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Unique Inflammatory Mediators and Specific IgE Levels Distinguish Local from Systemic Reactions after Anthrax Vaccine Adsorbed Vaccination

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Although the U.S. National Academy of Sciences concluded that anthrax vaccine adsorbed (AVA) has an adverse event (AE) profile similar to those of other adult vaccines, 30 to 70% of queried AVA vaccinees report AEs. AEs appear to be correlated with certain demographic factors, but the underlying immunologic pathways are poorly understood. We evaluated a cohort of 2,421 AVA vaccinees and found 153 (6.3%) reported an AE. Females were more likely to experience AEs (odds ratio [OR] = 6.0 [95% confidence interval {CI} = 4.2 to 8.7]; $P < 0.0001$). Individuals 18 to 29 years of age were less likely to report an AE than individuals aged 30 years or older (OR = 0.31 [95% CI = 0.22 to 0.43]; $P < 0.0001$). No significant effects were observed for African, European, Hispanic, American Indian, or Asian ancestry after correcting for age and sex. Additionally, 103 AEs were large local reactions (LLRs), whereas 53 AEs were systemic reactions (SRs). In a subset of our cohort vaccinated 2 to 12 months prior to plasma sample collection ($n = 75$), individuals with LLRs ($n = 33$) had higher protective-antigen (PA)-specific IgE levels than matched, unaffected vaccinated individuals ($n = 50$; $P < 0.01$). Anti-PA IgE was not associated with total plasma IgE, hepatitis B-specific IgE, or anti-PA IgG in individuals who reported an AE or in matched, unaffected AVA-vaccinated individuals. IP-10 was also elevated in sera of individuals who developed LLRs ($P < 0.05$). Individuals reporting SRs had higher levels of systemic inflammation as measured from C-reactive protein ($P < 0.01$). Thus, LLRs and SRs are mediated by distinct pathways. LLRs are associated with a vaccine-specific IgE response and IP-10, whereas SRs demonstrate increased systemic inflammation without a skewed cytokine profile.

Bacillus anthracis, the causative agent of anthrax infection, forms long-lived spores, which have previously been prepared and released as a biological weapon (1–4). Anthrax vaccine adsorbed (AVA), the only anthrax vaccine licensed in the United States, is administered to at-risk personnel to provide preexposure protection. AVA contains cell-free culture supernatant of *B. anthracis* adsorbed on an adjuvant, aluminum hydroxide (5–7). The primary immunogen of AVA is protective antigen (PA), a component of anthrax toxin that is nontoxic alone. In many animal models, the magnitude and toxin neutralization ability of the humoral response to PA is correlated with protection against *B. anthracis* spore challenge (8–12). Since AVA became mandatory for select populations in 1998, concerns have arisen regarding the relatively high rate of adverse events (AEs), especially in women (13, 14). Among AVA vaccinees, 0.5 to 1% report systemic AEs and 4 to 10% report local AEs to health care professionals (13, 15), similar to other adult vaccinations (15–17). However, when AVA vaccinees are queried after each vaccination, 30 to 70% report AEs (18), similar to various vaccines with higher rates of AEs (16).

While the etiology of vaccination-induced AEs is uncertain, local reactions may occur due to nonspecific activation of the immune system in response to large doses of aluminum adjuvants, mercurothiolate, formaldehyde, or various toxoids (19). In AVA vaccination specifically, intramuscular rather than subcutaneous administration may reduce the rate of local AEs but does not seem to influence the rate of systemic AEs (20). Additionally, certain types of reactions may be associated with sex, ethnicity, and body mass index (14, 16, 17). Despite the results of these studies, little is known about the measurable correlates or immune mechanisms that may underlie adverse events in response to AVA. In this study, we investigated demographic predictors of AEs, as well as serological markers in individuals who reported large local reactions (LLRs) and systemic reactions (SRs) to AVA. An understanding of the immunologic pathways that are associated with AEs may help identify those at risk for AEs and minimize AEs in the future.

