute sample collection (Table 1).

Of the 26 suspected CA cases in this study, there were 6 (pab4, pab16, pab23, pab25, pab27, and pab30) for which a sample was not available for analysis by culture/IHC/M'Fadyean (culture/observation) (Table 1). Of the 20 case patients for which a lesion sample was available, 6 (pab1, pab3, pab5, pab12, pab18, and pab21) were confirmed by culture/IHC/M'Fadyean (Figures 1 and 2). Serum from these 6 individuals was also reactive for LF toxemia, anti-PA IgG, and serum TNA. The LF concentrations for these 6 CA cases ranged from 0.041 to 1.26 ng/mL. There was not a consistent trend between the magnitude of toxemia and eschar reactivity by culture/IHC/M'Fadyean. One case patient (pab20) that was indeterminate by culture/IHC/M'Fadyean was nonreactive by any test except the anti-PA IgG ELISA. However, this individual was classified as indeterminate as there was not a 4-fold increase in anti-PA IgG between acute (<LLOQ) and convalescent (4.0 μg/mL) sera. There were 13 case patients (pab2, pab7, pab8, pab9, pab10, pab11, pab13, pab14, pab15, pab19, pab29, pab31,and pab32) for which there was a sample available but that were by negative for culture observation by either culture, IHC or M'Fadyean.

Twelve case patients (pab2, pab4, pab7, pab8, pab9, pab10, pab11, pab13, pab14, pab15, pab31, and pab32) were reactive by toxemia and serology only (Table 1). Three of these (pab14, pab31, and pab32) were reactive but did not meet the required 4-fold change between acute and convalescent paired sera for anti-PA and 1 (pab8) did not meet the ≥4-fold increase required for TNA reactivity. For pab14 and pab31, anti-PA IgG levels increased from <LLOQ to 10 μg/mL and from 4.0 μg/mL to 12.3 μg/mL between acute and convalescent sera, respectively. Thus, they did not meet the criterion for seroconversion. Pab32 had a high level of anti-PA IgG in the acute serum (679.5 μg/mL)
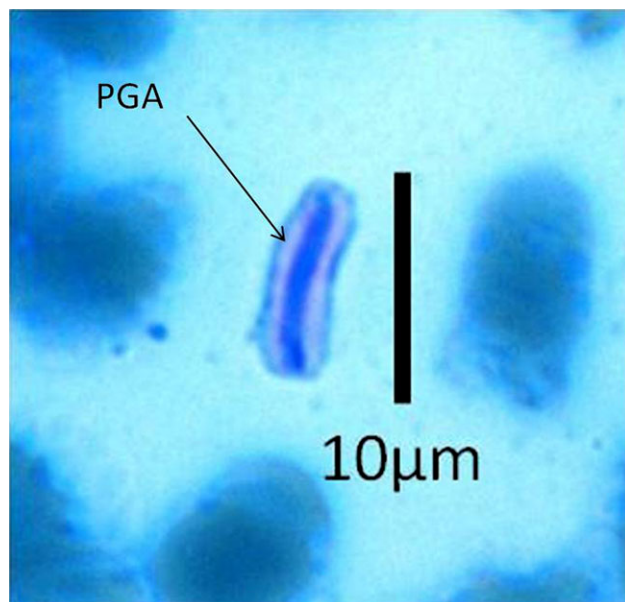
and convalescent serum (487.2 μg/mL) and therefore also did not achieve a 4-fold change in anti-PA required for seroconversion. Serum TNA for these 3 cases increased from <LLOQ to an ED50 titer of 40 and 45 for pab14 and pab31, respectively, and from 58 to 487.2 for pab32. LF levels in pab14, pab31 and pab32 were 0.040, 0.035, and 0.086 ng/mL, respectively. Case pab8, although not reactive by TNA assay, had measureable levels of both LF (0.11 ng/mL) and anti-PA antibody (convalescent, 26.8 μg/mL), indicating that this also was a true infection. The combined data from all 3 serological tests indicate that these were true infections.

