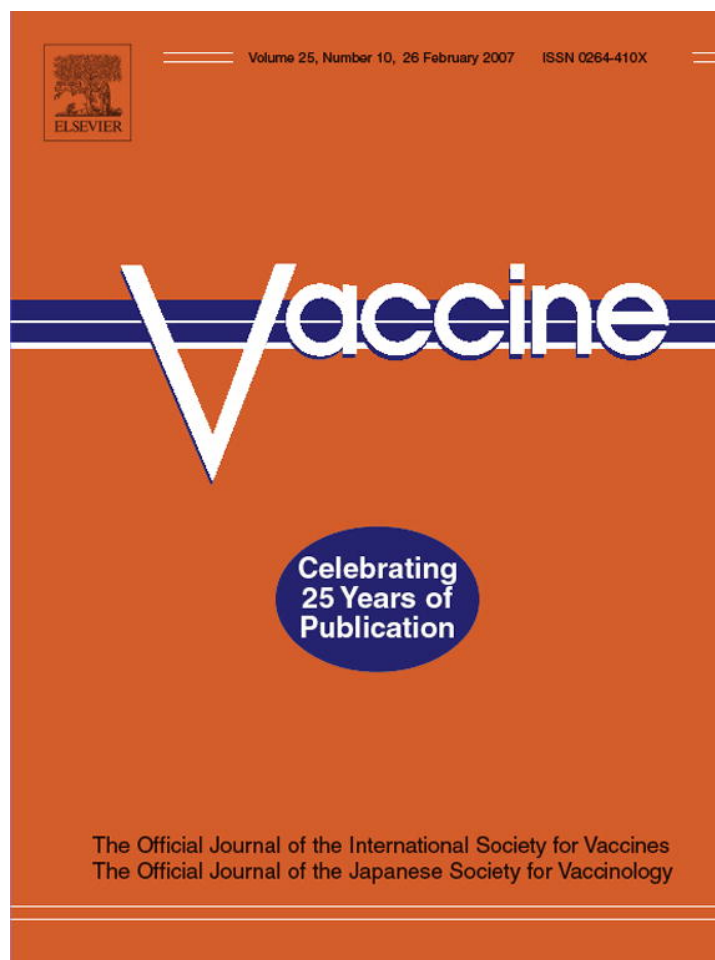


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Analysis of anti-protective antigen IgG subclass distribution in recipients of anthrax vaccine adsorbed (AVA) and patients with cutaneous and inhalation anthrax

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Abstract

The anti-PA IgG1, IgG2, IgG3, and IgG4 subclass responses to clinical anthrax and to different numbers of anthrax vaccine adsorbed (AVA, BioThrax®) injections were determined in a cross-sectional study of sera from 63 vaccinees and 13 clinical anthrax patients. The data show that both vaccination with three AVA injections and clinical anthrax elicit anti-PA IgG1, IgG2, and IgG3 subclass responses. An anti-PA IgG4 response was detected in AVA recipients after the fourth injection. The anthrax lethal toxin (LTx) neutralization efficacy of sera from recipients who received 4 to ≥ 10 AVA injections did not vary significantly in relation to changes in distribution of anti-PA IgG1 and IgG4 subclasses.

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Keywords: AVA; BioThrax®; Anti-PA antibody; IgG subclasses; Anthrax; *Bacillus anthracis*

1. Introduction

Protective antigen (PA) is the pivotal protein of the anthrax toxin complex and the principal immunogen of anthrax vaccines, including anthrax vaccine adsorbed (AVA, BioThrax®; BioPort Corp., Lansing, MI) [1–4]. Studies in animal models indicate that the immune response to PA is central to protection against *Bacillus anthracis* [3–5]. The anti-PA immunoglobulin G (IgG) antibody concentrations and dilutional titers are, therefore, the most commonly reported marker of human immune responses to anthrax vaccines and *B. anthracis* infection [6–8]. Previous reports on the human immune response to the licensed UK anthrax vaccine and

B. anthracis infection showed that there is a difference in anti-PA IgG subclass distributions between vaccine recipients and clinical patients [9]. However, quantitative analyses of human IgG subclass responses to AVA, recombinant PA (rPA) based vaccines or inhalation anthrax and their anthrax lethal toxin (LTx) neutralization efficacies have not been reported. Studies in guinea pigs have correlated the functional characteristics and anti-PA IgG subclass distribution in that genus with survival against virulent *B. anthracis* challenge [10]. Based on these observations and the knowledge that each of the four human IgG subclasses has a unique effector function relevant to the clearance and elimination of a foreign antigen [11,12], we hypothesized that determination of changes in the anti-PA IgG subclass distribution in response to AVA vaccination and human clinical anthrax will help characterize the humoral antibody responses to repeated antigen exposure during the full regimen of AVA vaccination at 0, 2, and 4 weeks and 6, 12, and 18 months. These data will provide new information on serological responses to PA that may be used as a marker for differentiation between

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clinical infection and AVA vaccination [1,13]. The objectives of this study therefore, were to analyze changes in the anti-PA IgG subclass distribution in response to different numbers of AVA injections, to compare these with anti-PA IgG subclass profiles in convalescent sera from humans with clinically confirmed bioterrorism-associated anthrax and to determine the effect of changing subclass proportions on the anthrax lethal toxin (LTx) neutralization activity in these sera.

