

# Immune Responses to *Bacillus anthracis* Protective Antigen in Patients with Bioterrorism-Related Cutaneous or Inhalation Anthrax

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Anti-protective antigen (PA) immunoglobulin (Ig) G, toxin neutralization, and PA-specific IgG memory B cell responses were studied in patients with bioterrorism-related cutaneous or inhalation anthrax and in a patient with laboratory-acquired cutaneous anthrax. Responses were determined for >1 year after the onset of symptoms. Eleven days after the onset of symptoms (15 days after likely exposure), anti-PA IgG was detected in 16 of 17 patients with confirmed or suspected clinical anthrax who were tested. Anti-PA IgG remained detectable 8–16 months after the onset of symptoms in all 6 survivors of inhalation anthrax and in 7 of 11 survivors of cutaneous anthrax who were tested. Anti-PA IgG levels and serum toxin neutralizing activity were strongly associated ( $R^2 = 0.83$ ). PA-specific IgG memory B cells were detectable in all 6 survivors of inhalation anthrax but in only 2 of 7 patients with cutaneous anthrax who were tested. Anti-PA IgG is an important diagnostic marker of anthrax, a predictor of serum anti-toxin activity, and a marker of immunological memory against anthrax.

The pathogenicity of *Bacillus anthracis* is primarily due to the action of its 3 main virulence factors, the  $\gamma$ -linked poly-D-glutamic acid capsule and the 2 protein exotoxins [1]. The exotoxins comprise 3 proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF). PA combines with EF and LF to form, respectively, the binary toxins edema toxin (ETx) and lethal toxin (LTx) [2]. ETx elevates intracellular cyclic-AMP levels, whereas LTx inactivates members of the

mitogen-activated protein kinase family, to effect an imbalance in the production or release of a range of cytokines that may contribute to the pathogenesis of anthrax [3, 4]. The combined effects of the toxins are local necrosis and edema during cutaneous anthrax and hemorrhagic mediastinal necrosis, hypoxic insult, and pleural edema during inhalation anthrax [4, 5]. LTx has been shown to be sufficient to cause death in a variety of animal models [4, 6–8]. Ameliorating the toxin effects, therefore, is thought to be central in host protection, and much evidence has accumulated indicating that protection is mediated by antibody responses, whether actively induced or passively administered [9–12]. Limited data are available on the human immune response to *B. anthracis* infection. Retrospective serum samples obtained from patients with cutaneous [13, 14] or gastrointestinal anthrax [15, 16] have been studied, but a prospective study of the onset, magnitude, and duration of serological and memory B cell immune responses to *B. anthracis* in the setting of bioterrorism has not been conducted.

From 4 October to 20 November 2001, 22 individuals

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with bioterrorism-related cutaneous (11 patients) or inhalation (11 patients) anthrax were identified in the United States [5, 17, 18]. The infections were known or were suspected to have resulted from mail containing or contaminated with *B. anthracis* spores. Another case of cutaneous anthrax occurred in March 2002, in a laboratory worker handling samples from this outbreak [19]. In the present study, sensitive serological, LTx neutralization, and memory B cell immunoassays were used for an immunological evaluation of the surviving cohort of these bioterrorist attacks. Anti-PA IgG responses were determined by ELISA [20], and a functional assessment of humoral immunity in the same serum samples was performed by use of an in vitro anthrax LTx neutralization activity (TNA) assay [21]. PA-specific IgG memory B cell responses ~1 year after the onset of symptoms were determined by enzyme-linked immunospot (ELISPOT) assay. These in vitro end points were used to evaluate the development of acquired immunity to this disease and the role played by anti-PA serological testing in the diagnosis of anthrax.

## MATERIALS AND METHODS

**Anthrax cases.** Anthrax cases were classified as confirmed or suspected [22]. A confirmed case required either (1) a compatible clinical syndrome together with isolation of *B. anthracis* from blood or another typically sterile site or (2) positive reactions in at least 2 *B. anthracis*-specific laboratory tests. A suspected case required either (1) a compatible clinical syndrome and a positive reaction in at least 1 *B. anthracis*-specific laboratory test or (2) a compatible clinical syndrome for a case epidemiologically linked to a confirmed environmental exposure [18, 22]. At least 1 serum sample was obtained from 10 patients with confirmed inhalation anthrax, 5 patients with confirmed cutaneous anthrax, and 6 patients with suspected cutaneous anthrax. A laboratory worker who acquired cutaneous anthrax when handling samples from the outbreak was also included in the present study. In many instances, no definitive exposure date could be established; time points in this study are reported as the number of days after the onset of symptoms. For determination of anti-PA IgG and TNA ED<sub>50</sub> levels, all serum samples were tested at least twice, in duplicate, by 2 blinded independent operators. None of the patients in the present study received anthrax vaccine. This study was approved by the Human Investigations Committee of the Centers for Disease Control and Prevention (CDC).

