Protective Antigen and Toxin Neutralization Antibody Patterns in Anthrax Vaccinees Undergoing Serial Plasmapheresis

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Recipients of licensed anthrax vaccine (AVA; Biothrax) could serve as a source of hyperimmune plasma and immunoglobulin for therapy and prophylaxis. We measured serum antibodies during serial weekly to biweekly plasmapheresis in 38 individuals previously vaccinated with 4 to 27 doses of AVA. Immunoglobulin G (IgG) to protective antigen (PA) and toxin neutralization assay (TNA) antibody levels were highly correlated (r = 0.86930 and P < 0.0001 for anti-PA concentration versus TNA concentration). Significant decreases in antibody titer and concentration were observed over time when compared for the number of days from the last AVA injection (P < 0.0001 for both anti-PA and TNA concentration) and for the number of days from the first plasmapheresis (P = 0.0007 for anti-PA concentration and P = 0.0025 for TNA concentration). The rate of the decrease in total IgG concentration (half-life [t1/2] = 198.90 days after first plasmapheresis) was significantly less than the decrease in anti-PA IgG (t1/2 = 63.53 days) (P < 0.0001), indicating that the reduction in anti-PA IgG was more likely due to natural decay than plasmapheresis. The time since the last injection and the time after initial plasmapheresis are important elements in considering an optimal schedule for collecting anthrax hyperimmune plasma. Good correlation between IgG to PA and TNA antibodies suggests that the anti-PA enzyme-linked immunosorbent assay can be used as a high-throughput screen for functional immune reactivity in donor plasma units.

In 2001, bioterrorism attacks in the United States resulted in 11 cases of inhalation anthrax and 11 cases of cutaneous anthrax (7, 17, 18). Among these, five deaths from inhalation anthrax occurred, despite the use of appropriate antibiotics and intensive supportive care. A critical need exists to develop adjunctive therapies for managing patients with systemic infection with Bacillus anthracis.

At a time when the threat to large populations of susceptible individuals from biological weapons has evolved from the hypothetical to the real, immune plasma and/or immune globulins have emerged as potentially important components to vaccine and antibiotic countermeasures (4). In the preantibiotic era, passive immunotherapy formed a mainstay for managing many infectious conditions (5). Today, immune plasma or purified immune globulins derived from hyperimmunized animals and previously vaccinated humans continue to be used as therapy and/or prophylaxis for a variety of viral (2, 10, 30, 37, 41) and bacterial (1, 6, 15, 42) diseases or intoxications.

The lethality of anthrax is primarily due to the effects of toxins (lethal toxin and/or edema toxin) elaborated by B. anthracis throughout the infectious cycle. Immuno-therapy, in conjunction with antibiotics, represents one option for addressing the extremely high case fatality rates associated with systemic anthrax by interfering with toxin activity at multiple stages in the pathogenic process. In 1965, as a result of observing the successful treatment of inhalation anthrax in nonhuman pri-mates using a combined regimen of penicillin, anthrax immune plasma of equine origin, and vaccine, Lincoln et al. proposed that antiserum be administered concurrently with antibiotics to counter toxin release as bacterial cells were lysed by antibiotics (21). In recent years, our understanding of toxin expression, the mechanisms of toxin activity, and the role of anthrax toxins in the various stages of infection have been greatly enhanced. With these insights, a role for specific antibody therapy in neutralizing anthrax toxins and recovery from intoxication has emerged (32, 39).

Recipients of anthrax vaccine are a potential source of hyperimmune plasma and fractionated immunoglobulin for therapy and prophylaxis. In 2002, a collaborative program involving the U.S. Centers for Disease Control and Prevention (CDC), the Department of Defense, and the National Institutes of Health (NIH) was established to procure anthrax immune plasma for assessing efficacy in animal models and to make available a therapeutic agent for contingency use in humans under an investigational protocol. We sought to characterize levels of neutralizing and immunoglobulin G (IgG) to protective antigen (PA) antibodies in this group of donors to better inform current and future collection and testing strategies for this potentially promising therapeutic modality.