2. Materials and methods

2.1. Human standard reference serum

A human reference serum AVR414 prepared from plasma of AVA recipients was used for quantification of anti-PA IgG subclasses using anti-PA IgG1–4 subclass-specific enzyme-linked immunosorbent assays (ELISA). The anti-PA IgG subclass concentrations in AVR414 are IgG1 = 79.6 µg/ml, IgG2 = 25.4 µg/ml, IgG3 = 3.2 µg/ml and IgG4 = 25.3 µg/ml [14]. The standard reference serum AVR414 was stored in 3 ml aliquots at ≤70 °C.

2.2. Antigen preparation

Purified recombinant protective antigen (rPA) with an amino acid sequence concurring with the *Bacillus anthracis* V770-NP1-R vaccine strain was provided by Dr. Stephen H. Leppla (National Institute of Allergies and Infectious Diseases, National Institutes of Health, Bethesda, MD). The antigen was purified as described previously [15,16] and stored in 100–500 µl aliquots (4.75 mg/ml) in 5 mM Hepes, pH 7.3 at ≤70 °C.

2.3. Monoclonal antibodies to human IgG subclasses

Monoclonal antibodies to human IgG1 (HP6069 γ1 Fc), IgG2 (HP6002 γ1 Fc), IgG3 (HP6047, anti-hinge region) and IgG4 (HP6025 γ1 Fc) [17,18] were purchased from the Hybridoma Reagent Laboratory (Baldwin, MD). The specificities of these reagents have been documented in an IUIS/WHO collaborative study [18].

2.4. Human sera for anti-PA IgG subclass analyses

The serum samples were obtained from 63 healthy adult volunteers (34 males and 29 females; median age 34.5 years, range 24–65 years). All vaccinees in this study had received a different number of subcutaneous injections of AVA with the licensed regimen of injections at 0, 2, and 4 weeks and 6, 12, and 18 months with annual boosters [1,13]. The serum samples were collected 2 weeks after the first two injections, within 1–3 weeks after the third injection and within 2–5 weeks after injections 4–7 and in also the group with multiple annual boosters (≥10 injections of

AVA). Peak individual anti-PA IgG concentrations were not determined.

Clinical serum samples were collected during the bioterrorism-associated anthrax outbreak of 2001 in the USA and consisted of 13 serum samples available for testing; 6 serum samples were obtained from patients with confirmed inhalation anthrax and 7 serum samples from patients with confirmed cutaneous anthrax. All clinical sera were collected between 13 and 197 days after onset of symptoms (Table 2) [8]. The specimens were aliquoted (100 µl) and stored frozen at –70 °C until testing was performed. All sera were tested in duplicate without heat inactivation. The collection and use of human sera was approved by the Human Investigations Committee of the Centers for Disease Control and Prevention.

2.5. Quantitative enzyme-linked immunosorbent assays (ELISA) for human anti-PA IgG subclasses

2.5.1. Assay procedures

Detection and quantification of each of the human anti-PA IgG subclasses were done in separate assays. Briefly, Immulon® 2 HB microtiter plates (Thermo Labsystems, Franklin, MA) were coated with purified recombinant PA (2 µg/ml) in 0.01 M phosphate buffered saline (PBS) pH 7.4 (Life Technologies, Gaithersburg, MD) and incubated overnight (16–24 h) at +4 °C. Plates were washed three times with PBS containing 0.1% (v/v) Tween 20 (ELISA wash buffer) and the first wells of each dilution series were loaded with 100 µl of each test serum diluted in PBS containing 5% (w/v) Skim Milk (Becton, Dickinson and Company, Sparks, MD) and 0.5% (v/v) Tween-20, pH 7.4 (Serum Diluent) without a separate blocking step. The first dilution of the standard and test sera was 1:50 for IgG1 and IgG4 subclasses, 1:25 for IgG2 and 1:16 for IgG3. The serum was mixed in the plate wells and serially transferred in two-fold dilutions down the plate to make an 8-point dilution series. Plates were incubated for 60 min at 37 °C and washed three times with ELISA wash buffer. In each of the separate subclass detection ELISAs unconjugated monoclonal antibody to each of the human IgG1–4 subclasses was added to the appropriate plates (100 µl/well, 1 µg/ml in PBS) and incubated at 37 °C for 60 min. Plates were then washed three times with ELISA wash buffer and then 100 µl/well of goat anti-mouse IgG-HRP conjugate (1:1000 dilution) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to all of the plates and incubated for 60 min at 37 °C. Plates were again washed three times with ELISA wash buffer and 100 µl/well of ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to all plate wells. After a 30-min incubation at 37 °C, 100 µl of ABTS peroxidase stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and the plates were read within 30 min with a MRX Revelation microtiter plate reader (Thermo Labsystems, Franklin, MA), at a wavelength of 410 nm with a 490-nm reference filter.

