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Anthrax Vaccination Induced Anti-Lethal Factor IgG: Fine Specificity and Neutralizing Capacity

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Abstract

The efficacy biomarker of the currently licensed anthrax vaccine (AVA) is based on quantity and neutralizing capacity of anti-Protective Antigen (anti-PA) antibodies. However, animal studies have demonstrated that antibodies to Lethal Factor (LF) can provide protection against *in vivo* bacterial spore challenges. Improved understanding of the fine specificities of humoral immune responses that provide optimum neutralization capacity may enhance the efficacy of future passive immune globulin preparations to treat and prevent inhalation anthrax morbidity and mortality. This study (n = 1000) was designed to identify AVA vaccinated individuals who generate neutralizing antibodies and to determine what specificities correlate with protection. The number of vaccine doses, years post vaccination, and PA titer were associated with *in vitro* neutralization, reinforcing previous reports. In addition, African American individuals had lower serologic neutralizing activity than European Americans, suggesting a genetic role in the generation of these neutralizing antibodies. Of the vaccinated individuals, only 69 (6.9%) had moderate levels of anti-LF IgG compared to 244 (24.4%) with low and 687 (68.7%) with extremely low levels of IgG antibodies to LF. Using overlapping decapeptide analysis, we identified six common LF antigenic regions targeted by those individuals with moderate levels of antibodies to LF and high *in vitro* toxin neutralizing activity. Affinity purified antibodies directed against antigenic epitopes within the PA binding and ADP-ribotransferase-like domains of LF were able to protect mice against lethal toxin challenge. Findings from these studies have important implications for vaccine design and immunotherapeutic development.

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Keywords

Bacillus anthracis; Anthrax; Anthrax Vaccine Adsorbed; Lethal Factor; Protective Antigen; correlate of protection

Introduction

Bacillus anthracis, a gram-positive, spore-forming bacterium, is the causative agent of anthrax infection. Infection can be initiated by cutaneous, gastrointestinal, or inhalational routes, with the inhalational route resulting in 45-90% mortality¹. Systemic infection is characterized by an extremely high blood concentration of bacilli, resulting in high concentrations of the secreted tripartite toxin. This toxin is composed of three polypeptides: protective antigen (PA), lethal factor (LF), and edema factor (EF). PA binds to its cellular receptor(s), Tumor Endothelial Marker 8 (TEM8) or Capillary Morphogenesis Protein 2 (CMG2)¹⁻³, and is cleaved by a furin-like membrane endoprotease. The resulting 63kDa fragment oligomerizes and, when endocytosed, carries EF and/or LF into the cell^{1, 4-5}. Edema toxin (ET), composed of PA and EF, is an adenylate cyclase that results in edema and can be lethal when injected into animals⁶. This toxin has also been shown to impair macrophage phagocytosis and increase cAMP levels^{1, 7}. Lethal toxin (LT), formed by the combination of PA with LF, is a zinc-dependent protease that causes lysis of intoxicated macrophages and is lethal in animal models^{1, 8}. Following PA-mediated translocation of LF into the cytosol, target cells such as macrophages release pro-inflammatory cytokines inducing endothelial cell death by apoptosis and leading to vascular collapse^{1, 8-14}.

The design of the current United States anthrax vaccine (Anthrax Vaccine Adsorbed, AVA) is predicated on the fact that PA serves as a crucial component of both LT and ET, and antibodies against PA are known to provide protection from disease in animals¹⁵⁻¹⁶. This vaccine is produced from a cell-free filtrate of an attenuated bovine isolate (V770-NP1-R) that produces a higher fraction of PA¹⁷. However, all three toxin components (PA, LF, and EF) are present in the product¹⁷⁻¹⁸. While it is clear that antibodies to PA are the primary method of protection generated following AVA immunization, studies with mouse models have demonstrated the protective significance of antibodies to LF alone¹⁹. Antibodies directed against LF have been shown to provide protection against challenge with toxin or bacteria in several experimental animal models^{14, 20-25}. Additionally, the protective capacity of neutralizing antibodies directed against PA can be greatly enhanced by the addition of LF neutralizing antibodies²¹. Limited human data exists characterizing the fine-specificity and potential for protection of antibodies to LF following AVA immunization. This study evaluated plasma from a large cohort (n = 1000) of AVA immunized individuals for the quantitative levels of LF specific antibodies as well as for the presence of binding to sequential B cell epitopes that contribute to functional protection. Antibodies directed against two antigenic regions of LF, one in the PA binding domain and one in the ADP-ribotransferase-like domain, are able to provide protection in an *in vivo* mouse model of lethal toxin challenge. These data suggest that development of new active and passive vaccination strategies that incorporate these LF antigenic regions will lead to improved protection against anthrax.