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Recipients of licensed anthrax vaccine (AVA, Biothrax) could serve as a source of hyperimmune plasma and immunoglobulin for therapy and prophylaxis. We examined serum antibody patterns during serial weekly to biweekly plasmapheresis in 38 individuals previously vaccinated with 4-27 doses of AVA. Immunoglobulin G (IgG) to protective antigen (PA) and toxin neutralization assay (TNA) antibody levels were highly correlated with $r = 0.86930$ and $P < 0.0001$ for anti-PA concentration versus TNA concentration). Significant decreases in antibody titer and concentration were observed over time when compared for the number of days from the first injection ($P = 0.0001$ for both anti-PA and TNA concentration) and for the number of days from the first plasmapheresis ($P = 0.0007$ for anti-PA concentration and $P = 0.0025$ for TNA concentration). The rate of the decrease in total IgG concentration (half-life $t_{1/2} = 198.90$ days after first plasmapheresis) was significantly less than the decrease in anti-PA IgG ($t_{1/2} = 63.53$ days ($P = 0.0001$), indicating that the reduction in anti-PA IgG was more likely due to natural decay than plasmapheresis. The time since the last injection and the time after initial plasmapheresis are important elements in considering an optimal schedule for collecting anthrax hyperimmune plasma. Good correlation between IgG to PA and TNA antibodies suggests that the anti-PA enzyme-linked immunosorbent assay can be used as a highly-throughput screen for functional immune reactivity in donor plasma units.
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**MATERIALS AND METHODS**

**Study design.** A protocol to collect plasma from individuals who had received at least four doses of anthrax vaccine (AVA) and were within 3 to 12 weeks of their last vaccination if they had received four to six inoculations or within 6 months of their last vaccination if they had received seven or more AVA inoculations was reviewed and approved by institutional review boards at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), the NIH, and the CDC, as well as the Human Subjects Research Review Board in the Office of the U.S. Army Surgeon General. Volunteers were recruited from the ranks of USAMRIID personnel at risk of laboratory exposure to anthrax. Prospective donors provided informed consent and then were screened and enrolled if they met allogeneic donor eligibility criteria in compliance with American Association of Blood Banks standards and U.S. Food and Drug Administration regulations. Weekly to biweekly plasmapheresis was performed at the NIH Clinical Center Department of Transfusion Medicine. Volunteers were asked to provide between 600 ml and 800 ml of plasma per donation, depending upon body weight. Serum samples for antibody measures were collected at the time of plasmapheresis. Informed consent was obtained from each individual before any procedure was performed. The study was performed in accordance with International Committee on Harmonisation guidelines for good clinical practice and with the Declaration of Helsinki.

**Laboratory studies.** Antibodies to *B. anthracis* PA were measured using a modification of a previously described indirect enzyme-linked immunosorbent assay (ELISA) (34). Briefly, twofold serial dilutions of serum from 1:800 to 1:1,02,400 were made in predefined regions of 96-well plates coated with recombinant protective antigen (rPA) (Science Applications International Corp., Frederick, MD). Twofold dilutions of an anti-AVA standard human reference serum (AVR414; CDC, Atlanta, GA) (40) from 1:200 to 1:1,280 were made in different wells of each coated plate. Positive serum controls with known high, medium, and low concentrations of anti-rPA IgG and a negative serum control were also included in each plate. Plates were incubated at 37°C for 60 min and washed three times. A peroxidase-conjugated goat anti-human IgG antibody (Kirkegaard and Perry, Gaithersburg, MD) was then added to detect bound antigen colorimetrically. Color development was after a 30-min incubation by adding peroxidase stop solution (Kirkegaard and Perry). Optical density (OD) values were read within 30 min by using a Bio-Tek ELx808 plate with 405-nm and 490-nm filters and KC4 software (Bio-TEK Instruments, Inc., Winooski, VT). OD values were converted to immunoglobulin concentration (in µg/ml) by using a standard curve calibration factor (36). Antibody concentration was determined at the dilution nearest the inflection point of a standard serum curve. Titers were expressed as the reciprocal of the highest dilution of the test serum yielding a mean OD value equal to or greater than the cutoff value (0.253) (as described in reference 36) for the assay.

*B. anthracis* lethal toxin neutralization activity was measured using a validated colorimetric toxin neutralization assay (TNA) (14, 34). In brief, confluent monolayers of J774A.1 (mouse macrophage) cells were grown in 96-well plates and used after overnight incubation. Twofold dilutions of test (from 1:50 to 1:1,024) and anti-AVA reference standard (AVR414) (from 1:100 to 1:1,280; initial dilution determined from antibody levels measured by ELISA) sera made in 96-well titration plates were combined with recombinant *B. anthracis* PA (rPA) (Science Applications International Corp.) and lethal factor (List Biologicals, Campbell, CA). After a 1-h incubation at 37°C, the serum-lethal toxin mixtures were then added to the cell monolayers and incubated at 37°C for 4 h. Cell viability after exposure to the serum-toxin mixtures was determined by adding thiazolyl blue (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) in 50% dimethyl formamide (Sigma) to each plate. After incubation at 37°C overnight, OD values were read at 570 nm by using a Bio-Tek ELx808 reader and KC4 software (Bio-TEK Instruments, Inc.).