2.5.2. Quantification of anti-PA IgG subclasses and total anti-PA IgG

The standard reference serum AVR414 was used as a calibrator to generate a 7-point standard curve in triplicate over a two-fold dilution series in each of the anti-PA IgG1-4 subclass-specific ELISAs [14]. Experiments were repeated a minimum of two times on different assay plates by two different operators. The anti-PA IgG subclass concentrations in test sera were calculated in $\mu\text{g/ml}$ by interpolation from the calibration curve using a four-parameter logistic-log model (4-PL) and ELISA for Windows software (Version 2.0) [19]. Total anti-PA IgG concentration was estimated by summation of data for each IgG subclass at each time point. The appropriateness of estimating total anti-PA IgG by summation of anti-PA IgG subclasses has previously been demonstrated by comparing the values obtained by summation of anti-PA IgG subclasses for nine sera of AVA recipients with the values obtained for these sera by total anti-PA IgG ELISA [7,14].

2.5.3. Performance characteristics of anti-PA IgG1-4 subclass specific ELISAs

Performance characteristics of the anti-PA IgG1-4 subclass ELISAs were evaluated by determination of parallelism between the standard reference serum and test serum dilution curves, precision (repeatability) of the 7-point dilution series of the standard reference serum in each IgG subclass ELISA, limits of detection and “goodness of fit” of the standard’s data for each IgG subclass [7]. Evaluation of parallelism was performed as described elsewhere using a within-assay coefficient of variation (CV) of $\leq 20\%$ as the acceptance criterion [20]. The precision in the anti-PA IgG1-4 subclass ELISAs was determined using positive quality control sera for each IgG subclass ELISA, which were selected from humans vaccinated with a minimum of four injections of AVA. The positive control sera with assigned anti-PA IgG subclass concentrations were used in duplicate as a series of two-fold dilutions. The ‘goodness of fit’ of the assay is, for comparative purposes, an indication of how closely the data points of the reference serum standard curve fit the 4-PL model. It is expressed as the regression coefficient (R^2) of the standard curve. An R^2 value that approaches unity is indicative of a ‘good fit’ for the data to the curve. The lowest concentration of anti-PA IgG detectable in a test serum (lower limit of detection, LLOD) was calculated using the 4-PL model applied to the 95% confidence interval (95%CI) of the AVR414 standard curve [7,21]. The LLOD values for IgG1–IgG4 subclass specific ELISAs were 5.1, 4.1, 0.4 and 1.6 $\mu\text{g/ml}$, respectively and were used as the reactivity thresholds for each relevant assay. Interassay precision of the assays for the four positive controls in each IgG subclass ELISA were all less than 20%; 6.3% for IgG1 positive control ($n=67$ tests), 8.7% for IgG2 ($n=80$ tests), 16.5% for IgG3 ($n=76$ tests) and 8.4% for IgG4 ($n=92$ tests). The goodness of fit (mean R^2) for the standard AVR414 in each of these assays was ≥ 0.99 .

2.5.4. Anthrax lethal toxin (LTx) neutralization activity

The J774A.1 macrophage/monocyte cell line (TIB-67, American Type Culture Collection, Manassas, VA) was cultured in D-MEM supplemented with high glucose (4.5 g/L), 4 mM L-glutamine (Gibco BRL, Gaithersburg, MD), 5% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan UT), 10 mM HEPES buffer solution (Gibco BRL), Penicillin (50 units/ml), streptomycin sulfate (50 $\mu\text{g/ml}$) and 1 mM sodium pyruvate (Gibco BRL). All incubations were at 37 °C in a 5% CO₂ atmosphere, 95% relative humidity. Cultures of J774A.1 cells were harvested into warmed growth medium and plated at 3×10^4 cells/well in 96 well flat bottom microtiter Plates 17–19 h prior to the assay. Test antisera were prepared in a separate 96-well microtiter plate as two-fold dilutions in triplicate and then incubated with anthrax lethal toxin (LTx; 50 ng/ml PA and 40 ng/ml LF) at 37 °C for 30 min. Spent medium was removed from the J774A.1 cells, the toxin-antiserum mix was then transferred (100 $\mu\text{l/well}$) to the J774A.1 cell plate and the incubation continued for 4 h. Cell viability was determined by the addition of 25 $\mu\text{l/well}$ of a 5 mg/ml stock solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemicals, St. Louis, MO) dissolved in 0.01 M phosphate buffered saline (PBS) pH 7.4 (Life Technologies, Gaithersburg, MD) and the incubation continued for 2 h. The assay was terminated by addition of 100 $\mu\text{l/well}$ of 20% (w/v) SDS (Sigma Fine Chemicals, St. Louis, MO), 50% (v/v) *N,N*-dimethyl formamide (DMF) (Fisher Scientific, Pittsburg, PA). OD values were read using a MRX Revelation™ microtiter plate reader (Thermo Labsystems, Franklin, MA) at 570 nm using a 690 nm reference filter.

Assay endpoints were calculated using SAS® version 9.0 (SAS Institute Inc. Cary, NC USA) running an endpoint calculation algorithm developed by the CDC [35]. The primary endpoint used in this study is the reciprocal of a serum sample dilution that results in 50% neutralization of anthrax lethal toxin cytotoxicity (ED50) and corresponds to the inflection point of a 4-parameter logistic log fit of the neutralization curve.

The TNA assay has a lower limit of detection (LLOD) ED50 of 12 and a lower limit of quantification (LLOQ) ED50 of 35.5 (percent error = 16.0%, CV = 13.5%) using human standard reference serum AVR414. The assay has a diagnostic sensitivity of 100% and a diagnostic specificity of 100%.