**Anti-PA IgG ELISA.** The quantitative ELISA for human anti-PA IgG has been described in detail elsewhere [20]. Immulon 2 HB microtiter plates (Thermo Labsystems) were coated with recombinant PA in 0.01 mol/L PBS (pH 7.4; Life Technologies) at a concentration of 2 µg/mL. (Recombinant PA and LF were obtained from S. H. Leppala, National Institutes of

Health, Bethesda, MD.) Plates were washed 3 times with ELISA wash buffer (PBS and 0.1% Tween 20), and each well was loaded with 100 µL of PBS containing 5% skim milk and 0.5% Tween 20 (pH 7.4; Dilution Buffer). Test serum was mixed on the plate and was serially transferred down the plate to make an 8-point dilution series. The final volume in each well was 100 µL. After washing, bound anti-PA IgG was detected by use of horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-human IgG Fc PAN clone HP6043 (Hybridoma Reagent Laboratory) and was color developed with ABTS substrate (Kirkegaard and Perry Laboratories). Data were analyzed by use of a 4-parameter logistic-log curve-fitting model with ELISA for Windows software [23]. A calibration factor (141.2 µg/mL anti-PA IgG) for the standard reference serum AVR414 was used to determine the concentration of anti-PA IgG, in micrograms per milliliter of test serum. The reactivity threshold of the anti-PA ELISA was determined to be 3.0 µg/mL anti-PA IgG in undiluted serum; diagnostic sensitivity was 98.6%, and diagnostic specificity was 94.2%.

**LTx neutralization activity.** The J774A.1 macrophage/monocyte cell line (ATCC; TIB-67) was cultured in Dulbecco's modified Eagle medium supplemented with 4.5 g/L high glucose, 4 mmol/L L-glutamine (Gibco BRL), 5% heat-inactivated fetal bovine serum (FBS; HyClone), 10 mmol/L HEPES buffer solution (Gibco BRL), 50 U/mL penicillin, 50 µg/mL streptomycin sulfate, and 1 mmol/L sodium pyruvate (Gibco BRL). All incubations were at 37°C in a 5% CO<sub>2</sub> atmosphere with 95% relative humidity. Cultures of J774A.1 cells were harvested into warmed growth medium and were plated at 3 × 10<sup>4</sup> cells/well in 96-well flat-bottom microtiter plates 17–19 h before the assay. Test antisera was prepared in a separate 96-well microtiter plate as 2-fold dilutions in triplicate and then was incubated with anthrax LTx (50 ng/mL PA and 40 ng/mL LF) for 30 min at 37°C. Spent medium was then removed from the J774A.1 cells, the toxin-antiserum mix was transferred to the J774A.1 cell plate (100 µL/well), and the incubation was continued for 4 h. Cell viability was determined by the addition of 25 µL/well of a 5 mg/mL stock solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) dissolved in 0.01 mol/L PBS (pH 7.4; Life Technologies), and the incubation was continued for 2 h. The assay was terminated by the addition of 100 µL/well of 20% (wt/vol) SDS (Sigma) and 50% (vol/vol) N,N-dimethylformamide (Fisher Scientific). Optical density values were read by use of an MRX Revelation microtiter-plate reader (Thermo Labsystems) at 570 nm, with a 690-nm reference filter.

Assay end points were calculated by use of SAS software (version 8.0; SAS Institute) running an end-point calculation algorithm developed by the CDC [24]. The primary end point for the present study was the reciprocal of a serum-sample dilution

**Table 1. Temporal patterns of anti-protective antigen (PA) IgG response, in patients with inhalation anthrax.**

Patient	Serum samples tested, no.	Level (days after onset of symptoms)			
		Nonreactive anti-PA IgG, by ELISA	Initial quantifiable anti-PA IgG, by ELISA	Peak anti-PA IgG, by ELISA	Peak TNA ED <sub>50</sub>
IA-1 <sup>a</sup>	1	<LLQ (5)	NA	NA	NA
IA-2	5	NA	10.8 (16)	37.1 (18)	242 <sup>b</sup> (17)
IA-3	8	<LLQ (5)	246.0 (12)	422.9 <sup>c</sup> (17)	424 (17)
IA-4	14	<LLQ (6)	20.1 (11)	299.6 (38)	899 (31)
IA-5 <sup>a</sup>	0	NA	NA	NA	NA
IA-6 <sup>a</sup>	1	<LLQ (5)	NA	NA	NA
IA-7	12	<LLQ (2)	30.2 (18)	314.5 (39)	1098 (32)
IA-8	5	<LLQ (6)	186.1 (22)	1449.5 (43)	2348 (43)
IA-9	4	<LLQ (10)	55.7 (18)	168.5 (58)	655 (58)
IA-10 <sup>a</sup>	1	<LLQ (4)	NA	NA	NA
IA-11 <sup>a</sup>	3	<LLQ (5, 6, 7)	NA	NA	NA

**NOTE.** Data are micrograms per milliliter, unless otherwise noted. <LLQ, less than the lower limit of quantification; NA, not applicable; TNA, lethal toxin neutralization activity.

<sup>a</sup> Patients who died.

<sup>b</sup> Insufficient volume of serum for TNA assay on day 18 (shown is the peak anti-PA IgG level, by ELISA).