Materials and Methods

Human Subjects and Sample Collection

Individuals who were vaccinated with the currently licensed AVA were enrolled in this study (n = 1000). Participants provided informed consent and information about vaccination history, sex, age, and ethnicity. One hundred non-vaccinated individuals were recruited to

provide control samples. Institutional Review Board approval was obtained from the Oklahoma Medical Research Foundation, Oklahoma University Health Sciences Center, Walter Reed Army Medical Center, Washington, DC and Womack Army Medical Center, Fort Bragg, NC, before the start of recruitment. Plasma was collected and stored at -20°C until further use.

Standard and peptide-specific ELISAs

Ninety-six well plates were coated overnight at 4°C with 1 µg/well of recombinant LF (rLF) or recombinant PA (rPA, List Biologicals, Campbell, CA) or multiple antigenic peptides (MAP) (OUHSC Molecular Biology Core Facility). The peptides sequences were as follows: ₂₅₇YIEPQHRDVL₂₆₆, ₂₈₆LSLEELKDQR₂₉₅, and ₅₃₉SPDTRAGYLENGKL₅₅₂. After washing with PBS-Tween and blocking with PBS/BSA, diluted plasma was added and incubated for 2 hours (h) at room temperature (RT). After washing, the plates were incubated with a 1:10,000 dilution of AP-labeled anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at RT, washed again and incubated with pNPP substrate (Sigma, St. Louis, MO) for 30 minutes. The optical densities (OD) at 410 nm were measured using a Dynex MRX II microplate reader (Dynex Technologies, Chantilly, VA). Endpoint titer was calculated based on the last dilution to yield a positive result, using the following formula: average OD plus 2 times the standard deviation (SD) of the unvaccinated control group at a 1:100 dilution. The concentration of antibodies to PA was calculated using reference serum AVR801 (Biodefense and Emerging Infections (BEI) Resources, Manassas, VA) containing antibodies to PA serially diluted 2-fold at a starting concentration of 109.4 µg/ml ²⁶. Plasma samples were tested at a dilution of 1:100, and samples that could not be interpolated at this dilution were re-tested at dilutions of 1:10 or 1:1000.

In vitro Lethal Toxin Neutralization Assay

Inhibition of LT activity by participant plasma was performed as previously described ^{23, 27-28}. Briefly, RAW264.7 macrophages (ATCC, Manassas, VA) were plated into a 96-well flat bottom tissue culture plate (100,000 cells per well) and cultured overnight at 37°C with 5% CO₂. Plasma samples were diluted 1:100 in culture medium and incubated for 1 h at room temperature with LT (comprised of 50 ng of rPA and 50 ng of rLF). After incubation, the medium was removed from the cultured cells and 100 µl of the plasma/toxin mix was added. Wells containing cells alone or cells with added rPA only, rLF only, or cells with rPA and rLF (LT) served as controls and quality control determinants. After addition of the plasma/toxin mixture, the cells were incubated at 37°C with 5% CO₂ for 2 h, followed by addition of 10 µl of WST-8 (CKK8, Dojindo Molecular Technologies, Rockville, MD). The Optical Density (OD) at 450 nm was detected at approximately 3 hours. Percent viability was calculated using the following formula: (OD of plasma-toxin mixture)/(OD of cells only)*100. A sample from a vaccinated individual was considered negative if the average viability from three independent experiments was below the cut-off as determined by the average viability plus two times the standard deviation of the unvaccinated controls (12% viability).