MATERIALS AND METHODS

Human subjects. U.S. military personnel ($n = 2,421$) who had received AVA vaccination at least once provided informed written consent and vaccination history, sex, age, and race information. Institutional Review

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Board approval was obtained from the Oklahoma Medical Research Foundation, Walter Reed National Military Medical Center Vaccine Healthcare Centers (VHC) Network/Allergy-Immunology, and Womack Army Medical Center/Fort Bragg Regional VHC. Peripheral blood samples were collected, and plasma, sera, and blood smears were stored until testing. Criteria for experiencing an AE included new onset of cephalalgia; myalgia, and/or arthralgia; fatigue that limited daily activities; large local swelling, redness, or soreness; or another new-onset medical condition that affected the ability to work, sleep, exercise, and/or enjoy leisure activities (see Tables S1 and S2 in the supplemental material). Subsequent serologic studies were performed in individuals who had experienced an adverse event within the last year.

Anthrax protective antigen IgG and IgE ELISAs. Ninety-six-well microtiter plates (Corning, Lowell, MA) were coated with 1 μg/well of recombinant PA (List Biological Laboratories, Campbell, CA). For each sample, plasma was diluted 1:10, 1:100, 1:1,000, and 1:10,000 and added to the plate, followed by conjugate (anti-human IgG [Jackson ImmunoResearch, West Grove, PA] or anti-human IgE [Genway Biotech, San Diego, CA]), with washing after each step. Anti-PA IgG enzyme-linked immunosorbent assays (ELISAs) were developed using paranitrophenylphosphate substrate (Sigma-Aldrich, St. Louis, MO); anti-PA IgE ELISAs were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) and stop solution (SureBlue TMB and TMB Stop Solution; Kirkegaard and Perry Laboratories, Gaithersburg, MD). The optical density (OD) at 405 or 450 nm was detected using a Dynex MRX II microplate reader (Dynex Technologies, Chantilly, VA). The concentrations of anti-PA IgG and IgE were calculated from standard curves of the reference serum AVR801 (BEI Resources, Manassas, VA) (21) and human IgE (Athens Research and Technology, Athens, GA), respectively.

Total plasma IgG and IgE. Total plasma IgG and IgE were assessed by commercial ELISAs (Bethyl Laboratories, Montgomery, TX, and GenWay Biotech, San Diego, CA, respectively) according to the manufacturer's protocol. Briefly, total plasma IgG was detected using anti-human IgG, followed by additions of blocking solution, diluted plasma sample (1:100,000) or standards, and conjugate, with washing between the steps. Total plasma IgE was detected by diluting plasma (1:200), transferring it to precoated plates, and adding the supplied conjugate. Total IgG and IgE ELISAs were developed with the supplied TMB substrate and stop solutions. Plasma IgG and IgE concentrations were determined using assay-specific 7-point calibration curves generated with the manufacturer-supplied standard. A value of 1.0 μg/ml was assigned for concentrations below the limit of detection.

High-sensitivity CRP (hs-CRP) testing. C-reactive protein (CRP) levels were measured in freshly thawed sera by a high-sensitivity commercial ELISA (Biomerica, Irvine, CA). The sera were diluted 1:100 and added to a precoated plate, followed immediately by addition of conjugate. After incubating, the serum-conjugate mixture was removed by washing, and the plate was developed with TMB and stop solution (Biomerica, Irvine, CA). Serum CRP concentrations were calculated against a 5-point standard curve.

Blood smear assessment. Blood smears were made at the time of sample collection and stored at room temperature with minimal light exposure. One hundred leukocytes per smear were counted and characterized by an American Society for Clinical Pathology (ASCP)-licensed medical laboratory technician (C. A. Velte).