<sup>c</sup> Patient underwent plasmapheresis on days 14–28.

that resulted in 50% neutralization of anthrax LTx cytotoxicity (ED<sub>50</sub>) and corresponded to the inflection point of a 4-parameter logistic-log fit of the neutralization curve. The TNA assay used has a working range of 0.07–0.30 µg/mL of neutralizing anti-PA IgG, a lower limit of quantification of 0.07 µg/mL, and a positive-negative threshold of 2.5 µg/mL anti-PA IgG in undiluted serum. The assay has a diagnostic sensitivity of 97% and a diagnostic specificity of 100% (H. Li, unpublished data).

**IgG-specific memory B cell assay.** A detailed protocol has been described elsewhere [25, 26]. In brief, peripheral-blood mononuclear cells (PBMCs) were isolated from cell preparation tubes (BD Biosciences) shipped overnight at ~4°C. PBMCs were cultured for 6 days in 24-well plates at 5 × 10<sup>5</sup> cells/well in RPMI 1640 with 10% FBS (HyClone) that was supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µmol/L L-glutamine, and 50 µmol/L β-mercaptoethanol (R-10). In addition, the following mixture of polyclonal mitogens was added to the cultures: 1/100,000 pokeweed mitogen extract (Emory University, Atlanta, GA), 6 µg/mL phosphorothioated CpG ODN-2006 [27], and 1/10,000 *Staphylococcus aureus* strain Cowan (Sigma). Samples were cultured in 6–24 wells/patient. A negative control was cultured in R-10 alone. In preparation for the ELISPOT, 96-well filter plates (MAHA N4510; Millipore) were coated with recombinant PA at a concentration of 1 µg/mL. Keyhole limpet hemocyanin (2.5 µg/mL) was used as a nonanthrax antigen control. To detect all IgG-secreting cells, a separate plate was coated with 10 µg/mL goat anti-human IgG (Caltag Laboratories). Before use, plates were washed and were blocked with RPMI 1640 and 1% bovine serum albumin (fraction V; Sigma) for 2–4 h at 37°C.

Cultured PBMCs were washed thoroughly and were plated

onto ELISPOT plates for 5 h. Plates were washed 3 times with ELISPOT wash buffer (EWB; 0.01 mol/L PBS [pH 7.2]) and then 3 more times with the same buffer containing 0.05% Tween 20 (EWBT). Plates were then incubated overnight in 1 µg/mL biotin-conjugated mouse monoclonal anti-human IgG Fc PAN clone HP6043B (Hybridoma Reagent Laboratory) in EWBT and 1% FBS. Plates were washed and were incubated with 5 µg/mL HRP-conjugated avidin-D (Vector Laboratories) in EWBT and 1% FBS. Plates were again washed and were then developed by use of 3-amino-9-ethyl-carbazole (Sigma). Developed plates were counted by inspection or by use of a plate reader (Cellular Technology). Results are presented as the percentage of the total number of IgG memory B cells that were PA specific. The lower limit of detection was 0.002–0.01 antigen-specific cells/10<sup>6</sup> PBMCs.

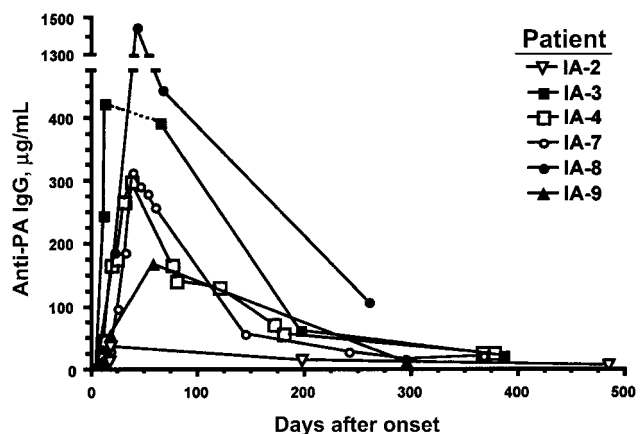
## RESULTS

**Anti-PA IgG responses.** Eleven patients with inhalation anthrax were identified during the bioterrorist attacks of October to November 2001 (table 1), and ≥1 serum sample was available from 10 of the patients. Five of the 11 patients died within the first week of hospitalization. Serum samples collected 4–7 days after the onset of symptoms from 4 of the 5 patients who died and 2–10 days after the onset of symptoms from 5 of the 6 patients who survived had anti-PA IgG antibody levels below the lower limit of quantification of the ELISA. In the 6 surviving patients, anti-PA IgG was first detected in serum samples collected 11–22 days after the onset of symptoms (15–28 days after likely exposure) (table 1). The ELISA responses of the patients

are shown in figure 1. Patient IA-3 received high-dose steroid therapy and daily plasmapheresis for 2 weeks beginning after serum-sample collection on day 14; data pertaining to serum samples collected during this treatment period were excluded from the figure, as indicated by the broken line connecting the data points. This patient also developed microangiopathic hemolytic anemia and thrombocytopenia during therapy, but the significance of this development with regard to his immune response is not known. Where serial serum samples were available, testing indicated that anti-PA IgG levels rose for 4–8 weeks after the onset of symptoms; peak responses occurred between 38 and 58 days after the onset of symptoms (figure 1), except for patient IA-2, who had a peak anti-PA IgG level (37.1  $\mu\text{g}/\text{mL}$ ) 18 days after the onset of symptoms but from whom there were no further serum samples until day 197. Peak detected anti-PA IgG levels ranged from 168.5 to 1449.5  $\mu\text{g}/\text{mL}$ . Although anti-PA IgG levels declined after the peak antibody response, all 6 of the survivors of inhalation anthrax had detectable anti-PA IgG levels 8–16 months after the onset of symptoms (range, 12.6–107.8  $\mu\text{g}/\text{mL}$ ).

