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Human Leukocyte Antigens and Cellular Immune Responses to Anthrax Vaccine Adsorbed

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Interindividual variations in vaccine-induced immune responses are in part due to host genetic polymorphisms in the human leukocyte antigen (HLA) and other gene families. This study examined associations between HLA genotypes, haplotypes, and homozygosity and protective antigen (PA)-specific cellular immune responses in healthy subjects following immunization with Anthrax Vaccine Adsorbed (AVA). While limited associations were observed between individual HLA alleles or haplotypes and variable lymphocyte proliferative (LP) responses to AVA, analyses of homozygosity supported the hypothesis of a "heterozygote advantage." Individuals who were homozygous for any HLA locus demonstrated significantly lower PA-specific LP than subjects who were heterozygous at all eight loci (median stimulation indices [SI], 1.84 versus 2.95, $P = 0.009$). Similarly, we found that class I (HLA-A) and class II (HLA-DQA1 and HLA-DQB1) homozygosity was significantly associated with an overall decrease in LP compared with heterozygosity at those three loci. Specifically, individuals who were homozygous at these loci had significantly lower PA-specific LP than subjects heterozygous for HLA-A (median SI, 1.48 versus 2.13, $P = 0.005$), HLA-DQA1 (median SI, 1.75 versus 2.11, $P = 0.007$), and HLA-DQB1 (median SI, 1.48 versus 2.13, $P = 0.002$) loci, respectively. Finally, homozygosity at an increasing number (≥4) of HLA loci was significantly correlated with a reduction in LP response ($P < 0.001$) in a dose-dependent manner. Additional studies are needed to reproduce these findings and determine whether HLA-heterozygous individuals generate stronger cellular immune response to other virulence factors (*Bacillus anthracis* LF and EF) than HLA-homozygous subjects.

Bacillus anthracis is a Gram-positive, spore-forming pathogen that acts through toxin production and infects both humans and animals. The organism's virulence factors include protective antigen (PA), lethal factor (LF), and edema factor (EF) (1). PA is divided into four different functional domains, with toxin-neutralizing antibody (Ab) epitopes mapping to specific domain 4 (residues 596 to 735) (1). PA is known to bind to a receptor on the cell surface and mediate the entry of both LF and EF into the cell. Recombinant PA-based vaccines have demonstrated protective properties in several animal models, including rhesus macaques, against *B. anthracis* subcutaneous (SQ) and aerosol challenges (2–4). Vaccines based on recombinant PA (rPA) are being considered as novel anthrax vaccine candidates (5, 6).

Anthrax Vaccine Adsorbed (AVA; Biothrax, Lansing, MI) was licensed in 1970 and is produced from an avirulent strain of *B. anthracis*. Given the role of PA in the pathogenesis of clinical disease, levels of antibody (Ab) to anthrax PA (AbPA) in humans have been generally accepted as the primary measure of immunogenicity following vaccination and/or exposure (3). While there is limited information available on the development of cell-mediated immunity (CMI) after AVA vaccination, data from experimental animal models suggest that PA-specific humoral responses are important for protection against anthrax disease (7, 8).

The Centers for Disease Control and Prevention (CDC) funded a multicenter, double-blind, placebo-controlled, randomized clinical trial that tested multiple regimens of AVA (clinicaltrials.gov identifier NCT00119067, here labeled AVA000) (9). Prior to AVA000, the licensed regimen for AVA consisted of SQ administration of six primary doses followed by annual booster doses. An interim analysis of the AVA000 data led the Food and Drug Administration (FDA) to approve a change to the licensed regimen, including a dose reduction from six to five doses and a switch to intramuscular (IM) administration (10).

Differences in the immune responses to AVA, particularly between individuals of European and African ancestry, have suggested a role for host genetic factors (9, 11, 12). Among the most likely candidate genes are the highly polymorphic human leukocyte antigen (HLA) loci on chromosome 6 with alleles that are known to bind a repertoire of naturally processed peptides presented to T cells (13). On the basis of the AVA000 data, Pajewski et al. (14) examined HLA haplotype associations with various antibody responses to AVA, finding significant associations between several DRB1-DQA1-DQB1 haplotypes (*15:01-*01:02-*06:02, *01:01-*01:01-*05:01, and *01:02-*01:01-*05:01) and lower production of AbPA (14). The objective of the present study was to further examine HLA polymorphisms within the specific context of PA-induced cellular immunity. To this end, we hypothesized