A four-parameter logistic fit of the OD data was used to determine serum TNA antibody titers (the dilution of serum resulting in 50% neutralization of anthrax lethal toxin, or ED₅₀). The neutralization capacity of each test serum in relation to that of the reference serum (50% neutralization factor, or NF₅₀, also known as the neutralization ratio) (14) was calculated from the quotient of the ED₅₀ of the reference serum and the ED₅₀ of the test serum. The concentration of immunoglobulin (in µg/ml) was determined by using a standard curve calibrating factor.

Total IgG antibody concentrations were measured by nephelometry under commercial contract (Esoterix, Inc., Austin, TX).

**Statistical analysis.** No outliers were identified in ELISA or TNA titer or concentration variables. After log₁₀ transformations were applied, the dependent variables met assumptions of normality and homogeneity of variance. Pearson product-moment correlation was used to compute correlation coefficients between ELISA and TNA values. Repeated measures mixed model analysis of variance (ANOVA) was used to compare values over time and between IgG measures. Days from last anthrax vaccine injection and days from first plasmapheresis were both used as measures of time. The rate of antibody decay was derived from the negative slopes of the regression lines for total IgG, IgG to PA, and TNA antibody concentrations versus time in days. Half-life (t₁/₂) was calculated as the time required for antibody concentrations to decrease by 50% from the initial value. All analyses were conducted using SAS version 8.2 (SAS OnlineDoc, version 8, 2000, SAS Institute Inc., Cary, NC).

**RESULTS**

**Donor characteristics.** Serum samples were collected during serial weekly to biweekly plasmapheresis of 38 subjects who met protocol inclusion criteria. The majority of donors were Caucasian (87%) and male (84%) (Table 1). The median age was 37 years (range = 20 to 59 years). The median number of AVA doses received by donors was seven (range = 4 to 27); 42.1% had received four to six doses and 31.6% had received 10 or more vaccinations. The median number of plasma donations per study volunteer was six (6 donors donated one to three times, 7 donated four to five times, and the remaining 25 donated six times each); a total of 196 procedures were performed. The time interval from the last AVA dose received to the initial plasmapheresis in donors ranged from 5 to 144 days (median = 29 days), and the time from the last AVA dose received to the final plasmapheresis in donors ranged from 28 to 197 days (median = 88 days). The length of time from initial plasmapheresis to last plasmapheresis was 0 (for one-time donors) to 118 days (median = 53 days). The median time between successive donations was 7 days (range = 6 to 63 days).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Range 20–59</td>
</tr>
<tr>
<td>Mean</td>
<td>37.60</td>
</tr>
<tr>
<td>Median</td>
<td>37</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 32 (84%)</td>
</tr>
<tr>
<td></td>
<td>Female 6 (16%)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>Caucasian 33 (87%)</td>
</tr>
<tr>
<td></td>
<td>African American 2 (5%)</td>
</tr>
<tr>
<td></td>
<td>Hispanic 3 (8%)</td>
</tr>
<tr>
<td>AVA dose no.</td>
<td>4–27</td>
</tr>
<tr>
<td>Mean</td>
<td>8.68</td>
</tr>
<tr>
<td>Median</td>
<td>7</td>
</tr>
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AVA is anthrax vaccine absorbed (Biothrax).

**Antibody levels.** IgG to PA and TNA antibody levels in serum from study subjects varied widely. IgG to PA dilutional titers ranged from 1:3,200 to 1:204,800 (geometric mean titer = 1:19,184), while IgG to PA concentrations ranged from 38 µg/ml to 1,991 µg/ml (geometric mean concentration = 186 µg/ml) (Table 2). The distribution of TNA antibody titers was 1:86 to 1:15,049 (geometric mean titer = 1:844), and that of TNA antibody concentrations was 13 µg/ml to 1,917 µg/ml.
Correlations between IgG to PA and TNA antibodies. While levels of antibody varied widely by plasma donor and over time, strong correlations for individual samples were observed with IgG to PA and TNA antibody titers \( r = 0.85251, P < 0.0001 \) as well as IgG to PA and TNA antibody concentrations \( r = 0.86930, P < 0.0001 \) (Fig. 4). There was no correlation between total IgG and IgG to PA concentrations \( r = -0.03, P = 0.6722 \) (data not shown).