Eleven patients with bioterrorism-related cutaneous anthrax and 1 patient with laboratory-acquired cutaneous anthrax were identified (table 2). All of the patients survived, although, in several patients, the clinical course was complicated by fever, chills, and other systemic symptoms. One patient (CA-7) had documented *B. anthracis* bacteremia, but it is unlikely that the peak anti-PA IgG response was captured (figure 2). At least 3 serum samples were available for testing from 11 of the 12 patients. Anti-PA IgG was detected as early as 12 days after the onset of symptoms (24 days after exposure). Where early serum samples were available, the latest time point at which a patient with cutaneous anthrax (CA-3) had nonreactive serum and then subsequently seroconverted was 18 days after the onset of symptoms (table 2). The apparent later responses of patients CA-7 and CA-11 were due to the nonavailability of serum samples from early or intervening time points (table 2 and figure 2). In patient CA-10, anti-PA IgG was not detected in acute or convalescent serum 4, 5, 47, and 253 days after the onset of symptoms.

Comparisons of responses for inhalation versus cutaneous anthrax were made by use of, first, the Shapiro-Wilk statistic test for Normality, followed by the *t* test for Normally distributed data and the Wilcoxon rank sum test otherwise. The maximum measured anti-PA IgG level was significantly lower in patients with cutaneous anthrax than in patients with inhalation anthrax (21.3  $\mu\text{g}/\text{mL}$  [ $n = 11$ ] vs. 286.4  $\mu\text{g}/\text{mL}$  [ $n = 5$ ] [ $P = .01$ , 2-sided unequal variance]). In serum samples collected >1 year after the onset of symptoms (range, 360–484 days), the mean anti-PA IgG level was also significantly lower in patients with cutaneous anthrax than in patients with inhalation anthrax (8.0  $\mu\text{g}/\text{mL}$  [ $n = 7$ ] vs. 19.9  $\mu\text{g}/\text{mL}$  [ $n = 4$ ] [ $P = .04$ , 2-sided



**Figure 1.** Anti-protective antigen (PA) IgG levels in patients with inhalation anthrax, 0–64 weeks after the onset of symptoms. In patients from whom serial serum samples were available, anti-PA IgG levels increased for 4–8 weeks after the onset of symptoms. The apparent peak responses ranged from 168.5 to 1449.5  $\mu\text{g}/\text{mL}$  38–65 days after the onset of symptoms and remained detectable until 240–480 days after the onset of symptoms (range, 12.6–107.8  $\mu\text{g}/\text{mL}$ ). The dotted line for IA-3 spans the period during which this patient underwent high-dose steroid therapy and daily plasmapheresis. The reactivity threshold of the assay is 3.0  $\mu\text{g}/\text{mL}$  anti-PA IgG in undiluted serum. Data points represent the mean of at least 2 tests, run in duplicate.

unequal variance]). Of 10 patients with cutaneous anthrax from whom serum samples were available, 4 had nonreactive convalescent serum samples >32 weeks (range, 249–413 days) after the onset of symptoms.

**LTx neutralization.** All of the patients with quantifiable anti-PA IgG levels 3–14 months after the onset of symptoms also had quantifiable TNA ED<sub>50</sub> levels, which peaked 4–8 weeks after the onset of symptoms. Several early serum samples from patients with inhalation anthrax and from patients with cutaneous anthrax had quantifiable anti-PA IgG levels without achieving 50% neutralization (data not shown). Linear regression analysis of TNA ED<sub>50</sub> levels versus anti-PA IgG levels for all reactive serum samples at all time points (excluding serum samples collected from the patient during high-dose steroid therapy and daily plasmapheresis) indicated a positive correlation ( $R^2 = 0.83$ ) (figure 3). The role played by anti-PA IgM or other toxin components is currently under investigation, but the similar degree of correlation between anti-PA IgG levels and TNA ED<sub>50</sub> levels in serum samples collected early ( $R^2 = 0.77$ , for <4 weeks after the onset of symptoms) and late ( $R^2 = 0.79$ , for >4 weeks after the onset of symptoms) suggests that any anti-PA IgM present in these serum samples did not make a significant contribution to LTx neutralization in this assay.