Solid-Phase Peptide Construction and Assay

To determine the fine specificity of the anti-rLF antibodies, a solid-phase peptide ELISA assay was used. Peptides, 10 amino acids in length and overlapping by 8 amino acids, covering the entire length of the LF protein (Pub Med Protein accession number AAY15237.1) were covalently synthesized onto polyethylene rods in a 96-well format as previously described ²⁹⁻³⁰. These peptides were incubated with a 1:200 dilution of plasma for 2 h at room temperature, washed with PBS-Tween, and then incubated with a 1:20,000 dilution of a HRP-labeled anti-human IgG conjugate (KPL, Gaithersburg, MD) overnight at

4°C. After washing, the solid-phase peptides were incubated with SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) for 5 min at room temperature, and the reaction was terminated by the addition of TMB Stop Solution (KPL, Gaithersburg, MD) and the OD at 450 nm was detected. A region was defined as commonly antigenic when more than 50% of the anti-rLF positive plasma from vaccinated individuals contained antibodies to the region and the average vaccinated OD for a single anti-peptide response was greater than the average OD plus two times the SD of the unvaccinated control group (average cut-off value for a single peptide: 0.16 ± 0.088).

Column Absorption

Multiple antigenic peptides (₂₅₇YIEPQHRDVL₂₆₆, ₂₈₆LSLEELKDQR₂₉₅, and ₅₃₉SPDTRAGYLENGKL₅₅₂) were bound to cyanogen-preactivated Sepharose 4B beads as previously described³¹⁻³³. To absorb the peptide-specific antibodies, plasma (1 ml) was re-circulated six times over the beads, eluted with 100 mM glycine (pH 2.5), and concentrated to a volume equal to the original specimen. Column-absorbed samples were then used in standard ELISAs and peptide-specific ELISAs to test for reactivity to rLF and the peptide used for absorption, respectively.

In vivo toxin challenge

Peptide-specific antibodies were enriched via column absorption as described above. After absorption the amount of IgG in each sample was quantified via OD at 280nm (NanoDrop Technologies Inc, Wilmington, DE) and then used in the standard ELISA and the peptide-specific ELISA to test for reactivity to rLF and the synthesized peptide, respectively. Next, 30 µg of antibody/mouse was given by i.p. injection into 6-week-old A/J mice (Jackson Laboratories, Bar Harbor, ME). Control mice received either saline or 30 µg of total IgG that was enriched from a non-vaccinated control. Three hours after the passive transfer the mice were challenged with $3 \times LD_{50}$ of LT i.p. as previously described (300 µg PA + 125 µg LF^{23, 28}). Mice were then monitored for 7 days and mortality recorded. Survival curves and percent survival were generated by using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). Animal experiments were reviewed and approved by the Oklahoma Medical Research Foundation (OMRF) Institutional Animal Care and Use Committee (IACUC).

Statistical analysis

With the exception of the survival data, multiple group comparisons were tested for overall significance using Kruskal-Wallis one-way ANOVA. Secondary p values, to determine which groups contributed to the differences, were generated using the Dunn's multiple comparisons post-hoc test. Mann-Whitney t tests were used in comparisons to the variables of the years post vaccination, number of vaccinations, and PA endpoint titer in the matched European and African American groups. Linear regression was used to compare rPA endpoint titer and anti-rPA concentration. Multivariate analysis was performed with SAS software using a mixed model with fixed effects. Log transformation of variables was performed when necessary to correct for non-normality of the data. For the *in vivo* survival data, survival curves were generated and tested using a log-rank test (Mantel-Cox) to determine the overall p value. Mann-Whitney t tests were then used to compare each treatment group to the control group. All statistical analyses except the multivariate analysis were performed using GraphPad Prism 4.0.

Results

AVA vaccination results in the development of antibodies directed against LF in a small subset of individuals

Individuals who had received at least the first three doses of the AVA anthrax vaccine series were recruited and provided plasma. Participants provided vaccination history and self-reported demographics, including sex, age, and ethnicity. European Americans comprised the majority of the cohort (63.7%), but other racial/ethnic groups were well represented, with 134 African Americans, 75 Hispanics, 23 Asians, and 131 individuals of American Indian, Pacific Island or mixed ethnicity (Table 1). Of the cohort, 87.4% (n=874) were male (Table 1). As a whole, the participants were young with an average age of 30.1 (median: 28), although the range was from 18 to 62 years (95% CI: 29.58, 30.56). The average number of anthrax vaccine doses received was 4.5 with a median of 4.0 (range: 3 to 12 doses). While yearly vaccinations are recommended, the average number of years post vaccination was 1.4 in our cohort (median of 0.7, 95% CI 1.36, 1.54). The range of time since last vaccination was broad, from 7 days to 17 years (Table 1), and 60.3% had received a vaccination within the past year.