**DISCUSSION**

Immunotherapy for anthrax has a long-standing historical precedent. During the first half of the 20th century, parenteral administration of equine hyperimmune anthrax antiserum was used successfully to cure cutaneous disease in humans (12, 16, 19, 27, 28, 29). Therapeutic response was dose dependent, but no controlled studies were performed, and other than volume, details of the antibody preparations used (concentration, potency, etc.) were not reported. Although largely supplanted by antimicrobial agents, in some parts of the world equine antibody preparations continue to be used in managing this condition (9).

The utility of immune antiserum in therapy of inhalation anthrax has been studied for many years in animal systems. Polyclonal equine antiserum raised against the *B. anthracis* Sterne strain proved effective in treating inhalation anthrax in nonhuman primates when administered intramuscularly once (day 1) after aerosol exposure or twice (days 1 and 6) after exposure; 40 to 45% survival (compared with 90% lethality in controls) was achieved, and mean time to death was delayed from 5 to more than 20 days (13). In later studies, 69% cure rates were achieved in lethally challenged rhesus macaques treated with equine antiserum in conjunction with penicillin (22), and 84% cure rates were achieved when antiserum was combined with antibiotics and vaccine (21).

Improved survival and delays in time to death in guinea pigs were demonstrated after administration of polyclonal rabbit serum raised against *B. anthracis* PA up to 96 h after lethal anthrax spore challenge (20, 23). Protective effects appeared to correlate with toxin neutralization activity in this model (20, 38). Of interest, similar success with murine monoclonal antibodies to PA selected for high toxin neutralization activity was not achieved in guinea pigs (20, 23). Murine monoclonal anti-PA and anti-lethal factor did prove effective in neutralizing lethal toxin in an in vivo rat model, however (25, 26). Recently, recombinant antibody fragments engineered by fusing toxin-neutralizing, monoclonal, single-chain variable fragments to a human constant \( \alpha \) domain (31) and by generating phage display libraries bearing Fab fragments derived from blood or bone marrow of AVA-vaccinated donors (44) were shown to be capable of protecting rats from lethal toxin challenge.

We examined the dynamics of polyclonal IgG antibodies to PA in humans previously vaccinated with anthrax vaccine adsorbed who subsequently underwent serial plasmapheresis. Although wide (>10-fold) variations in IgG to PA and TNA antibody titers and concentrations were observed among plasma donors in this study, we were able to discern patterns in antibody dynamics that should prove useful in designing future plasma collection strategies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Maximum</th>
<th>Minimum</th>
<th>Median</th>
<th>Geometric mean</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG to PA</td>
<td>Reciprocal titer</td>
<td>204,200</td>
<td>3,200</td>
<td>12,800</td>
<td>19,184</td>
</tr>
<tr>
<td></td>
<td>Concentration (( \mu g/ml ))</td>
<td>1,991</td>
<td>38</td>
<td>170</td>
<td>186</td>
</tr>
<tr>
<td>TNA</td>
<td>Reciprocal titer (( ED_{50} ))</td>
<td>15,049</td>
<td>84</td>
<td>897</td>
<td>844</td>
</tr>
<tr>
<td></td>
<td>Concentration (( \mu g/ml ))</td>
<td>1,917</td>
<td>13</td>
<td>140</td>
<td>126</td>
</tr>
</tbody>
</table>
centrations, we were, unfortunately, unable to perform these analyses. In addition, we were unable to assess the contribution of IgM to our study findings. It is noteworthy, however, that in the study by Quinn et al. of anthrax antibodies in victims of bioterrorism-associated anthrax, strong correlations between IgG to PA and TNA antibodies were observed in both early (<4 weeks after infection) and late (>4 weeks after infection) serum samples, suggesting that any IgM to PA present did not make a significant contribution to neutralizing activity (35).