2.6. Statistical analysis

Datasets were created for the concentration of each IgG subclass ($\mu\text{g/ml}$) and for Lethal Toxin (LTx) neutralization activity (ED50) for AVA recipients and clinical anthrax cases, respectively. For each group (Table 1), proportions of total IgG were computed and treated as continuous random variables. Absolute IgG concentration levels, ED50 values, and anti-PA IgG1-4 subclass proportions were used in the analysis. Data for each IgG subclass in each

Table 1
Changes in IgG subclass distribution and LTx neutralization (ED50) with different numbers of AVA injections

Number of AVA injections	Nominal injection week (serum collection) ^a	Group size (n)	Anti-PA IgG subclass % of total concentration ^b (mean anti-PA IgG concentration in µg/ml ± 1 S.E.)				Mean total IgG (µg/ml) ± 1 S.E.	Mean ED50 ± 1 S.E.
			IgG1	IgG2	IgG3	IgG4		
1	0 (2)	6	<LLOD ^c	<LLOD ^c	<LLOD ^c	<LLOD ^c	<LLOD ^c	<LLOD ^c
2	2 (4)	5	67.2% (81.5 ± 35.1)	26.5% (41.2 ± 23.6)	6.2% (5.6 ± 1.6)	128.6 ± 59.9 ^d	207 ± 80	
3	4 (5–7)	8	66.5% (66.0 ± 22.2)	29.4% (29.4 ± 11.2)	4.0% (5.1 ± 1.9)	100.8 ± 34.8	379 ± 65	
4	26 (28–33)	9	66.0% (125.8 ± 50.2)	23.2% (38.2 ± 35.2)	3.0% (5.3 ± 1.9)	180.4 ± 63.4	1657 ± 507	
5	52 (55–57)	10	67.2% (156.4 ± 51.1)	21.0% (46.7 ± 14.0)	2.8% (6.3 ± 2.0)	223.4 ± 68.6	1108 ± 307	
6	78 (80–83)	7	52.9% (99.2 ± 13.3)	20.4% (37.0 ± 2.8)	3.0% (5.4 ± 0.8)	187.2 ± 18.3	1065 ± 171	
7	130 (132–135)	9	53.0% (152.4 ± 39.7)	21.7% (77.5 ± 31.0)	2.9% (9.4 ± 4.2)	336.6 ± 119.8	1293 ± 280	
≥10	>130 ^e (>130)	9	36.0% (80.8 ± 18.4)	19.6% (43.9 ± 7.9)	2.1% (4.7 ± 1.0)	223.4 ± 35.8	1412 ± 294.7	

^a Results represent the range of serum collection in weeks.

^b Values in are means of each subject's percentage of each subclass total anti-PA IgG. These do not, in general, equate to the overall percentage that might be computed from the mean IgG subclass levels presented in the table.

^c One sample from one participant had measurable anti-PA IgG and LTx neutralization. The group mean value of total IgG and IgG subclasses was however, <LLOD.

^d Discrepancies between the sum of the mean of IgG1, IgG2 and IgG3 subclasses and the average of the sum of total IgG subclasses are due to rounding and excluding values <LLOD.

^e For analytical purposes recipients of ≥10 injections of AVA were grouped and assigned a nominal time point >130 weeks.

group were tested for Normality (Shapiro–Wilcoxon and Kolmogorov–Smirnov tests). For tests where Normality was rejected, non-parametric Wilcoxon or Kruskal–Wallis tests were performed to compare respective groups. In addition, Wilcoxon or Kruskal–Wallis tests were used to compare anti-PA IgG immune response in AVA recipients and clinical patients. For time points and groups where Normality was not rejected, *t*-tests were used. Significance levels were set at $\alpha = 0.05$.

3. Results

3.1. Statistical analysis

The hypothesis that the data were selected from a Normal distribution was rejected for many of the groups of AVA recipients and clinical serum samples. Accordingly, we made comparative tests using the non-parametric Wilcoxon test. The wider range of individuals across the various numbers of injections introduced a wider range of demographics and individual variation, yet captures the statistically measurable changes across time. Even for those groups where the Normality was not formally rejected by statistical test, we performed only the Wilcoxon test, rather than the *t*-test which applies only to Normal distributions. The Wilcoxon is sufficiently robust to be a valid test regardless of the distribution. Analysis of demographics and antibody subclass response found no particular association between age and subclass distribution, or between sex and subclass distribution.

3.2. Anti-PA IgG subclass distribution and neutralization activity in the serum of AVA recipients

Vaccination with AVA elicited all four anti-PA IgG subclasses in human serum. The subclass distribution however, depended on the number of injections received (Table 1). Of six recipients of a single injection of AVA, one had a detectable anti-PA IgG1 response (16.0 µg/ml). The PA-specific IgG subclass response to AVA after the second and third injections primarily consisted of IgG1 (mean concentrations 81.5 µg/ml and 66.0 µg/ml; 67.2% and 66.5% of total IgG, respectively) and IgG2 (mean concentrations 41.2 µg/ml and 29.4 µg/ml; 26.5% and 29.4% of total IgG, respectively) with a low proportion of IgG3 (mean concentrations 5.6 µg/ml and 5.1 µg/ml or 6.2% and 4.0%, respectively) (Table 1). The concentration of IgG1 subclass increased after the fourth injection (mean IgG concentration of 125.8 µg/ml), however, the percentage of IgG1 of total IgG remained similar to that observed after the third injection (66.0%). Recognizing that these may not be peak responses, the concentration of IgG1 continued to increase after the fifth injection (mean concentration 156.4 µg/ml). The IgG1 percentage of total IgG (67.2%); however, remained similar to those after 2–4 injections. IgG1 remained the most abundant subclass after administration of a series of six