Samples from these vaccinated individuals, along with unvaccinated controls, were first tested for antibodies directed against recombinant protective antigen (rPA) and lethal factor (rLF) by standard ELISAs. Consistent with prior reported data ²⁸, over 85% of the vaccinated individuals generated antibodies against rPA (Figure 1A). In contrast, the majority of vaccinated individuals (68.7%) had no detectable antibodies directed against rLF in their plasma. However, a small subset of individuals (n = 69) developed rLF-specific antibodies of moderate titers (1:100 and 1:1000) following vaccination (Figure 1B). No significant correlation was found between the magnitude of the anti-rPA response and the anti-rLF response, but of the 69 individuals with moderate rLF antibodies, 38 (55%) had high titers of antibodies to rPA and no individuals generated an anti-rLF response in the absence of an anti-rPA response. As expected, unvaccinated controls demonstrated no antibody response to either rPA or rLF (data not shown).

LF antibody titer is not associated with *in vitro* protection

A plasma sample from each individual was next tested for ability to neutralize lethal toxin (LT) by an *in vitro* neutralization assay. Individuals were classified into four groups based on their neutralizing activity: high (51-100% cell viability), medium (26-50%), low (12%-25%), and very low (less than 12%). By this criterion, 43% of vaccinated individuals were classified as having very low neutralization activity; in effect, possessing the same neutralizing capacity as unvaccinated individuals. Of the remaining participants, 16% had low neutralizing capacity, 16% had medium neutralizing capacity, and 26% had greater than 50% *in vitro* neutralization (Figure 1C).

To determine if rPA or rLF antibody titer associated with *in vitro* neutralization, the relationship between endpoint titer and the percent viability was examined. Anti-rLF titer is not associated with *in vitro* protection (Figure 2A). However, individuals that demonstrated low, medium, or high neutralization activity had significantly higher average rPA endpoint titers than individuals with very low neutralization activity (Figure 2B). Average and standard errors of rPA endpoint titers for very low, low, medium, and high neutralizers are 310 ± 53.5 , 652 ± 92.1 , 1905 ± 242.2 , and 3595 ± 257.2 , respectively ($p \leq 0.0001$ by one-way ANOVA). The anti-rPA concentration was also quantified in each titer-defined group by the use of the reference standard AVR801 ²⁶; the mean ($\pm$ SEM) microgram content in each titer group was 5.54 ± 0.89 μ g/ml for titers $\leq 1:10$, 19.09 ± 1.04 μ g/ml for titers of 1:100, 85.82 ± 5.74 μ g/ml for titers of 1:1000, and 232.1 ± 28.33 μ g/ml for titers of 1:10000.

As expected, the anti-rPA concentration was correlated with average rPA endpoint titer ($p < 0.001$, $R^2 = 0.2$ by linear regression).

The ability to neutralize toxin *in vitro* was also associated with the years post vaccination and the number of vaccinations received. As shown in Figure 2C, samples with low and very low neutralization capacity were collected from individuals significantly longer post vaccination than those samples with medium or high neutralization capacity (average of 1.9 and 1.5 years post for very low and low groups compared to 1.3 and 0.81 years post for medium and high neutralizers, $p \leq 0.0001$ by one-way ANOVA). In addition, the number of vaccinations received was significantly associated ($p < 0.001$ by one-way ANOVA) with the ability to neutralize toxin *in vitro*. Figure 2D shows that medium and high neutralizers had received an average of 5 vaccinations compared to only 4 vaccinations in the very low neutralizer group. These data support and extend previous findings that rPA titer, years post vaccination, and number of vaccinations received is highly associated with neutralization capacity²⁸.

Vaccination history and demographic characteristics are associated with *in vitro* neutralization but not LF endpoint titer

When neutralization activity was compared to demographic information, age or sex had no effect. On average, Hispanics had the highest neutralization activity, followed by individuals of mixed ancestry, European Americans, Asian Americans, and finally African Americans (Figure 3A). African Americans always demonstrated a lower percent macrophage viability in the presence of plasma/toxin mixtures as compared to other ethnic groups (Figure 3A, $p = 0.0004$ by one-way ANOVA).