Four case patients (pab3, pab15, pab31, and pab32) had detectable levels of anti-PA IgG in their acute stage sera. Acute stage samples for pab3, pab15, and pab31 were obtained at days 8, 3, and 5 after reported onset of symptoms, respectively. The onset of an anti-PA antibody response in these 4 individuals was earlier than the day 12 onset reported previously for bioterrorism associated cutaneous anthrax [8]. The acute stage sample for pab32 was obtained at day 14 and had a high level of anti-PA IgG (679.5 μg/mL), which declined to 487.2 μg/mL in the convalescent serum (day 25). This demonstrates that the immune response to infection had passed its peak level and that exposure may have been considerably earlier than assessed from the development of the cutaneous lesion. Of note is that this was the only case for which an acute serum sample had a detectable TNA titer and the titer increased ≥4-fold as discussed above. Linear regression analysis indicated a positive correlation ($r^2 = 0.41$) between log10 transformed TNA ED50 titer and anti-PA IgG
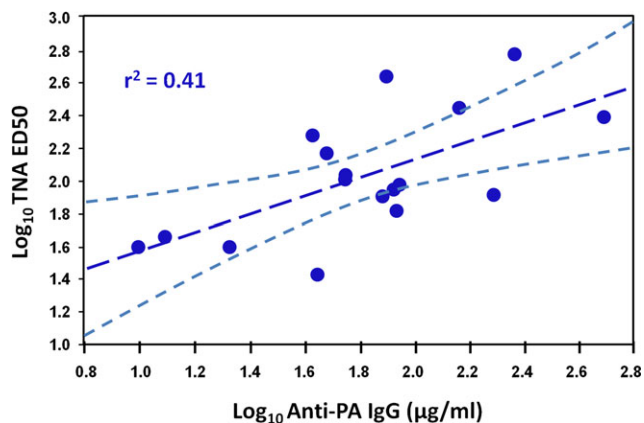
Figure 3. Linear regression analysis of $\log_{10}$ TNA ED50 titers with $\log_{10}$ anti–protective antigen (anti-PA) immunoglobulin G (IgG) (µg/mL) for all values greater or equal to the assays' lower limits of quantification indicated a positive correlation ($r^2 = 0.41$). In humans, PA is a major toxin neutralizing–antibody determinant for both cutaneous and inhalation anthrax.

Figure 4. Box Plot comparisons of maximum determined anti–protective antigen (anti-PA) immunoglobulin G (IgG) responses from bioterrorism (BT) associated and naturally occurring cutaneous anthrax cases. Geometric means are 25.0 µg/mL (n = 13) and 52.6 µg/mL (n = 21) respectively, which are statistically significantly different ($P = .046$). Comparisons were made using first the Shapiro-Wilk statistic test for normality, followed by the t test for normally distributed data.

concentration for all values $\geq$ LLOQ (Figure 3). This correlation is lower than that reported previously for inhalation anthrax ($r^2 = 0.83$). The reason for this difference is unclear and may be a consequence of the different disease presentations. Of particular interest to note is that no convalescent stage sample contained detectable LF and all case patients that seroconverted to anti-PA had detectable acute stage toxemia.

There are few opportunities to compare laboratory diagnostic data for human anthrax. We compared the anti-PA responses for naturally acquired CA in this study with those previously reported for bioterrorism (BT) associated CA [8]. The BT and naturally acquired CA samples were evaluated using the same validated anti-PA ELISA, reference standards, and QC criteria. The long-term performance stability of this assay has also been described [17] and provides confidence in the comparison. Comparisons were made using first the Shapiro-Wilk statistic test for normality, followed by the t test for normally distributed data. Both the bioterrorism-acquired and the naturally acquired maximum anti-PA IgG values were normally distributed in log space. The maximum measured anti-PA IgG level was significantly lower in BT associated CA than in naturally acquired CA (25.0 µg/mL [n = 13] vs 52.6 µg/mL [n = 21] [$P = .046$, 2-sided unequal variance] (Figure 4).

DISCUSSION

More than 95% of all naturally occurring *B. anthracis* infections reported worldwide are cutaneous [7]. Cutaneous anthrax is associated with handling infected animals or contaminated items such as meat, wool, hides, leather, or hair products from infected animals [18]. Historically, case-fatality rates for this form of the disease are 20% without treatment and <1% with appropriate antimicrobial therapy [19]. The human serum
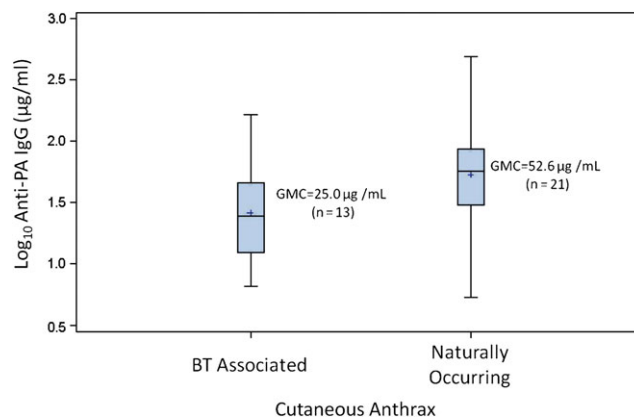
samples in this study were all from suspect cutaneous anthrax cases associated with handling infected cattle and goats. Anthrax was confirmed by detection of *B. anthracis* in cutaneous lesions by culture, immunohistochemistry (IHC), and M'Fadyean staining (MFad). There were no fatalities (Chakraborty et al, submitted).