doses and one annual booster (seven injections in total), but the mean concentrations decreased from 156.4 $\mu\text{g/ml}$ after the fifth injection to 99.2 $\mu\text{g/ml}$ after the sixth injection. The percentage of IgG1 also decreased between the fifth and sixth injections (67.2% and 52.9%) and remained essentially unchanged (53%) in response to the seventh injection of AVA. The proportion of IgG4 in response to injections six and seven followed a similar trend (23.7% versus 22.4%) (Table 1). The proportion of IgG2 ranged from 19.6 to 29.4% with the highest proportion detected in response to the first three injections and of these the highest mass value was 41.2 $\mu\text{g/ml}$ (Table 1). Both the concentration and the proportion of IgG3 during administration of six primary injections and one annual booster of AVA were consistently low, but detectable (5.1–9.4 $\mu\text{g/ml}$, 2.8–6.2%, respectively). The highest proportion of IgG3 was detected in response to the first two injections (6.2%) and the highest mass value was detected after the seventh injection (9.4 $\mu\text{g/ml}$) (Table 1). An anti-PA IgG4 response was detected only after the fourth injection (11.0 $\mu\text{g/ml}$ or 7.8% of total anti-PA IgG) (Table 1). Both the concentration and the proportion of anti-PA IgG4 trended upwards following the fifth, sixth and seventh injections of AVA (14.0 $\mu\text{g/ml}$, 45.6 $\mu\text{g/ml}$ and 97.3 $\mu\text{g/ml}$, 9.0%, 23.7% and 22.4%, respectively). The percentage of serum IgG4 in recipients of seven injections of AVA increased from 22.4 to 42.3% after 10 injections, although the mass values of IgG1 remained similar (mean of 97.3 and 94.0 $\mu\text{g/ml}$, respectively). The observed increase in percentage of IgG4 coincided with the reduction of IgG1, whose overall proportion decreased from 67.2% after the second AVA injection to 36.0% in the recipients with ≥ 10 injections of AVA ($p < 0.0001$, Kruskal–Wallis test) (Fig. 1).

The mean concentration of total anti-PA IgG after the second and the third injections was 128.6 and 100.8 $\mu\text{g/ml}$, respectively (Table 1). It continued to increase after the fourth and fifth injections (180.4 $\mu\text{g/ml}$ and 223.4 $\mu\text{g/ml}$, respectively). The mean concentration of total anti-PA IgG decreased from 223.4 $\mu\text{g/ml}$ after the fifth injection to 187.2 $\mu\text{g/ml}$ after the sixth injection. The maximum total anti-PA IgG concentration (336.6 $\mu\text{g/ml}$) was observed after the seventh injection of AVA.

Notwithstanding the changes in distribution of anti-PA IgG1 and IgG4 subclasses during the AVA vaccination regimen, the ability of vaccinee sera to neutralize LTx did not change significantly. Of six recipients of a single injection of AVA, the one serum with detectable anti-PA IgG also had detectable LTx neutralizing activity ($\text{ED}_{50} = 27$). The levels of LTx neutralizing activity in sera increased consistently after the second and third injection of AVA (mean $\text{ED}_{50} = 207$ and 379, respectively). The ED_{50} reached maximum values after the fourth injection of AVA (mean $\text{ED}_{50} = 1657$) and remained relatively stable over the remaining vaccination schedule and in the group of recipients with ≥ 10 injections of AVA (mean $\text{ED}_{50} = 1412$, $p = 0.85$, Wilcoxon test) (Table 1).

3.3. Anti-PA IgG subclass distribution and LTx neutralization activity in clinical anthrax sera

Serum samples from six patients with inhalation anthrax and seven patients with cutaneous anthrax were available for IgG subclass analysis. In patients with inhalation anthrax the anti-PA IgG immune response consisted of IgG1, IgG2 and IgG3 subclasses. There was a wide range of concentrations of the detected anti-PA antibodies for each IgG subclass