Because the number of Hispanics, Asian Americans, and individuals of mixed ancestry was small, we compared African Americans and European Americans specifically. African Americans had, on average, significantly lower anti-rPA endpoint titers than European Americans ($p = 0.016$ by Mann-Whitney t test, Figure 3B). In addition, plasma from the African Americans individuals were collected longer post vaccination than from European Americans ($p = 0.003$ by Mann-Whitney t test, Figure 3C). Both of these differences, rPA endpoint titer and time post vaccination, might account for the lower neutralization activity in African Americans individuals. However, African American individuals also had a higher average number of vaccinations than European Americans ($p = 0.002$ by Mann-Whitney t test, Figure 3C).

To control for the confounding factors of time post vaccination and the number of vaccinations, we first performed a subset analysis in which we compared a group of African American men who were less than one year post vaccination and had received three to four vaccinations ($n = 23$) to a group of European American men matched on age, number of vaccinations, and time post vaccination ($n = 229$). We examined both rPA titer and neutralization capacity in this matched group, and found no significant difference in the anti-rPA titer in individuals of African American descent ($1:650 \pm 126$) compared to individuals of European American descent ($1:1,487 \pm 115$). However, while 26% of the vaccinated European Americans had high neutralization capacity, only 14% of the African Americans had high neutralization ($p = 0.003$ by exact Chi-square, Figure 3D). To corroborate these findings, a mixed model multivariate analysis was performed on all individuals within the cohort specifically examining the effect of rPA end titer, rLF end titer, years post vaccination, number of vaccinations, and ethnicity. While ethnicity was correlated with all of these factors, ethnicity alone was found to contribute significantly to predicting neutralization capacity ($p = 0.005$). The reason for this contribution of ethnicity to vaccine response is unclear but may be related to genetic variations, such as HLA haplotype.

Vaccination history and demographic characteristics were analyzed to determine the relationship between these factors and rLF endpoint titer. Unlike with rPA endpoint titer, we found no significant association between rLF endpoint titer and number of vaccinations received, years since last vaccination, age, sex, or ethnicity (data not shown).

Six common antigenic regions of lethal factor were associated with *in vitro* neutralization

The results above suggest that anti-rLF titer alone is not associated with *in vitro* neutralization. However, it is possible that the humoral fine specificity, or quality, of the vaccination response is more important for protection than the sheer quantity of that response. To explore this hypothesis, the fine specificity of the LF response was determined using solid-phase peptide ELISA with plasma from both rLF positive, highly neutralizing individuals as well as rLF positive, very low neutralizers. Using this methodology, six antigenic regions of LF were identified in the high responders that were not recognized by unvaccinated controls or individuals with low neutralization (Figure 4A and 4B). Based upon previous reports^{1, 10}, the functional domain and amino acid number were determined for each epitope (Figure 4C and 4D). Most epitopes clustered in the central region of LF with two localizing to Domain I, two within Domain II, and two within Domain III. No common antigenic regions were found within domain IV.

Focusing on antibodies that are potentially neutralizing in individuals with high, *in vitro* neutralization capacity, epitope #1 (aa257-266, Figure 4D) was of particular interest since it was bound by 71% of the individuals within the high neutralizing group. Sites within the Epitope #1 region have been identified as being within the PA binding domain, which is critical for toxin formation^{1, 10}. Another epitope within the PA binding domain (epitope #2, aa286-295) was noteworthy since antibodies directed against this region were found only in the individuals within the high neutralizing group. A third potentially important epitope was identified in 57% of the individuals with high neutralization and is located within the ADP-ribotransferase-like domain of LF (epitope #6, aa539-548, Figure 4D).

To confirm these antigenic regions using a second method, we synthesized the epitopes of interest as soluble multiple antigenic peptides (MAP) and characterized the reactivity of plasma to these peptides in peptide-specific ELISAs. We identified 30 individuals (43.5% of the 69 rLF positive individuals) with antibodies directed against the PA binding epitope #1, a mostly unique set of 30 individuals with antibodies directed against the PA binding epitope #2, and 19 individuals with antibodies directed against ADP-ribotransferase-like epitope #6 (Figure 4). Of the 69 LF positive individuals, seven (10%) had antibodies directed against all three antigenic regions. To confirm specificity to these epitopes in LF positive individuals, we then tested a group of 69 LF negative individuals, matched by years post vaccination, age, sex, ethnicity, and PA titer to the LF positive group by the same method. Only one of 69 LF negative vaccinated individuals had antibodies directed against the PA binding domain (epitope #1) and no unvaccinated controls had antibodies directed against these antigenic regions (data not shown).