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TABLE 1 Demographic characteristics of subjects from the AVA000 clinical trial[a]

Parameter	Value	*n*	Mean SI	SD	Median SI	Q1	Q3	*P* value[b]
Overall		275	5.5	8.3	1.9	1.2	6.1	
Age group (yr)	<30	63	6.6	8.2	2.9	1.3	8.5	0.025
	30–39	64	5.6	8.7	1.9	1.2	4.1	
	40–49	83	6.0	8.8	2.1	1.2	7.4	
	50–62	65	3.7	7.1	1.5	1.0	3.6	
Sex	Female	139	5.9	8.6	2.0	1.2	6.8	0.433
	Male	136	5.1	7.9	1.9	1.2	5.5	
Race	Other race/ethnicity	57	4.9	7.1	1.9	1.2	5.1	0.555
	White	218	5.7	8.5	2.0	1.2	6.2	
Clinical site name	Baylor	62	4.3	5.0	2.4	1.3	5.1	<0.001
	Emory	43	11.1	12.0	6.7	2.2	15.4	
	Mayo	40	8.6	11.3	2.5	1.3	11.0	
	UAB	57	4.2	6.8	1.5	1.1	2.6	
	WRAIR	73	2.6	3.7	1.5	1.0	2.7	
Arm/group	TRT-4IM (3IM)	56	4.5	6.7	1.4	1.1	4.8	0.600
	TRT-5IM (3IM)	55	6.2	9.3	2.4	1.1	5.5	
	TRT-7IM (3IM)	53	6.2	9.2	1.9	1.3	7.4	
	TRT-8IM (4IM)	56	4.9	7.0	2.1	1.2	5.3	
	TRT-8SQ (4SQ)	55	5.9	8.9	1.9	1.2	6.7	

[a] See Marano et al. for a description of the study groups (9). Prior to sampling at week 30, the 3IM arms (TRT-4IM, TRT-5IM, and TRT-7IM) received 3 intramuscular (IM) doses of AVA (0.5 ml) at 0 and 4 weeks and 6 months with 0.5 ml placebo at 2 weeks; the 4IM arm (TRT-8IM) received 4 IM doses of AVA (0.5 ml) at 0, 2, and 4 weeks and 6 months; and the 4SQ (TRT-8SQ) arm received 4 doses SQ AVA 0.5 ml at 0, 2, and 4 weeks and 6 months. Q1 and Q3, the first and third quartiles, respectively. SQ, subcutaneously; TRT, treatment; SI, stimulation index; Baylor, Baylor College of Medicine; Emory, Emory University School of Medicine; Mayo, Mayo Clinic; UAB, University of Alabama at Birmingham; WRAIR, Walter Reed Army Institute of Research.

[b] Data represent the results of analysis of variance using log-transformed SI values.

that polymorphisms within the HLA genes are associated with cellular immune responses to AVA. We specifically sought to study the role of HLA alleles, haplotypes, and homozygosity in lymphoproliferative responses to PA following vaccination with AVA.

MATERIALS AND METHODS

Study subjects. Details of AVA000 have been published elsewhere (9, 14). In brief, 1,564 healthy subjects between 18 and 61 years of age were enrolled at five sites, including the Mayo Clinic (Rochester, MN); Walter Reed Army Institute of Research (Silver Spring, MD); Baylor College of Medicine (Houston, TX); Emory University School of Medicine (Atlanta, GA); and the University of Alabama at Birmingham (Birmingham, AL). We examined a cross-sectional convenience sample of 331 AVA000 participants, measuring the immune response approximately 1 month following the 6-month vaccination (week 30), which corresponds to either the third or fourth dose of AVA depending on the study arm within which the subject participated. Subjects in this convenience sample agreed to participate in a substudy of HLA and lymphocyte proliferation requiring additional blood draws. Of these 331 subjects, 56 were randomly assigned to the saline placebo arm and thus were excluded from the present analyses. Table 1 lists demographic characteristics for the remaining 275 subjects as well as their distribution across the five active treatment arms. The Institutional Review Boards of the Mayo Clinic, CDC, Walter Reed Army Institute of Research, Baylor College of Medicine, Emory University School of Medicine, and University of Alabama at Birmingham approved the study, and written informed consent from each subject was obtained before enrollment. The present study includes only those volunteers who expressly approved (with written consent) the use of their samples and data for further research.