Concerns over the potential impact of serial plasmapheresis on immune competence and overall health have prompted critical assessments by regulatory agencies to ensure the well-being of donors. Studies have shown that weekly plasma

FIG. 1. IgG antibody to B. anthracis PA concentrations in sera from plasma donors. Antibody levels declined significantly over time for days from last AVA vaccination ($P < 0.0001$) (A) and for days from initial plasmapheresis ($P < 0.0007$) (B).
donors may experience declines in total IgG, IgA, and IgM concentrations during the initial 4 to 6 months of serial plasmapheresis, with subsequent stabilization (8, 11). Considerable individual variability exists, however (3), and no significant impact on humoral and cellular immunity has been documented (43). We observed a small but consistent and statistically significant reduction in total IgG and IgG to PA antibody levels during the period after initial plasmapheresis in this study cohort. Comparing IgG to PA and TNA with total IgG concentrations in the same study subjects revealed differential rates of decline, indicating that the observed temporal changes in anti-PA and TNA levels were due primarily to natural an-

FIG. 2. *B. anthracis* TNA antibody concentrations in sera from plasma donors. Antibody levels declined significantly over time for days from last AVA vaccination (*P* < 0.0001) (A) and for days from initial plasmapheresis (*P* < 0.0001) (B).
FIG. 3. Comparative rates of decline between IgG antibody to B. anthracis PA concentrations and total IgG antibody concentrations in sera from plasma donors. The rate of decrease in total IgG concentration was significantly less than that for the IgG to PA concentration over the interval from the last AVA injection ($P < 0.0001$) (A) and over the interval from initial plasmapheresis ($P < 0.0001$) (B). Regression lines and respective equations are included.
FIG. 4. Correlations between IgG to B. anthracis PA and TNA antibody titers (A) and concentrations (B). Regression lines and correlation coefficients (R) are included for each plot.
tibody decay rather than plasmapheresis. We were unable to assess the impact of weekly versus biweekly plasmapheresis due to limited sample size and the uncontrolled nature of the study. Nevertheless, our findings that IgG to PA and TNA antibody concentrations decreased relatively slowly after initial plasmapheresis ($t_{1/2} = 63.35$ days and 57.30 days, respectively) and that geometric mean antibody concentrations after repeated donations generally remained above levels correlating with protection against aerosol challenge in the rabbit model (24, 33) suggest that plasmapheresis conducted at weekly intervals is not likely to influence the degree of humoral immune protection afforded vaccinees.

Initial measures of total IgG concentrations in our study subjects were in the normal range (784 mg/dl to 1,610 mg/dl; mean = 1,032.19 mg/dl), and the prolonged rate of decay observed ($t_{1/2} = 198.90$ days) was not unexpected because of continual stimulation of new and different antibody-producing cells over time. Similarly, the $t_{1/2}$ observed for IgG to PA (63.53 days) was considerably longer than that reported for human IgG (7 to 23 days, depending upon subclass). While the continued production of antibodies specific for PA following vaccination would be expected to result in prolonged decay rates compared with measures of decay under static (e.g., postinfection) conditions, additional factors may also have contributed to this finding. Possibilities include the depot effect of the vaccine’s alhydrogel adjuvant, which resulted in low-level release of antigen and thus prolonged immune stimulation; promotion of a Th2-type response by alhydrogel which, with multiple vaccinations, would be expected to manifest as a significant increase in IgG4 subclass and apparent prolongation of the response; IgG subclass switching as a consequence of repetitive immunization (57.9% of plasma donors had received seven or more AVA injections), resulting in antibodies with high functional affinity for PA that may have extended half-lives; or other, as yet undefined, factors.

Caution must be exercised in extrapolating findings from this sample to other populations. The relatively limited sample size ($n = 38$ plasma donors), the wide variability in donor characteristics, and the absence of a control group are factors that may have biased our results and limit our ability to predict an optimal time for collecting plasma from immunized vaccinees.

We found significant correlations between IgG to PA and TNA antibody titers and concentrations. Similar correlations were reported among vaccinees participating in a dose reduction and route change study of AVA (34) and for survivors of natural cutaneous and inhalation anthrax (35). These observations provide evidence that, for AVA recipients and B. anthracis infection, a relatively simple biochemical assay (i.e., enzyme immunoassay) can serve as a valid surrogate for functional immune reactivity (i.e., lethal toxin neutralization). It should be possible, therefore, to apply the anti-PA enzyme immunoassay as a high-throughput screen in assessing functional antibody activity contained in donor plasma.

The efficacy and potency of anthrax immune plasma and/or immune globulin in treating systemic anthrax disease in humans has not yet been demonstrated in controlled studies. Additional efforts to more completely understand correlates and determinants of immunity against anthrax, as well as controlled studies to define anti-PA and TNA antibody kinetics, are needed to inform use of hyperimmune plasma and fractionated immune globulin for therapy and/or prophylaxis of this deadly infection.

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REFERENCES