Select epitope-specific anti-LF antibodies can provide protection against *in vivo* toxin challenge

To ensure that the neutralization detected was due to the anti-LF response and to test the protective capability of antigen-specific antibodies to LF, antibodies specific for each of the three epitopes of interest were affinity purified from the plasma of AVA vaccinated individuals. The concentration of antibody in column-absorbed samples was measured and these antibodies were confirmed by standard ELISA to be reactive to whole rLF and the peptide used for purification (data not shown). These epitope-specific antibodies were then passively transferred into A/J mice. Three hours after transfer the mice were challenged with

3×LD₅₀ of lethal toxin and the mortality was recorded (Figure 5). Seventy percent of the mice receiving antibodies directed toward one of the PA binding epitopes (epitope #2, Figure 4D) survived the lethal toxin challenge ($p = 0.02$ compared to controls by Mann-Whitney test). While the survival rates were not significantly different, only 50% of the mice receiving another PA binding antibody (epitope #1) survived the challenge, indicating that epitope specificity plays a critical role in protection. The antibody directed to epitope #6, a region found within the ADP-ribotransferase-like domain, was able to provide 60% protection against lethal challenge ($p = 0.05$ compared to controls by Mann-Whitney test, Figure 5). These data indicate that select LF peptide-specific antibodies generated following AVA vaccination can provide protection against *in vivo* lethal toxin challenge.

Discussion

The predominant antibody response generated following at least 3 doses of US anthrax vaccine was, as expected, targeted against PA, although a small subset (6.9%) also generated LF-specific antibodies. In contrast to the US vaccine, the UK vaccine contains both a significant amount of PA and LF, but individuals vaccinated with the UK vaccine typically develop an anti-LF response which is at least one log lower than their anti-PA response³⁴. In a number of studies, monoclonal antibodies directed against LF have been found to neutralize LT by *in vitro*^{14, 20-24, 35} and *in vivo* testing^{20, 24}. In addition, several studies have found that when neutralizing antibodies directed against LF are administered with neutralizing antibodies directed against PA *in vivo*, they can provide synergistic protection against both toxin and spore challenge²¹⁻²². Together, these studies suggest the induction of antibodies to LF following vaccination may provide more protection than PA antibodies alone. Identification of protective epitopes of LF will inform the selection of truncated protein fragments or peptides of LF most likely to induce responses leading to enhanced protection.

As described in a previous study, anti-PA titer is not a perfect predictor of neutralization activity. Indeed, a large proportion of AVA vaccinated individuals (43%), many of which possess high anti-PA titers, have very low neutralizing capacity²⁸. Elucidating other factors beyond anti-PA titer which correlate with protection would therefore be a more reliable measure of AVA-induced immunity and a more precise measure of vaccine efficacy. The current study further supports the observation that the number of vaccinations an individual has received and how much time has passed since the last vaccination, in addition to PA titer, are associated with the development of a neutralizing response (Figure 2 and²⁸). In contrast, there were no clear predictors of developing a response to LF, and anti-LF titer was not associated with *in vitro* neutralization. However, because of the much higher amount of PA in the vaccine, it is possible that anti-LF responses simply could not be isolated from the response to PA. Indeed, we found no individuals with moderate LF responses that did not have antibodies directed against PA as well. Further studies will focus on raising LF-specific antibodies in isolation for this purpose. It should be noted that this study was not designed to address the impact of time interval between doses, which is known to affect at least the short term response in UK vaccination³⁶. Future studies will address this phenomenon in individuals vaccinated with AVA.

In addition to confirming previously reported correlates of protection, this more extensive study suggests that ethnicity may play a role in the development of neutralizing antibodies following anthrax vaccination. A previous study of AVA vaccination with a similar cohort size ($n=1564$) also found a racial/ethnic difference in the AVA response³⁷. When anti-PA IgG antibody responses were determined at two time-points during a standard vaccination schedule, antibody levels were significantly higher in European Americans compared with African Americans at week 8 (during the immunization schedule) but not at month 7 (one

month after the last vaccination). In a recent study of elderly individuals of European (n=33), African (n=39), and Hispanic (n=41) descent, influenza-induced proliferation of peripheral blood mononuclear cells (PBMCs) was increased post vaccination in European American and Hispanic individuals, but was not increased in African American individuals³⁸.