**PA-specific IgG memory B cells.** Serum samples from the survivors of anthrax were obtained 8–14 months after the onset of symptoms, to assess the development of long-term immune memory. PA-specific IgG memory B cells were detected in se-

**Table 2.** Temporal patterns of anti-protective antigen (PA) IgG response, in patients with cutaneous anthrax.

Patient	Serum samples tested, no.	Level (days after onset of symptoms)			
		Nonreactive anti-PA IgG, by ELISA	Initial quantifiable anti-PA IgG, by ELISA	Peak anti-PA IgG, by ELISA	Peak TNA ED <sub>50</sub>
CA-1	4	NA	163.4 (24)	163.4 (24)	364 <sup>a</sup> (35)
CA-2	5	NA	84.0 (21)	84.0 (21)	ND <sup>a</sup>
CA-3	5	<LLQ (16, 18)	8.0 (34)	14.4 (391)	152 (391)
CA-4	6	<LLQ (13)	5.6 (14)	23.5 (31)	127 (14)
CA-5	0	NA	NA	NA	NA
CA-6	5	NA	34.3 (16)	34.3 (16)	199 <sup>a</sup> (27)
CA-7 <sup>b</sup>	3	NA	56.9 (95)	56.9 (95)	354 (95)
CA-8	4	NA	3.8 (12)	5.6 (57)	61 (57)
CA-9	3	<LLQ (13)	17.5 (29)	17.5 (29)	106 (29)
CA-10	4	<LLQ (4, 5, 47, 253)	NA	NA	NA
CA-11	3	<LLQ (7)	9.6 (56)	9.6 (56)	<LLQ
CA-12 <sup>c</sup>	7	NA	12.9 (26)	45.0 (53)	122 (53)

**NOTE.** Data are micrograms per milliliter, unless otherwise noted. <LLQ, less than the lower limit of quantification; NA, not applicable; ND, not done; TNA, lethal toxin neutralization activity.

<sup>a</sup> Insufficient volume of serum for TNA assay on day 24 (shown is the peak anti-PA IgG level, by ELISA).

<sup>b</sup> Patient was bacteremic.

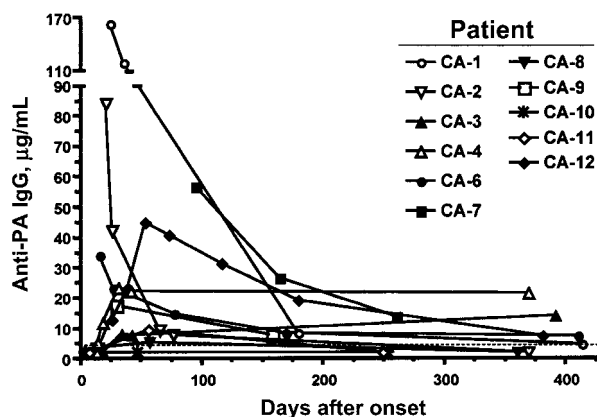
<sup>c</sup> Patient with laboratory-acquired cutaneous anthrax.

rum samples from all 6 of the patients with inhalation anthrax who survived and from 2 of the 7 patients with cutaneous anthrax who were tested (we were unable to culture PBMCs from patient CA-1) (figure 4A). The observed difference in detectable immune memory between patients with inhalation versus cutaneous anthrax was significant ( $P = .02$ , 2-tailed Fisher's exact test). PA-specific IgG memory B cell frequencies observed in patients with inhalation anthrax were comparable to or higher than frequencies of PA-specific immune memory observed in persons vaccinated with the US-licensed anthrax vaccine adsorbed (AVA) (BioPort) (figure 4A and authors' unpublished data). One of the patients with cutaneous anthrax (CA-7) in whom PA-specific IgG B cell memory was detected was also the only patient with cutaneous anthrax in whom *B. anthracis* bacteremia was detected. The sizes and spot intensities of PA-specific antibody-secreting cells in the ELISPOT assay were comparable in the patients with cutaneous and the patients with inhalation anthrax for whom responses were detected (figure 4B), suggesting that there was no qualitative difference in the memory B cells detected.

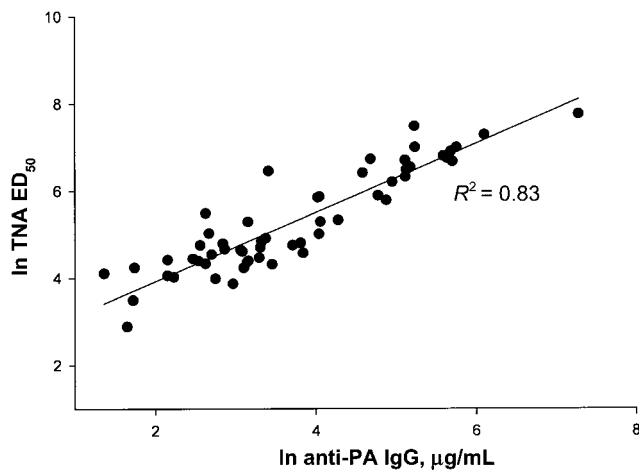
Linear regression analysis indicated a positive correlation ( $R^2 = 0.74$ ) between peak anti-PA IgG levels and the development of B cell memory ~12 months after the onset of symptoms (figure 5A), even though the number of available serum samples was limited. Anti-PA IgG levels and PA-specific IgG memory B cell frequencies obtained from the same venipuncture also revealed a positive correlation between peak anti-PA IgG levels and the development of B cell memory ( $R^2 = 0.57$ ) (figure 5B).