Multiplex bead-based assays for cytokines, chemokines, and other soluble mediators. Fifty cytokines, chemokines, and other soluble mediators were assessed by xMAP multiplex assays (Panomics/Affymetrix, Santa Clara, CA), as previously described (22). Multiplex soluble-mediator data were analyzed on the BioPlex 200 array system (Bio-Rad Technologies, Hercules, CA) with a lower boundary of 100 beads per analyte per sample. The median fluorescence intensity (MFI) for each analyte was used to interpolate concentration values from 5-parameter logistic nonlinear-regression standard curves. Analytes below the detection limit were assigned a value of 0.001 pg/ml. A known control serum was included on each plate (Cellgro human AB serum; catalog number 2931949; L/N M1016). The well-specific validity was assessed with AssayCheX QC microspheres (Radix BioSolutions, Georgetown, TX, USA) to evaluate nonspecific binding. The mean interassay coefficient of variance (CV) of multiplexed bead-based assays for cytokine detection has previously been shown to be 10 to 14% (1, 2, 23, 24), and a similar average CV (11%) was obtained across the analytes in this assay using healthy-control serum. The intra-assay precision of duplicate wells averaged <10% CV in each 25-plex assay. Soluble mediators were eliminated from further analysis if >80% of the samples had out-of-range counts or if ≥20% of the samples had normalized MFI values of <0. The plate-to-plate consistency was determined by comparing controls from the individual plates. Soluble mediators were also eliminated if the normalized MFI values of the controls differed significantly across plates (adjusted P value < 0.0001). The following cytokines did not meet these quality control standards and were excluded from further analyses: interleukin 1β (IL-1β), IL-4, IL-6, IL-10, transforming growth factor β (TGF-β), gamma interferon (IFN-β), IL-13, IL-23, IFN-γ, tumor necrosis factor alpha (TNF-α), IFN-α2, IL-17a, IL-2RA, MIG, PAI, RANTES, MCP-1, eotaxin, VEGF-A, TNFR1, TNFR2, resistin, and sVCAM. The following cytokines met the quality control standards and were retained for analysis: IL-2, IL-5, IL-7, IL-12, granulocyte colony-stimulating factor (G-CSF), IL-1α, IL-1RA, IL-15, IL-21, ICAM-1, NGF-β, leptin, SCF, SDF-1, MIP-1α, MCP-3, sFasL, IFN-γ-inducible protein of 10 kDa (IP-10; also known as CXCL10), platelet-derived growth factor (PDGF), MIP-1β, LIF, TRAIL, GROα, sE-selectin, and sCD40L.

Lethal toxin neutralization assay. The ability of plasma to neutralize lethal toxin (LT) was assessed as previously described (25–27) using RAW 264.7 macrophages (ATCC, Manassas, VA). Plasma samples were diluted 1:100 in culture medium and incubated with LT (500 ng/ml PA, 500 ng/ml lethal factor [LF]) for 1 h at room temperature before being added to cell cultures. Control wells included cells alone or cells with PA only, LF only, or LT. Viability was assessed by addition of 10 μl of WST-8 (CCK8; Dojindo Molecular Technologies, Rockville, MD). The OD at 450 nm was detected when the ODs of wells containing only cells were 3.0. *In vitro* neutralization of LT was determined as the percent viability in treated wells relative to wells containing only cells.

Statistical analysis. Statistical analyses included tests for correlation (Spearman), population differences (Mann-Whitney U), and differences of proportion (chi-square with Yate's correction) as appropriate. For cytokine comparisons, P values were adjusted for multiple comparisons by the false-discovery rate method. Analyses of predictors of AEs were performed for each variable independently; the variables analyzed were sex, race, and age at AE occurrence. The number of vaccinations and the time since the last vaccination were not analyzed, as vaccination schedules were often stopped after an AE. The age at last vaccination is approximate and was calculated from the age at sample draw. All analyses of proportion compared the population of interest to all other individuals (e.g., individuals of Asian descent versus all non-Asian individuals). Multivariate analyses were performed with generalized linear models. In analyses of association, trends in data were visualized with a linear regression line. Within the subset of the cohort that was used for serological measurements, each individual who reported a specific AE (an LLR or SR) was cohort matched with two vaccinated individuals without reported events (VIs) so that the number of vaccinations, time since last vaccination, age, race, and sex were consistent between the group of AEs (LLRs or SRs) and the group of compared VIs. VIs were matched within 6 months and 2 vaccinations. Due to individuals not matching person to person in demographic and vaccination history factors, comparisons of the median values were performed using unpaired statistical tests.

RESULTS

Sex, ethnicity, and age are associated with AEs. More than 2,000 consenting human subjects who received at least one dose of the currently licensed U.S. anthrax vaccine were included in this study

TABLE 1 Demographic, vaccination history, and vaccine response information for vaccinated individuals