Lower responses to the AVA vaccine in African American subjects could be due to a variety of genetic effects such as HLA haplotype, polymorphisms in cytokine or cytokine receptor genes, or variations in cell surface molecules. Several HLA alleles have been associated with non-responsiveness to vaccination including HLA-DRB1*07³⁹, HLA-B alleles 46, 15, and 08 as well as DRB1*03⁴⁰. In addition to HLA alleles, variations in IL-1 family member genes have been associated with differences in either the magnitude or the kinetics of the antibody response to the hepatitis B vaccine⁴¹⁻⁴⁴. Polymorphisms in cytokine and cytokine receptor genes, as well as in HLA and pattern recognition receptor mannose-binding lectin-2 genes have all been associated with differing response to the yearly influenza vaccine^{40, 45}. Finally, variants of TLR-2, 3, 4, 5, and 6, as well as MyD88 and MD2, SLAMF, and CD46, are associated with humoral and cellular immunity to measles, including differences in antibody titers, proliferative responses, and cytokine secretion⁴¹⁻⁴². Most of the studies detailed above were done with a limited cohort of one race or ethnicity; however, as genes are often segregated by race or ethnicity, the effect of these polymorphisms on a particular group could be significant.

While anti-rLF titer was not found to be associated with *in vitro* neutralization, fine specificity mapping of the LF response demonstrated that individuals with high neutralization activity contained antibodies directed against at least six specific antigenic regions of LF. Plasma from unvaccinated controls and LF-positive individuals with low neutralization activity did not bind these regions, suggesting that specificity of the anti-LF response is more important to neutralization than the overall antibody quantity. While the *in vitro* neutralization observed in these samples might be due to antibodies directed against PA, *in vivo* data with column purified antibodies demonstrates that antibodies directed against select regions of LF can provide protection against toxin challenge. The locations of the six antigenic regions were equally distributed between Domains I, II, and III. Monoclonal antibodies directed against Domain I, which is responsible for PA binding and is necessary for LF entry into the cell, are historically the most likely to be neutralizing^{14, 20-21, 25, 35}. Monoclonal antibodies have also been found to bind Domains II, III, and IV, which together form the catalytic site of MAPKK cleavage. Site-directed mutagenesis of Domain II can decrease the ability of LF to compete with the phosphorylation of MEK, suggesting a role of Domain II in MAPKK binding. Domain III contains residues that make specific contact with the amino termini of most of the members of the MAPKK family, suggesting it is responsible for substrate recognition. Finally, Domain IV of LF binds zinc, and insertional mutagenesis within this domain can eliminate LF toxicity without eliminating PA binding.

Binding of antibodies to Domain I is often experimentally determined by gel mobility shift assays or radiolabeled LF attachment to PA-incubated cells; this technique, however, may be overstating the contribution of the Domain I-specific response to protection. The technique of fine specificity mapping provides a more accurate localization of binding, and therefore of protective epitopes. Several antibodies that were assumed to bind Domain I (LF8, 10G3) as well as at least one antibody that had not been characterized (9A11) bind epitopes in Domains III and IV²³. Indeed, when the binding sites of previously described LF-specific monoclonal antibodies are determined by indirect or solid-phase peptide ELISAs, highly antigenic regions are most often found in Domains III and IV²³. The method of fine-specificity mapping detailed in this study is also reproducible; several of the

antigenic regions of LF found in this report (using human plasma following vaccination) coincide with those found in previous studies using sera or plasma from LF immunized mice, most notably epitopes 2, 4, and 5 (which overlap with epitopes 2, 3, and 4 of ²³). To further address the neutralization capacity of antibodies directed specifically toward the novel epitopes found here, future studies will isolate monoclonal antibodies specific to these epitopes and determine their neutralization capacity alone and with anti-PA.

In summary, lethal toxin neutralizing responses elicited by the AVA vaccine are specifically directed against select LF peptides. The antibodies generated by AVA vaccination and specific to these peptides can confer *in vivo* protection from toxin challenge. Indeed, if used in combination with antibodies to PA and antibiotics, these LF specific antibodies could contribute to effective treatment strategies following anthrax infection. In combination with previous findings correlating the anti-PA response with protection, the identification of protective anti-LF responses following AVA vaccination suggests that inclusion of portions of LF should be considered as a component of further anthrax vaccine formulations.