## DISCUSSION

The diagnosis of anthrax is based on characteristic clinical features in combination with appropriate environmental exposure, supplemented, if antibiotic treatment has not been initiated, by culture isolation of *B. anthracis* from the patient [28] or by



**Figure 2.** Anti-protective antigen (PA) IgG levels in patients with cutaneous anthrax, 0–64 weeks after the onset of symptoms. At least 3 serum samples were available from 11 of the 12 patients. Anti-PA IgG was detected as early as 12 days after the onset of symptoms (24 days after exposure). The latest time point at which a patient with cutaneous anthrax (CA-3) had an initial detectable anti-PA IgG level was 34 days after the onset of symptoms (41 days after his confirmed exposure date). In patient CA-10, anti-PA IgG was not detected in acute or convalescent serum 4, 5, 47, and 253 days after the onset of symptoms. Data points represent the mean of at least 2 tests, run in duplicate.

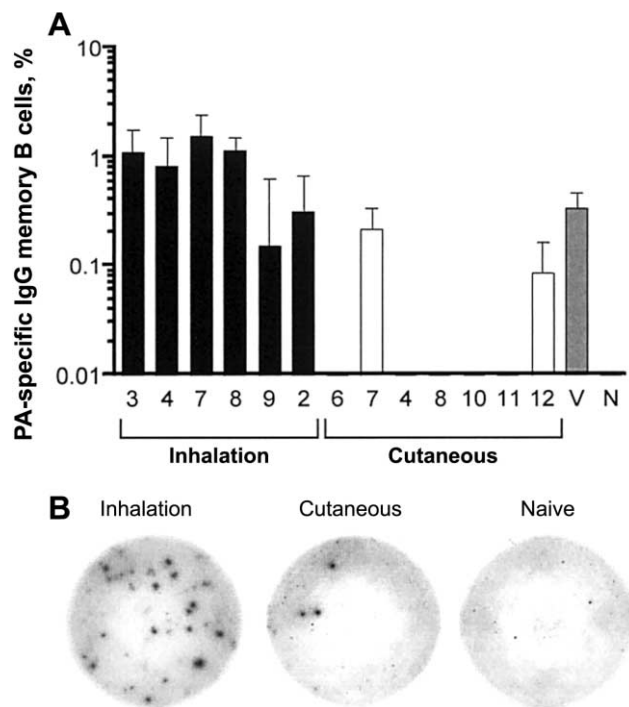


**Figure 3.** Relationship between anti-protective antigen (PA) IgG levels and lethal toxin neutralization activity (TNA) ED<sub>50</sub> levels, in patients with cutaneous and inhalation anthrax who survived. There was a strong positive correlation between anti-PA IgG levels and TNA in both the patients with cutaneous and the patients with inhalation anthrax ( $R^2 = 0.83$ ). In humans, PA is a major toxin neutralizing-antibody determinant for both cutaneous and inhalation anthrax. Patient IA-3 received high-dose steroid therapy and daily plasmapheresis for 2 weeks beginning after serum-sample collection on day 14; data pertaining to serum samples collected during this treatment period were excluded from this figure.

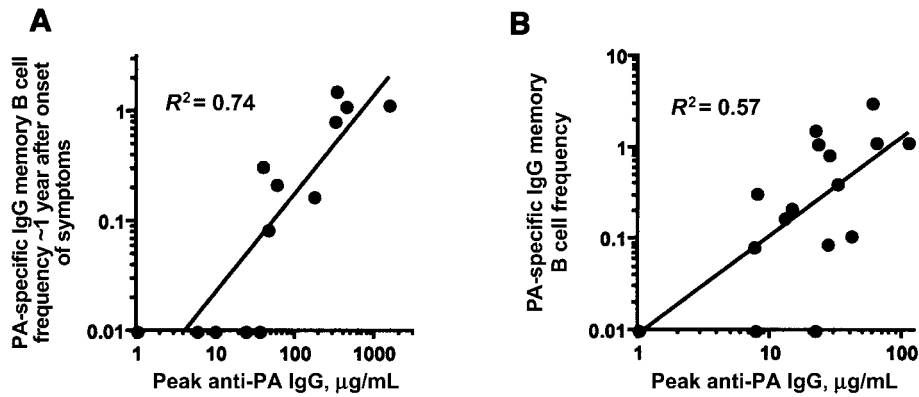
detection of *B. anthracis* antigens or nucleic acid [18, 29]. Because of the acute and often fatal nature of untreated inhalation or systemic anthrax, the measurement of an immune response has not been a prominent feature of diagnosis. Immunodiffusion assays for anthrax were developed in the 1950s, to support evaluation of anthrax vaccines for human use, but were rarely used clinically. Subsequently, an indirect microhemagglutination assay and ELISAs with varying degrees of specificity and sensitivity were developed for the detection of anti-PA antibodies [13–15, 30–35]. The value of these tests as retrospective diagnostic or epidemiologic tools for studies of cutaneous and gastrointestinal anthrax has been demonstrated [13–16]. There is, however, still a general paucity of information on the onset, magnitude, and duration of the human anti-PA immune response to clinical anthrax [16].