Characteristic[a]	Value		
	Entire cohort (n = 2,421)	Unaffected vaccinees (n = 2,268)	Vaccinees reporting an adverse event (n = 153)
Ethnicity [no. (%)]			
EA	1,614 (66.6)	1,510 (66.6)	104 (68.0)
AA	265 (10.9)	251 (11.1)	14 (9.2)
H	149 (6.2)	146 (6.4)	3 (2.0)
A	26 (1.1)	21 (1.0)	5 (3.3)
Other	367 (15.2)	340 (15.0)	27 (17.6)
Sex [no. (%)]			
M	2,190 (90.5)	2,089 (92.1)	101 (66.0)
Age (yr) at last vaccination			
Avg (SD)	27.7 (6.8)	27.3 (6.6)	32.2 (8.0)
Median	25.7	25.5	31.6
Range	18–61	18–61	19–54
Yr since last vaccination			
Avg (SD)	1.2 (1.3)	1.2 (1.1)	2.3 (2.6)
Median	0.8	0.7	1.5
Range	0.0–17.0	0.0–11.0	0.0–17.0
No. of vaccinations			
Avg (SD)	4.9 (1.8)	4.9 (1.8)	4.9 (2.1)
Median	5	5	5
Range	1–13	2–13	1–10
Plasma anti-PA IgG (μg/ml)			
Avg (SD)	153.9 (411.2)	155.3 (413.2)	132.8 (381.0)
Median	42.5	43.2	29.9
Range	0–5,505.5	0–5,505.5	0–3,132.8
LT neutralization (% viability)			
Avg (SD)	31.3 (29.6)	31.2 (29.6)	32.9 (30.2)
Median	16.3	16.2	19.2
Range	0.0–128.7	0.0–128.7	2.6–112.7

[a] EA, European American; AA, African American; H, Hispanic; A, Asian; M, male.

(n = 2,421) (vaccination histories and self-reported demographic information are presented in Table 1). Of these, 153 individuals (6.3%) reported reactions that were classified as either LLRs or SRs. Individuals were removed from the vaccination protocol after experiencing an AE; therefore, individuals were unlikely to report more than one AE. Of all the AEs, 103 (67.3%) were LLRs, defined as local redness or swelling >2 in. in diameter, a nodule, or numbness or burning at the vaccination site lasting >24 h. Forty-five AEs (32.7%) were SRs, defined as >24 h of headache, muscle or joint pain, fatigue, or other systemic response that interfered with work or recreation.

Previous clinical trials identified sex and race as factors associated with developing an AE. Following vaccination, females were three times more likely to develop swelling, pain, and activity limitations than men. Interestingly, individuals of African American descent were significantly less likely to report an AE than non-African Americans (14). To assess the influence of sex or other demographic factors on the prevalence of AEs in this real-world cohort of AVA vaccinees, we determined the relative ratios of total AEs (both LLRs and SRs) occurring in multiple demographic groups. AVA-vaccinated females (52/231; 22.5%) were markedly more likely to develop an AE than males (101/2,190; 4.6%) (P < 0.0001; odds ratio [OR] = 6.0 [95% confidence interval {CI} = 4.2 to 8.7]) (Fig. 1A). Hispanic individuals appeared less likely to report an AE (3/149; 2.0%) than non-Hispanics (160/2,421; 7.0%) (P = 0.03; OR = 0.27 [95% CI = 0.09 to 0.86]) (Fig. 1B). Individuals of Asian descent appeared more likely to report an AE (5/26; 19.2%) than non-Asians (158/2,421; 6.6%) (P = 0.03; OR = 3.39 [95% CI = 1.26 to 9.10]) (Fig. 1B). However, the effects of Hispanic (P = 0.07) and Asian (P = 0.4) ancestry were not significant after adjusting for age and sex.

Although AVA safety studies have been conducted with various age groups, an evaluation of age as a risk factor for AEs has not been done. We observed a significant increase of AEs in individuals over the age of 30 years. Specifically, individuals 18 to 29 years of age were less likely to report an AE (69/1,713; 4.0%) than anyone 30 years old or older (84/708; 11.9%) (P < 0.0001; OR = 0.31 [95% CI = 0.22 to 0.43]) (Fig. 1C). Increased age was incrementally associated with increased risk. Individuals 30 to 39 years old were more likely to report an AE than all other age groups (11.4% versus 5.0%; P < 0.0001; OR = 2.55 [95% CI = 1.82 to 3.57]), as were individuals 40 to 49 years old compared to all other age groups (12.3% versus 6.0%; P = 0.01; OR = 2.20 [95% CI = 1.25 to 3.87]) (Fig. 1C). Finally, the greatest difference was observed