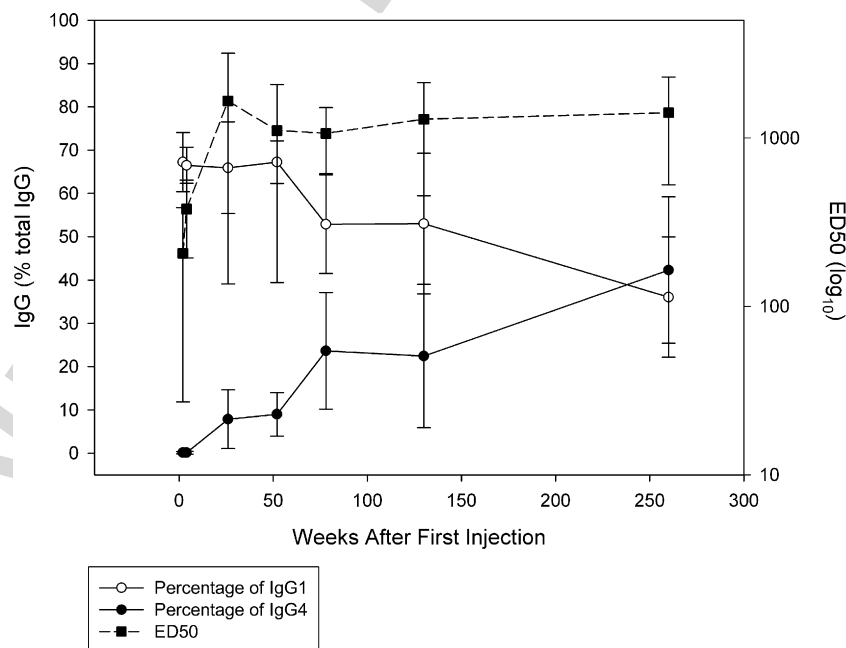


Fig. 1. Relationship between changing IgG1 and IgG4 subclass proportions and LTx neutralization (ED_{50}) over time. All sera were tested in duplicate in two separate tests. Error bars indicate one standard deviation from the mean (IgG1: open circles; IgG4: closed circles; ED_{50} : dashed lines).

Table 2

Percent of total IgG and mean anti-PA IgG subclass concentrations in sera from inhalation and cutaneous anthrax cases

Patient ID ^a	Serum collection (days post onset of symptoms)	Anti-PA IgG subclass % of total concentration ^b and (mean anti-PA IgG concentration in $\mu\text{g/ml} \pm 1 \text{ S.D.}$)				Mean total IgG ($\mu\text{g/ml}$)	Median ED50
		IgG1	IgG2	IgG3	IgG4		
IA-2	197	67.7% (11.9 \pm 0.3)	20.5% (3.4 \pm 0.4)	11.8% (2.0 \pm 0.4)	<LLOD ^c	17	67
IA-9	18	78.5% (32.9 \pm 1.0)	16.7% (7.0 \pm 0.2)	4.8% (2.0 \pm 0.2)	<LLOD	42	333
IA-4	41	57.8% (143.4 \pm 4.2)	29.6% (73.8 \pm 6.1)	12.9% (32.3 \pm 9.2)	<LLOD	250	783
IA-7	46	60.6% (169.4 \pm 17.4)	16.9% (47.1 \pm 1.5)	22.5% (63.0 \pm 13.8)	<LLOD	280	851
IA-8	43	81.5% (837.5 \pm 53.2)	14.6% (150.2 \pm 25.5)	3.9% (40.0 \pm 5.1)	<LLOD	1028	2550
IA-3	13	76.2% (109.8 \pm 28.7)	22.3% (32.2 \pm 9.5)	1.5% (2.2 \pm 0.6)	<LLOD	144	359
CA-4	41	52.9% (6.5 \pm 1.9)	27.6% (3.4 \pm 1.3)	19.5% (2.4 \pm 1.1)	<LLOD	12	75
CA-6	27	78.9% (12.0 \pm 0.3)	<LLOD	21.1% (3.2 \pm 2.5)	<LLOD	15	188
CA-1	179	100% (6.9 \pm 0.7)	<LLOD	<LLOD	<LLOD	7	76
CA-7	95	67.0% (28.8 \pm 3.8)	23.7% (10.2 \pm 2.7)	9.3% (4.0 \pm 0.2)	<LLOD	43	296
CA-9	29	50.8% (9.8 \pm 0.4)	30.1% (5.8 \pm 0.2)	19.1% (3.7 \pm 0.6)	<LLOD	19	74
CA-3	34	59.5% (4.4 \pm 0.6)	40.5% (3.0 \pm 0.9)	<LLOD	<LLOD	7	32
CA-8	57	100% (2.5 \pm 2.1)	<LLOD	<LLOD	<LLOD	3	37

Percent total anti-PA IgG is based on summation of total detected IgG subclasses. Anti-PA IgG subclass concentrations are expressed in $\mu\text{g/ml}$.

ED50 = LTx neutralization activity expressed as the reciprocal of the dilution of serum providing 50% neutralization in vitro.

^a IA = inhalation anthrax case; CA = cutaneous anthrax case.

^b Values are means of each subject's percentage of each subclass to total anti-PA IgG. These do not, in general, equate to the overall percentage that might be computed from the mean IgG subclass levels.

^c Values <LLOD were not included in total anti-PA IgG concentration.