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Figure 1.

AVA vaccination results in antibodies directed against PA and LF, but only a subset of vaccinated individuals develop high titers of neutralizing antibodies ($n = 1000$). The endpoint titer or last dilution at which a plasma sample was positive for antibodies to PA (A) or LF (B) by recombinant protein ELISA is presented. Shown is the number of individuals positive at each endpoint titer. (C) All vaccinated individuals were tested by *in vitro* LT neutralization, and are grouped by percent viability of macrophages after incubation with LT in the presence of plasma (diluted 1:100). The number of individuals whose samples were considered high (>50%), medium (25-50%), low (12-24%), or very low (<12%) neutralizers is indicated.

Figure 2.

In vitro LT neutralization is associated with anti-PA titer, the number of years post vaccination, and the number of vaccinations received. The anti-LF titer (A) or anti-PA titer (B) of all vaccinated individuals (n = 1000), grouped based on neutralization activity as determined by the *in vitro* LT neutralization assay is presented. Shown is the number of individuals positive at each endpoint titer. No significant differences were observed with respect to LF titer and neutralization. Average PA titer was significantly associated with neutralization (overall $p \leq 0.0001$ and average of each group was significantly different from the others, $p \leq 0.01$). The average with SEM of years post vaccination (C) and number of vaccinations (D) is shown for each neutralization group. For both years post vaccination and number of vaccination, the association with neutralization was significant (overall $p \leq 0.0001$). * = $p \leq 0.0001$ compared to <12% group, # = $p \leq 0.0001$ compared to 12-24% group, & = $p \leq 0.0001$ compared to 25-50% group.

Figure 3.

Differences in neutralization activity, vaccination history, and anti-PA titer are associated with ethnicity. The neutralization activity of all individuals as determined by LT neutralization, grouped by ethnicity is shown (A). African Americans (AA) had on average, lower neutralizing activity than Hispanics, European Americans (EA), and individuals of mixed ancestry (other, overall $p = 0.0004$). Secondary p values are represented on the graph with * $p \leq 0.05$ and *** $p \leq 0.001$. The two most highly represented ethnic groups, EA and AA, are shown with their average anti-PA endpoint titer (B), number of vaccinations and years post vaccination (C), and neutralizing activity (D).

Figure 4.

Six common antigenic regions of lethal factor, found in three of the four LF domains, were identified using solid phase epitope mapping. Plasma collected from vaccinated individuals with an LF endpoint titer of greater than or equal to 1:100 (n=36) were tested for peptide reactivity in a standard solid-phase peptide assay. Shown are plasma antibody responses from individuals with high neutralization activity (n = 18, panel A) and very low neutralizing activity (n = 18, panel B). The locations of the six identified antigenic regions (defined as those regions at least two standard deviations above the unvaccinated controls and which were bound by at least half of the individuals that were high neutralizers) are mapped onto the crystal structure of LF (C). The table includes the amino acid number, sequence, functional domain, and isoelectric point of each common antigenic region found in individuals with neutralizing antibodies (D).

Figure 5.

Peptide-specific antibodies mediate protection against *in vivo* LT challenge. Plasma samples from AVA-vaccinated participants were affinity purified to isolate peptide specific antibodies and tested for *in vivo* neutralization. Six-week-old A/J mice (n = 10 mice/group, combined from two independent experiments with 5 mice per group) were injected intraperitoneally with 30 μ g of peptide specific antibodies, then challenged three hours later with 3 $\times$ LD₅₀ of LT. Control mice received either saline or IgG purified from unvaccinated control plasma. Antibodies directed to epitope #2 and epitope #6 provided protection (p = 0.02 and p = 0.05, respectively), as compared to control mice.

Table 1

Demographics and vaccination history of AVA-vaccinated individuals.

AVA Vaccinated individuals (n = 1,000)	
Age, average (SD)	30.1 (7.9)
Sex:	
Male	874
Female	126
Ethnicity:	
European American	637
African American	134
Hispanic	75
Asian	23
Other ^a	131
Number of Vaccinations:	
Average (SD)	4.5 (1.7)
Median	4.0
Years post Vaccination:	
Average (SD)	1.4 (1.5)
Median	0.7
Range	7 days – 17 years
Samples <1 year post Vaccination	603 (60.3%)

^a Other includes individuals of self-reported American Indian, Pacific Island, and mixed descent