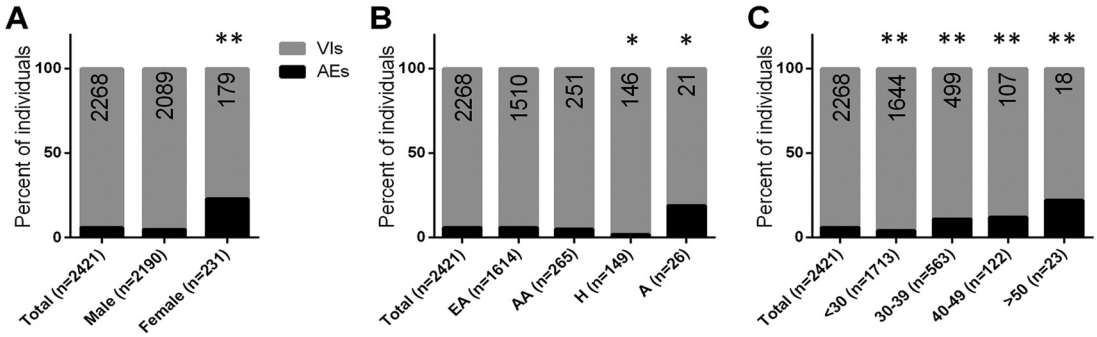

FIG 1 AVA-related AEs are associated with female sex and increased age. Self-reported demographic data were analyzed for associations with the prevalence of AEs. In individual comparisons of sex, ethnicity, and age, males (A), Hispanic individuals (B), and individuals less than 30 years of age (C) were less likely to experience AEs than other demographic groups. Conversely, females (A); individuals of Asian descent (B); and individuals 30 to 39, 40 to 49, and 50 or more years of age (C) were more likely to experience an adverse event. The numbers within the bars indicate the number of unaffected VIs within each demographic group. **, $P < 0.01$; *, $P < 0.05$ versus all other demographic groups by chi-square test with Yates' correction. In multivariate analysis, sex and age, but not race, remained significantly associated with AEs. EA, European American; AA, African American; H, Hispanic; A, Asian.

between the number of AEs reported among individuals 50 years of age or older and all other age groups (21.7% versus 6.2%; $P = 0.009$; OR = 4.22 [95% CI = 1.55 to 11.54]) (Fig. 1C). Notably, increased age was also associated with an increased number of vaccinations (Spearman $r = 0.37$; $P < 0.0001$), suggesting that older individuals may simply have more opportunities for a potential AE. After adjusting for sex and number of vaccine doses with generalized linear models, adverse events remained significantly more likely in individuals 30 to 39 years old ($P < 0.0001$; OR = 3.25 [95% CI = 2.17 to 4.86]), 40 to 49 years old ($P < 0.0001$; OR = 4.95 [95% CI = 2.92 to 8.38]), and 50 years old or older ($P < 0.0001$; OR = 9.68 [95% CI = 4.29 to 21.85]) than in individuals 18 to 29 years of age. These results suggest that the risk for AEs after AVA vaccination increases with age, independent of the total number of vaccinations.

Elevated anti-PA IgE levels are associated with LLRs. IgE specific for tetanus and diphtheria toxoids (Td) is common after Td vaccination in children, adolescents, and adults (28–30). Although rare, elevated levels of IgE antibodies to a vaccine component in human papillomavirus (HPV), varicella-zoster virus (VZV), and rubella have been correlated with an immediate allergic reaction (31). We therefore measured the level of IgE specific to PA in this cohort. Individuals reporting an LLR ($n = 33$) had higher median levels of PA-specific IgE than their matched controls, termed vaccinated individuals (LLR-VIs) (88.3 versus 60.5 ng/ml; $P < 0.01$) (Fig. 2A). In contrast, PA-specific IgE did not