In an outbreak setting, when time- or treatment-sensitive diagnostic methods (e.g., culture, polymerase chain reaction, and biopsy) may not be applicable, serological testing may be the only confirmatory diagnostic tool that can be used. Consequently, due to the importance of identifying anthrax cases (and to the implications with regard to treatment and public-health interventions that became apparent during the bioterrorist anthrax attacks of October to November 2001), an anti-PA IgG ELISA was developed that had increased sensitivity, specificity, accuracy, and precision, compared with those of the ELISAs previously available [20]; subsequently, it was validated

in accordance with the guidelines of the International Conference on Harmonization [36]. In the present study, this novel anti-PA IgG ELISA contributed to the diagnosis of 12 of the 22 confirmed or suspected cases of anthrax and was a critical component in the confirmation of 6 cases; in 3 patients with cutaneous anthrax, elevated anti-PA IgG levels in convalescent serum samples were the single confirmatory laboratory finding [18]. In addition to contributing to confirmatory diagnosis, the serological data from this ELISA indicated that the initial mail attack occurred more than a week earlier than was first estimated and placed the location of the initial attack ~1200 miles north of the original estimate—in New York, New York, rather than in Palm Beach County, Florida.



**Figure 4.** Enzyme-linked immunospot (ELISPOT) assay analysis of the frequency of protective antigen (PA)-specific IgG memory B cells, in patients with inhalation and cutaneous anthrax. Surviving patients with bioterrorism-related anthrax were tested for PA-specific IgG B cell memory 8–14 months after the onset of symptoms, by screening peripheral-blood mononuclear cells (PBMCs) using a novel ELISPOT-based functional memory B cell assay. *A*, PA-specific IgG memory B cells were detected in serum samples from all 6 of the patients with inhalation anthrax who survived and in 2 of the 7 patients with cutaneous anthrax who were tested. Patients are indicated on the abscissae by their cutaneous- or inhalation-anthrax case no. Data for a representative vaccinated individual ( $\geq 4$  doses of anthrax vaccine adsorbed) who had received a booster dose within the last year are shown for comparison (V). Data for a naive individual (N) are shown as a negative control. The lower limit of detection was 0.002–0.01 antigen-specific cells/ $10^6$  PBMCs. *B*, ELISPOT images of PA-specific IgG memory B cells, from CA-7, from a representative patient with inhalation anthrax, and from a naive individual (shown as a control).



**Figure 5.** A, Relationship between peak anti-protective antigen (PA) IgG levels and the subsequent magnitude of PA-specific IgG B cell memory ~1 year after the onset of symptoms ( $R^2 = 0.74$ , linear regression). Nonresponders are graphed at the origin. B, Comparison of anti-PA IgG levels and PA-specific IgG memory B cell frequencies, obtained from the same venipuncture. A positive correlation was observed ( $R^2 = 0.57$ , linear regression). Data represented in this graph were from venipuncture taken between 3 and 14 months after exposure. Nonresponders are graphed at the origin.

The TNA assay used in the present study was developed to measure the functional ability of antibodies to neutralize the LTx cytotoxicity of J774A.1 cells or other suitable cell lines or cells [21, 37]. It was applied here to determine the relationship between anti-PA IgG levels and LTx neutralization during clinical anthrax and to evaluate the neutralizing potential of serum before the emergence of a specific IgG response. The human memory B cell assay, which is based on strategies used to quantify murine memory B cells [38], was also developed to quantify PA-specific IgG memory B cells in individuals receiving AVA.

The unprecedented availability of serial serum samples from patients with inhalation and cutaneous anthrax, in combination with the development of sensitive, specific, and quantitative serological and IgG memory B cell assays, allowed us to analyze the onset, magnitude, and duration of the antibody response to the pivotal anthrax protein, PA, and the relationship between antibody function and the establishment of immunological memory against clinical anthrax. Serum samples collected  $\leq 10$  days after the onset of symptoms (the acute phase) did not prove to be useful diagnostically when tested for anti-PA IgG or LTx neutralization. In serum samples for which an anti-PA IgG response was measurable, it was first quantifiable 11 days after the onset of symptoms in the patients with inhalation anthrax who survived and 12 days after the onset of symptoms in the patients with cutaneous anthrax. The peak anti-PA IgG responses in the patients with inhalation anthrax were high, compared with those in the patients with cutaneous anthrax as well as with antigen-specific responses observed for other infectious diseases and vaccines for which similar antibody-quantification technologies are used [39–42], and were comparable to those in individuals receiving up to 4 doses of AVA (V. Semenova, unpublished data).

LTx neutralization was observed only in serum samples in which anti-PA IgG was quantifiable by ELISA. In these serum

samples, there was a positive association ( $R^2 = 0.83$ ) between anti-PA IgG and TNA  $ED_{50}$  levels. The correlation between anti-PA IgG and TNA  $ED_{50}$  levels was similar in patients with cutaneous and patients with inhalation anthrax (data not shown). In the current TNA assay, LF is present in a stoichiometric excess over the minimum needed to form an active LTx (PA plus LF) complex [43]. We interpret the strong correlation between anti-PA IgG and TNA  $ED_{50}$  levels as evidence that the assay emphasized the anti-PA component of the antibody immune response in these serum samples.