(Table 2). The anti-PA IgG1 subclass concentration in inhalation anthrax patients ranged from 11.9 to 837.5 $\mu\text{g/ml}$ (mean of 217.5 $\mu\text{g/ml}$, 95%CI of mean: -107.8 to 542.8 $\mu\text{g/ml}$), IgG2 ranged from 3.4 to 150.2 $\mu\text{g/ml}$ (mean of 52.3 $\mu\text{g/ml}$, 95%CI of mean: -5.0 to 109.6 $\mu\text{g/ml}$) and IgG3 ranged from 2.0 to 63.0 $\mu\text{g/ml}$ (mean of 23.6 $\mu\text{g/ml}$, 95%CI of mean: -3.3 to 50.5 $\mu\text{g/ml}$) (Table 2). IgG1 was the most abundant subclass (mean of 70.4% of total anti-PA IgG), followed by IgG2 (mean of 20.1%) and IgG3 (mean of 9.6%). The patient IA-4 had the lowest percentage of IgG1 (57.8%) and the highest percentage of IgG2 (29.6%). Patient IA-7 had a similar percentage of IgG1 (60.6%), a low percentage IgG2 (16.9%) and the highest percentage of IgG3 (22.5%) in this cohort. Patient IA-8 had the highest level of IgG1 (837.5 $\mu\text{g/ml}$, 81.5%) with the highest ED50 (median of 2550). The lowest ED50 (median of 67) was in-patient IA-2 with 11.9 $\mu\text{g/ml}$ (67.74%) anti-PA IgG1 at 197 days. With the exception of patient IA-7 the relative magnitudes of the subclass proportions in IA sera at all time points tested were IgG1 > IgG2 > IgG3 (Table 2).

PA specific IgG subclasses IgG1, IgG2 and IgG3 were also detected in patients with cutaneous anthrax. However, the profiles differed between patients. All three anti-PA IgG subclasses were detected in the sera of patients CA-4, CA-7 and CA-9. In-patient CA-6 only IgG1 and IgG3 subclasses were detected. In-patient CA-3 only IgG1 and IgG2 were detected. In the sera of patients CA-1 and CA-8 only the IgG1 subclass was detected (Table 2). Recognizing the difference in sample collection time points, the anti-PA IgG1 subclass concentration in cutaneous anthrax patients ranged from 2.5 to 28.8 $\mu\text{g/ml}$ (mean of 10.1 $\mu\text{g/ml}$, 95%CI of mean: 2.8 to 18.4 $\mu\text{g/ml}$), IgG2 ranged from <LLOD: 10.2 $\mu\text{g/ml}$ (mean of 3.2 $\mu\text{g/ml}$, 95%CI of mean: -0.3 to 6.7 $\mu\text{g/ml}$) and IgG3 ranged from <LLOD: 4.0 $\mu\text{g/ml}$ (mean of 1.9 $\mu\text{g/ml}$, 95%CI

of mean: 0.2 to 3.6 $\mu\text{g/ml}$) (Table 2). The IgG1 subclass was also present in the highest concentration and proportions (mean of 72.7% of total anti-PA IgG), followed by IgG2 (mean of 17.4%) and IgG3 (9.9%). In CA sera where IgG1, IgG2 and IgG3 were all detected, the relative magnitudes of the subclass proportions at the time points tested were IgG1 > IgG2 > IgG3 (Table 2).

All of the CA patients with quantifiable anti-PA IgG subclass concentrations also had quantifiable LTx neutralization ED50 levels. The highest ED50 was in the serum sample from patient CA-7 which also had the highest total anti-PA IgG concentration of 43 $\mu\text{g/ml}$ (ED50 = 296) (Table 2). Analysis of the anti-PA IgG subclass profiles between these two forms of anthrax showed that there was a statistical difference in the mean concentration for all IgG1-3 subclasses ($p = 0.0066$, 0.097 and 0.046, respectively). The comparison of anti-PA IgG immune responses between patients with inhalation anthrax and AVA recipients at week 26 after emergence of the IgG4 subclass showed that, at these time points in these cohorts, there was no statistical difference in the mean concentration of all IgG1-3 subclasses ($p = 0.48$, 0.64 and 0.48, respectively), thus indicating that the serum antibody responses to both AVA vaccination and recovery from inhalation anthrax are quantitatively and qualitatively similar.

4. Discussion

The role and significance of antigen specific IgG subclasses has been the subject of considerable discussion in the literature. In human adults IgG responses induced by viral and bacterial protein antigens during infection are mainly confined to IgG1 with antigen specific IgG3 and IgG4 making

only small contributions [22–26]. IgG2 subclass responses to infection are usually directed against polysaccharide antigens [24,27]. The distribution of IgG subclasses induced by vaccination against protein/peptide antigens is also usually limited to IgG1, IgG3, and IgG4 [28–31]. In this study, we analyzed the anti-PA IgG subclass-specific immune response and LTx neutralization activity in sera from 63 humans vaccinated with the licensed AVA and from 13 patients with confirmed bioterrorism-associated or laboratory acquired anthrax. Because of the rarity of human anthrax together with the regulated and limited distribution of the vaccine, the number and distribution of available serum donors for the study constrained us to a cross-sectional analysis. A comparative evaluation of the anti-PA IgG subclass changes in a single cohort of human vaccinees would require a study of significant duration (>10 years) and is beyond the scope of this manuscript. The data from this cross-sectional observational study show that these cases of clinical anthrax and vaccination with three AVA injections elicit anti-PA IgG1, IgG2 and IgG3 subclass responses. An anti-PA IgG4 response was also detected in recipients of AVA after the fourth injection and became the most abundant IgG subclass in recipients with ≥ 10 AVA injections. Serum from volunteers who received the six primary doses and one annual booster contained the highest levels of anti-PA IgG1, but the proportion of this subclass decreased from a maximum of 67.2% after the second injection of AVA to 53.0% after the first annual booster (AVA injection 7) (Fig. 1). The data also indicate that AVA vaccination and *B. anthracis* infection both elicit an anti-PA IgG2 subclass response of similar proportions, reaching approximately 30% of total anti-PA IgG. The percent distribution of IgG2 over the full regimen of AVA vaccination was very stable and decreased from 26.5% after the second AVA injection to 19.6% in the recipients with multiple boosters (≥ 10 injections of AVA). The proportion of IgG2 in the sera of clinical patients ranged from 14.6% to 29.6% and <LLOD to 41.0% for inhalation and cutaneous anthrax, respectively, with no significant difference between inhalation and cutaneous (Wilcoxon $p = 0.38$). The levels of anti-PA IgG3 were low, but consistently detectable over the course of AVA vaccination and in the sera of clinical patients. The presence of IgG2 and IgG3 may suggest their contribution in neutralization of LTx, however, the specific function of these subclasses in LTx neutralization have yet to be elucidated.