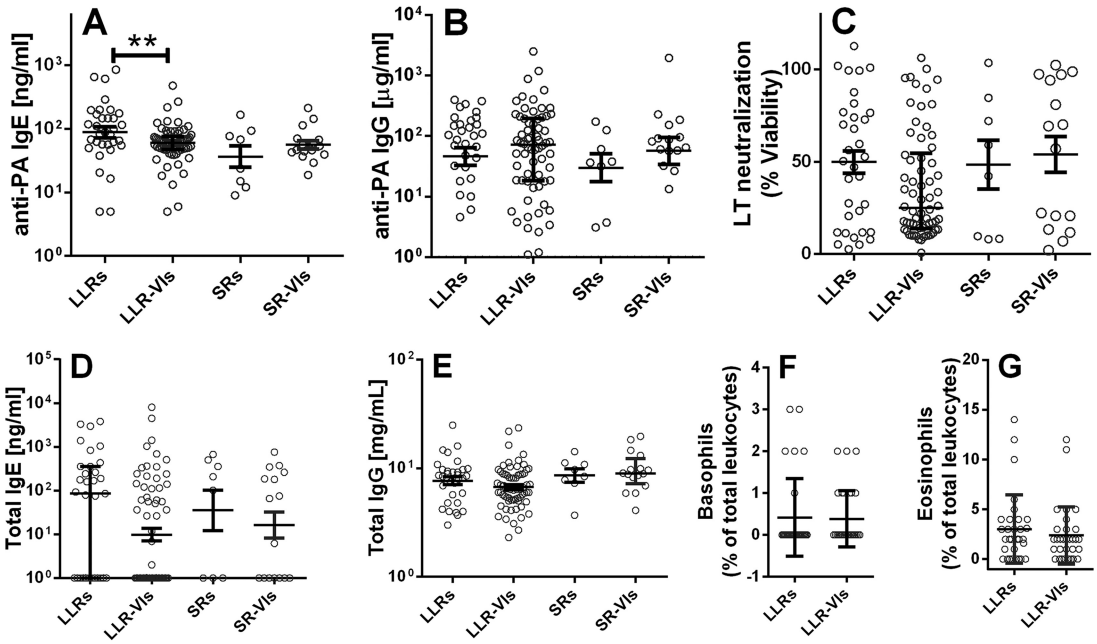

FIG 2 AVA-related LLRs are associated with anti-PA IgE. PA-specific IgE (A) was assessed by ELISA, along with anti-PA IgG (B), total plasma IgE (D), and IgG (E). The ability of plasma to neutralize LT (C) was assessed by *in vitro* assay, and the percentages of leukocytes that were basophils (F) and eosinophils (G) were assessed in blood smears by a trained medical technician. Individuals who reported an LLR had higher levels of anti-PA IgE than their unaffected, matched, vaccinated counterparts. There were no significant differences between the groups in any other measure. Each symbol represents a single individual; the horizontal bars indicate means, and the error bars indicate standard deviations. **, $P < 0.01$ between the indicated groups by Mann-Whitney U test.

TABLE 2 Demographic, vaccination history, and vaccine response information for matched AE and VI groups

Characteristic[a]	Value			
	LLRs ($n = 33$)	LLR-VIs ($n = 66$)	SRs ($n = 8$)	SR-VIs ($n = 16$)
Ethnicity [no. (%)]				
EA	20 (60.6)	39 (59.1)	5 (62.5)	12 (75.0)
AA	2 (6.0)	6 (9.0)	1 (12.5)	2 (12.5)
H	1 (3.0)	7 (10.6)	0 (0)	0 (0)
Mixed	10 (30.3)	14 (21.2)	2 (25.0)	2 (12.5)
Sex [no. (%)]				
M	29 (87.9)	59 (89.4)	6 (75)	10 (62.5)
Age at sample collection (yr)				
Avg (SD)	30.5 (6.6)	29.8 (6.6)	36.4 (7.2)	33.0 (6.2)
Median	30	28	37	34
Range	21–48	21–48	21–44	21–41
Yr since last vaccination [no. (%)]				
Avg (SD)	0.5 (0.2)	0.5 (0.2)	0.6 (0.2)	0.6 (0.2)
Median	0.5	0.5	0.7	0.6
Range	0.2–0.9	0.2–1.3	0.3–0.9	0.1–0.9
No. of vaccinations				
Avg (SD)	4.8 (2.0)	5.0 (1.6)	5.5 (1.9)	5.3 (1.3)
Median	4	5	6	5.5
Range	1–9	3–8	3–8	3–8
Plasma anti-PA IgG (μg/ml)				
Avg (SD)	115.6 (113.4)	172.4 (353.6)	59.1 (60.3)	199.3 (471.2)
Median	86.5	72.0	37.2	73.2
Range	0.1–395.8	1.1–2494.3	3.1–173.6	0.1–1951.1
LT neutralization (% viability)				
Avg (SD)	49.8 (34.6)	36.9 (29.1)	48.4 (37.5)	54.0 (38.8)
Median	50.3	24.8	50.7	63.2
Range	2.6–112.7	0.6–106.3	7.9–103.6	2.0–102.4

[a] EA, European American; AA, African American; H, Hispanic; A, Asian; M, male.

differ between individuals reporting an SR and their matched controls (SR-VIs) (45.9 versus 50.3 ng/ml) (Fig. 2A).