Standard diagnostic protocols for cutaneous anthrax include culture from vesicular fluid, swabs, or punch biopsy of the lesion. Culture from blood samples is recommended only if the patient has evidence of systemic anthrax and has not received antimicrobial agents (http://www.bt.cdc.gov/agent/anthrax/lab-testing/recommended_specimens.asp) [20]. The value of serological tests, including anti-LF antibody measurements, as retrospective diagnostic or epidemiologic tools in studies of cutaneous, gastrointestinal, and inhalation anthrax is well established [3, 8, 12, 19, 21, 22, 23]. However, the evaluation of toxemia in human cutaneous anthrax has not to our knowledge been previously reported. The mass spectrometry-based method used in this study for quantitative determination of LF toxemia is gaining increasing prominence as a high throughput, high sensitivity diagnostic tool [10, 24]. This method can provide confirmatory diagnosis up to 24 hours earlier than other methods in animal models and can detect and quantify LF for 12-days post-antimicrobial therapy and 7 days post-anthrax immune globulin intravenous (AIGIV) treatment in human clinical samples [13, 22]. The persistence of LF in serum after clearance of bacteremia by antimicrobial treatment is a key advantage of this method. Of particular interest from this study is that in all cutaneous anthrax cases confirmed by at least 1 other diagnostic test, LF toxemia was detectable up to day 14 after symptom onset (pab32) but was absent in all convalescent sera the earliest of which was obtained on day 16.

Measuring anti-PA antibody levels is valuable for confirming infection, because exposure to toxin requires spore germination and vegetative cell growth. This is demonstrated by the current data where all case patients that seroconverted also had detectable toxemia in their acute stage sera. As indicated for pab32, the magnitude of the antibody response and changes between acute and convalescent sera may be indicative of the onset of disease and need not always be concurrent with onset of discernable symptoms. In human cutaneous anthrax the peak anti-PA IgG response levels are considered to be significantly lower and take longer to develop than in inhalation anthrax [8]. In this study the maximum anti-PA antibody levels were significantly higher than those reported previously for bioterrorism associated cutaneous and inhalation anthrax. This may be a consequence of the different types of exposure to *B. anthracis*, the naturally acquired cases potentially having a greater degree of contact with more heavily contaminated materials.

The validated toxin neutralization assay (TNA) measures the ability of the antibodies present in serum to neutralize lethal toxin activity on a macrophage cell line [11]. Toxin neutralization activity (TNA) was observed only in sera from which anti-PA IgG was quantifiable by ELISA. In this study there was only a very low correlation ($r^2 = 0.42$) between serum anti-PA IgG concentration (μg/mL) and TNA ED50, which is in contrast to previous reports for bioterrorism associated inhalation anthrax [8]. The reason for these differences is not known, but it is tempting to speculate that it is related to the type of exposure and a lower level of antigen load that elicits production of lower affinity antibody that is less efficient at toxin neutralization. The single case patient (pab8) that was confirmed by at least 2 other tests but did not have detectable TNA is confirmation that 100% diagnostic sensitivity for an antibody-based diagnostic test for anthrax remains elusive. However, it must also be noted that in this study at least, the anti-PA ELISA produced no false positive or false negative results and only 1 indeterminate result.

In this study only 6 of 20 biopsy samples tested positive for the presence of *B. anthracis* by culture, M'Fadyean stain of vesicle/lesion smears, or IHC. This may be due in part to the use of antimicrobial therapy since 8 case patients with negative results had received antimicrobial therapy from 3 to 7 days prior to sample collection. In contrast, both acute LF and convalescent anti-PA were positive in 18 samples of which 9 had received antimicrobial agents from 2 to 7 days prior to first sample collection, showing little interference from antimicrobial use as observed previously [2, 22].

These results collectively indicate that while the infection may remain localized to the lesion during early cutaneous infection, in most cases toxins are secreted into the blood in sufficient quantity to be measured and subsequently induce effective toxin neutralizing antibody responses. This study reports the first documented evidence of measureable anthrax toxin LF in the blood during clinical cutaneous anthrax and illustrates a direct association of the presence of LF and the subsequent development of anti-PA and TNA. These results confirm the value of serological measurements for confirmation of anthrax, when *B. anthracis* cannot be cultured or visualized by staining methods.

Notes

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