The serological response curves for the 3 highest responding patients with cutaneous anthrax (CA-1, CA-2, and CA-7) were incomplete, and their peak responses were not determined. Of the patients with cutaneous anthrax, CA-1 had the highest measured anti-PA IgG level (163.4  $\mu\text{g/mL}$ ) and was approaching the level of magnitude observed in patients with inhalation anthrax. CA-7 had documented evidence of *B. anthracis* bacteremia. In another patient with cutaneous anthrax, however, anti-PA IgG was not detected in either acute or convalescent serum; for this patient, diagnosis was confirmed by exposure history, clinically compatible lesions, and immunohistochemical detection of *B. anthracis*-specific cell wall and capsule antigens in skin-biopsy samples. This patient received antibiotics within 2 days of the onset of symptoms, which may have minimized *B. anthracis* toxin antigen expression and influenced the magnitude and duration of the anti-PA IgG response [44]. Due to differences in treatment regimens and the small number of samples available, it was not feasible to evaluate the effect that antibiotic treatment has on anti-PA IgG responses.

*B. anthracis*-associated antigens other than PA have been proposed to contribute to a protective immune response to anthrax [45, 46]. Nonetheless, the immune response to PA is considered to be central to neutralization of anthrax toxin activity and protection from *B. anthracis* infection [45]. In addition, PA is the

primary active component of AVA [9, 10, 33], recombinant PA is the antigenic component for anthrax vaccines currently in development, and antibody to PA has been used as the protective correlate for licensed and research anthrax vaccines. However, the threshold or magnitude of anti-PA IgG response that correlates with protection against anthrax in humans is not known. Defining the relationship between anti-PA IgG levels and LTx neutralizing responses in individuals with bioterrorism-related cutaneous and inhalation anthrax is, therefore, important for understanding protective immunity to *B. anthracis* in humans. Although recognizing that treatment regimens might modulate the measurable immune responses to clinical anthrax, the data from the present study support the conclusion that there is a consistent positive correlation among the magnitude of the peak anti-PA IgG response, detectable frequencies of PA-specific IgG memory B cells, and LTx neutralization.

Although anti-PA antibodies are an important immunological correlate of protection against anthrax [9, 37, 47], persisting PA-specific IgG memory B cells may also contribute, due to their ability to proliferate and differentiate rapidly into anti-PA antibody-secreting plasma cells. For example, in rhesus macaque models of anthrax, serum anti-PA IgG levels have been shown to decrease over time to low or undetectable levels with only a partial loss of protection against aerosol challenge with virulent *B. anthracis* [48]. This finding may indicate that persisting memory B cells capable of replenishing neutralizing antibody after *B. anthracis* challenge are responsible for protection at late time points. It has been demonstrated that memory B cells persist in smallpox vaccine-immunized individuals for >50 years [25]. Our data suggest that quantitative analysis of PA-specific IgG B cell memory may prove to be a useful predictor of the duration of protection against anthrax and that peak anti-PA IgG levels after infection or vaccination may be a surrogate marker for the presence and magnitude of PA-specific IgG memory B cells.

PA-specific IgG memory B cell frequencies observed in patients with inhalation anthrax were comparable to or higher than those observed in the AVA vaccinees, suggesting that both survivors of inhalation anthrax and vaccinees develop long-term protective immunity to anthrax. The detection of PA-specific IgG B cell memory was significantly lower in patients with cutaneous anthrax. The lack of detectable PA-specific IgG B cell memory (<0.01 cells/10<sup>6</sup> PBMCs) in most of the patients with cutaneous anthrax may suggest that these individuals did not develop long-term protective immunity. Alternatively, this observed difference in PA-specific IgG B cell memory in the blood may have been a reflection of systemic versus local immunity, in which case the patients with cutaneous anthrax with undetected memory B cells in the blood were still unlikely to have acquired protective immunity.

In the present study, we have demonstrated that, in bioter-

rorism-related *B. anthracis* infection of humans, a strong positive correlation between anti-PA IgG levels and in vitro serum anti-toxin activity exists throughout acute infection and convalescence, implying that the potential contribution of anti-PA IgM or IgA to neutralization was negligible. In vitro TNA ED<sub>50</sub> declined with anti-PA IgG levels. Our data indicate that anti-PA IgG levels peaked 4–8 weeks after the onset of symptoms in the patients with inhalation anthrax and 7–14 weeks after the onset of symptoms in the patients with cutaneous anthrax and that anti-PA IgG levels remained detectable, although declining, in both cohorts for >1 year after the onset of symptoms. The correlation between peak anti-PA IgG levels and detectable anti-PA IgG memory B cells also suggests that peak anti-PA IgG levels may be a readily quantifiable surrogate marker for the development of protective immune memory. These relationships may have significant implications for the identification of surrogate markers of protection against anthrax and for the establishment of efficacy criteria for an anthrax vaccine regimen.

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