Due to the short half-life of IgE, a consistent time lapse between vaccination and blood draws was maintained between individuals reporting an AE (LLRs or SRs) and their matched controls (LLR-VIs and SR-VIs) (total $n = 123$) (Table 2). Sample collection occurred 2 to 11 months (average, 6 ± 1 month) after vaccination for all individuals reporting an AE and 1 to 15 months (average, 6 ± 1 month) after vaccination for VIs. All comparisons were performed between the subset of AEs (LLRs or SRs) and their matched VIs (LLR-VIs and SR-VIs).

Notably, median PA-specific IgG levels did not differ between the LLR and LLR-VI groups (86.5 versus 72.0 μg/ml) or between the SR and SR-VI groups (37.2 versus 73.3 μg/ml) (Fig. 2B). Similarly, the ability of plasma to neutralize LT *in vitro* did not differ between the LLR and LLR-VI groups (50.3 versus 24.9% viability) (Fig. 2C) or between the SR and SR-VI groups (50.7 versus 63.2% viability) (Fig. 2C). However, in the LLR group, anti-PA IgE levels were associated with anti-PA IgG levels (Spearman's $r = 0.53$; $P < 0.01$) and the number of vaccinations (Spearman's $r = 0.41$; $P < 0.02$), suggesting increased vaccination may induce both PA-specific IgG and IgE.

In general, the highest levels of toxoid-specific IgE after toxoid vaccination may be found in atopic individuals (19). To determine if PA-specific IgE levels were reflected in overall immunoglobulin levels, we measured total plasma IgE and total plasma IgG. Total plasma IgE levels were below the limit of detection in 53% (35/66) of LLR-VIs but similar in the remaining LLR-VIs and individuals with LLRs (Fig. 2D). Median total plasma IgE levels were nonsignificantly higher in the LLR group than in LLR-VIs (85.3 versus 1.0 μg/ml; $P = 0.07$) but did not differ between the SR and SR-VI groups (154.8 versus 36.0 μg/ml) (Fig. 2D). Anti-PA IgE levels were associated with total IgE levels, but only in the LLR group (Spearman's $r = 0.34$; $P < 0.05$). Median total plasma IgG levels were very similar across all groups (Fig. 2E). Additionally, complete blood counts performed on blood smears from 31 individuals with LLRs and 31 matched LLR-VIs showed no significant differences in levels of basophils (Fig. 2F), eosinophils (Fig. 2G), or any other leukocyte type evaluated.

IP-10 levels are associated with LLRs. Multiple studies have previously demonstrated that increased levels of particular cytokines are associated with increased allergic symptoms (32, 33). We therefore hypothesized that levels of soluble inflammatory mediators would be associated with the occurrence of an AE. To test this hypothesis, we determined the levels of 50 soluble mediators by multiplex bead-based assays in freshly thawed plasma from all

individuals with LLRs or SRs and matched VIs. After correcting for multiple comparisons, the levels of IP-10 remained significantly associated with experiencing an LLR. In addition, median IP-10 levels were increased in the LLR group compared to LLR-VIs (MFI = 251.3 versus 183.2; first degree relative [FDR]-adjusted $P = 0.03$), while no differences in IL-4, IL-5, or eotaxin levels were noted.

C-reactive protein levels are elevated in patients with SRs. We hypothesized that widespread inflammation would be associated with elevated CRP levels in individuals reporting an SR. Indeed, we found that mean CRP levels were significantly higher in the SR group than in the SR-VIs (6.88 versus 1.86 mg/liter; $P = 0.02$). Mean serum CRP levels did not differ between the LLR group and the LLR-VIs (3.88 versus 3.05 mg/ml).

DISCUSSION

The possible use of *B. anthracis* as a biological warfare agent necessitates a safe, effective anthrax vaccine. In 1998 the Department of Defense implemented mandatory AVA vaccination of the U.S. military and certain civilian personnel, making AEs in response to AVA vaccination an important consideration for this population. In this study, we assessed whether AVA-related AEs could be predicted by vaccination history, demographic factors, or serological factors in otherwise healthy adult vaccinees.