Only after the fourth injection of AVA were there detectable levels of anti-PA IgG4. The increasing percentage of IgG4 (23.7%) after the sixth injection coincided with a decreasing proportion of IgG1 (52.9%). IgG4 became the most abundant subclass in serum from recipients of multiple annual boosters (≥ 10 injections of AVA) (42.3% of total IgG). Despite these changes in distribution of IgG1 and IgG4 subclasses in AVA recipients after the fourth injection (Fig. 1), the functional ability of sera to neutralize LTx did not change significantly ($p = 0.85$, Wilcoxon test) (Fig. 1). LTx neutralization activity correlated with total anti-PA IgG ($r^2 = 0.56$) in all AVA recipients, but did not appear to be either depen-

dent upon, or significantly affected by, the changes in anti-PA IgG subclass distribution.

In the sera from the inhalation and cutaneous anthrax patients studied here, anti-PA IgG1 was present in the greatest proportion, and in some patients was the only detectable subclass. Anti-PA IgG4 was not detectable in any of the clinical sera tested. We have previously reported a strong positive correlation ($r^2 = 0.83$) between total anti-PA IgG concentration and LTx neutralization ED50 values in patients with cutaneous and inhalation anthrax [8]. In this study, we see a similar relationship ($r^2 = 0.99$).

There is limited information about anti-PA IgG subclass distribution in humans with anthrax or who have been immunized with anthrax vaccines. Prior to this study, the specific anti-PA IgG subclass responses in serum from AVA recipients at different stages of the vaccination regimen and from survivors of inhalation anthrax have not been characterized. Baillie et al. [9,22] compared human anti-PA IgG subclass responses in individuals with cutaneous anthrax with those of individuals immunized with the UK licensed anthrax vaccine. In those studies individuals with cutaneous anthrax produced anti-PA IgG1 and IgG3 while immunization with the UK licensed anthrax vaccine elicited anti-PA IgG1, IgG2, IgG3 and IgG4. The subclass with highest dilutional titers in both groups in that study was IgG1. The distribution of anti-PA IgG subclasses in response to vaccination with the UK anthrax vaccine and with AVA seem therefore to be qualitatively similar in that vaccine-induced anti-PA IgG4 responses were detected only after the fourth injection. The observation that neither the inhalation nor cutaneous anthrax study groups exhibited PA-specific IgG4 suggests that this IgG subclass could be potentially used as a qualitative marker to differentiate responses to clinical anthrax from those elicited by the full regimen of AVA vaccination or vaccination with at least four AVA injections. The absence of such an IgG4 response in individuals with cutaneous anthrax was also found by Baillie et al. [9]. In contrast to Baillie et al., however, the present study shows that some patients with bioterrorism associated cutaneous anthrax produced detectable levels of anti-PA IgG2 (Table 2).

The structural differences between IgG1 and IgG4 reside for the most part in the hinge and constant heavy chain C_H2 regions. Thus, the functional differences between these subclasses primarily relate to different interactions with the C1_q component of complement or Fc receptors on various cell types. It might therefore be speculated that a shift in anti-PA response from predominantly IgG1 to a significant level of IgG4 may have a detrimental effect on any opsonic activity conferred by an anti-PA response [32,33]. Progressive increases in affinity maturation of anti-PA IgG subclasses with ongoing vaccination are, however, unlikely to be affected by class switching [34]. This is relevant particularly for serum from recipients of ≥ 10 AVA injections for which the persistence of high levels of anti-PA IgG1 together with the continued high ED50 values were clearly indicative that the LTx neutralization efficacy of the anti-AVA

antibody response was not compromised by the emergence and increasing proportion of anti-PA IgG4.

In conclusion, based on this observational, cross-sectional study with the limited number of sera available, we found that vaccination with AVA elicits all four anti-PA IgG subclasses in human sera. Subclass distribution, however, was dependent on the number of AVA injections received. The anti-PA IgG4 response in our study was detected only after the fourth injection of AVA. The anti-PA IgG subclass distribution in AVA recipients who received three doses was similar to that in patients with inhalation anthrax and consisted of anti-PA IgG1, IgG2, and IgG3 subclasses. The functional ability of sera of AVA recipients to neutralize LTx increased during the first three injections of AVA and remained relatively constant throughout the remaining vaccination regimen up to ≥ 10 injections of AVA. The changes in IgG1 and IgG4 subclass distribution during the course of immunization did not affect the functional ability of sera to neutralize LTx.

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