Demographic factors are correlated with AEs; in the Vaccine Adverse Event Reporting System (VAERS), AEs after AVA vaccination typically occurred among individuals 30 years of age and older (34). Likewise, our results show that the rate of AEs incrementally increases with age, independent of other known risk factors. In addition, multiple studies have demonstrated that females have an increased incidence of AEs following AVA vaccination (18, 34). A 2003 study of U.S. Army medical personnel reported 3.9% of men were temporarily unable to perform one or more of their normal duties due to an AE compared to 5.8% of women. AEs were most common following the initial vaccination, affecting 6.0% of males and 12.2% of females (35). Among U.S. soldiers deployed in Korea and served by the same clinic, a higher percentage of females reported an AE after the first (males, 39.6%; females, 67.9%), second (males, 39.7%; females, 66.1%), and third (males, 31.5%; females, 59.9%) vaccinations (18). Consistent with these studies, a higher proportion of AVA-related VAERS reports were from females, even though more males were immunized with AVA in the military health care system (34). Our results highlight the need for additional studies to elucidate the causes of AEs in female AVA vaccinees and AVA vaccinees over the age of 30 and to identify modified vaccination schedules or other measures to reduce the rate of AEs in at-risk populations.

Several mechanisms have been suggested to lead to AEs. One of the mechanisms is linked to an increase in IgE (31, 36). Our findings show an increase in PA-specific IgE in individuals who develop LLRs but not SRs. IgE has a half-life of 2 days; therefore, relatively elevated levels of IgE in patients reporting LLRs versus LLR-VIs is intriguing. Furthermore, this study reports a 46% increase of anti-PA IgE in individuals suffering from LLRs. An increase in IgE levels of this magnitude is likely to be biologically and clinically significant and may be related to the etiology of the LLRs. For instance, a similar increase in HIV-1-specific IgE correlates with reduced virus production (37), decreased opportunistic infections, and a better quality of life for individuals infected with HIV (38). Proving a direct causal relationship between an antigen-specific IgE and an AE is difficult, and further investigations are necessary to determine a possible mechanism (39).

Both SRs and LLRs are likely mediated by chemokines. In this study, IP-10 (CXCL10) was also associated with LLRs. Various cell types produce IP-10 upon stimulation with the proinflammatory cytokine IFN-γ (40, 41). In addition to recruiting leukocytes to sites of inflammation (42, 43), IP-10 interferes with *B. anthracis* spore germination and causes extensive cell wall and cell membrane disruption *in vitro* (44). Moreover, neutralization of IP-10 increases host susceptibility to pulmonary *B. anthracis* infection *in vivo* (45). Previous studies have identified IP-10 as one of the molecules correlated with trivalent influenza vaccine-associated AEs; however, despite consistent clinical features shared by the patients, a biological mechanism could not be identified (46).

A recent study showed increased levels of CRP, but not of IP-10, in 17 individuals (not stratified by AEs) within 7 days after AVA administration (47). In the same study, individuals receiving an AVA formulation with CPG 7909 adjuvant had early increases in both CRP and IP-10. We observed increased CRP levels months after vaccination in individuals with SRs. Others have noted that a moderate increase in CRP accompanied other proinflammatory molecules in healthy term infants and adults following hepatitis B and influenza vaccine administration, respectively (48, 49). However, these studies reported that CRP levels decreased 48 to 72 h after vaccination. Here, we observed elevated CRP levels 2 to 11 months postvaccination in individuals reporting SRs compared to their paired controls (SR-VIs). It is plausible that one or more of the AVA components are capable of instigating CRP production in these individuals for an extended period. Consequently, prolonged upregulation of proinflammatory molecules, including CRP, likely contributed to a systemic adverse reaction.

Together, these results support a role for IP-10 and CRP in LLRs and SRs, respectively, after AVA vaccination. We have identified likely relationships between types of adverse events and their possible triggers that could be applied to future vaccine design and current vaccination guidelines. Using demographic information, along with serological markers, the ability to differentiate individuals likely to have a specific type of adverse reaction may enable future vaccination guidelines to recommend less frequent immunization or coadministration of anti-inflammatories for individuals at risk for an adverse event. However, further studies should be done to identify the direct relationship between CRP and SRs, as well as IP-10, elevated PA-specific IgE levels, and LLRs.

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We declare that there are no conflicts of interest.

We all materially participated in the research, contributed to manuscript development, and approved the final manuscript.

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