Lymphocyte proliferation (LP) to rPA of *Bacillus anthracis*. Blood samples were collected from each subject at week 30 (~1 month postvaccination) into CPT Vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were harvested as previously described (15). PBMCs were plated in quadruplicate into 96-well round bottom microtiter plates containing 200 μl of either cell culture media alone or media containing 1.25 μg/ml recombinant protective antigen (rPA) (BEI Resources, Manassas, VA). The positive control was phytohemagglutinin (PHA; 10 μg/ml). PBMCs were incubated for 96 h at 37°C in a 5% CO_2 atmosphere. Cultures were then pulsed with 20 μl of a 50 μCi/ml [^{3}H]thymidine solution and incubated for 18 h at 37°C in a 5% CO_2 atmosphere. Cells were harvested onto filter discs (Fisher, Pittsburgh, PA) and counted on a Packard scintillation counter (Packard, Meriden, CT). Stimulation indices (SI) were assessed as the quotient of mean counts per minute of PA-stimulated cells divided by the mean counts per minute of unstimulated cells. A positive lymphoproliferative response was defined empirically as the mean baseline SI across all enrolled individuals (1.1) plus twice the standard deviation (0.53), or 2.16.

HLA genotyping. HLA genotyping was carried out with high-resolution A-SSP, C-SSP, DQA-SSP, DQB1-SSP, DPA1-SSP, and DPB1-SSP Unitray typing assays (Invitrogen), as previously described (16, 17). Class I HLA-B and class II DRB1 locus typing was performed using high-resolution automated reference strand conformation analysis (RSCA) and SSP Unitray typing kits (Invitrogen), as previously described (17).

Statistical methods. Our statistical approach and the description of the statistical analyses and methods are nearly identical to what we have previously published (16, 18–22). Data were descriptively summarized using frequencies and percentages for all categorical variables and medians and interquartile ranges (IQR) for all continuous variables. Associations of SI values with demographic and clinical variables of interest were assessed using analysis of variance methods.

Separate HLA allelic analyses were carried out for each locus. Alleles were grouped by HLA genotype, and descriptive summaries for the measures of SI were first obtained using medians and interquartile ranges. Individuals contributed two observations to these descriptive summaries: one for each allele. Associations between HLA alleles and SI were then formally evaluated using linear regression models. In these models, regression variables for each allele were coded 0, 1, or 2, according to the number of copies of the allele that a subject carried. Separate analyses were carried out using both four-digit and two-digit allele types. Differences in SI among all alleles of each HLA locus were first assessed globally by inclusion of all but one of the ordinal allele variables in a multivariable linear model and simultaneously testing the significance of the entire set of alleles from that HLA locus. Following these global tests, we examined individual allele associations with SI. Each allele was tested individually by inclusion of only the variable corresponding to that allele in separate linear models, effectively comparing the effect of the allele of interest on SI relative to those of all other alleles combined. These series of tests were performed in the spirit of Fisher's protected least-significant-difference test; individual allele associations were not considered statistically significant in the absence of locus-specific global significance.

We next examined associations between SI and HLA haplotypes. Two sets of haplotypes were considered: one for the three class I loci (A, B, and C) and one for the five class II loci (DRB1, DQA1, DQB1, DPA1, and DPB1). Because each individual's linkage phase is unknown, there may be multiple pairs of haplotypes that are consistent with the observed HLA alleles. Posterior probabilities of all possible haplotypes for an individual, conditional on the observed genotypes, were estimated using an expectation-maximization (EM) algorithm similar to the method outlined by Schaid et al. (23). Haplotype design variables were then created using these posterior probabilities. As with the allelic variables, values for each haplotype variable ranged from 0 to 2 and can be considered to be the estimated number of that haplotype carried by the individual after accounting for phase ambiguity. We retained only those haplotypes occurring at least five times in the cohort, pooling all lower frequency haplotypes into an "other" category. Formal analyses then proceeded with these design variables, using the same two-step approach as was used with the allelic analyses, by first assessing associations on a global level and then proceeding with individual haplotype tests only in the presence of a statistically significant global test. Due to phase ambiguity, haplotype-specific descriptive summaries using medians and interquartile ranges could not be obtained using the same approach we applied for individual alleles. Thus, descriptive summaries were represented using the t-statistics obtained from the linear regression analyses. Although not displayed using the original units of measurement, these t-statistics can still be used to assess the magnitude and direction of the haplotype effect since negative values correspond to haplotype-specific decreases in SI and positive values correspond to increases in SI.

We also compared measures of SI in homozygous versus heterozygous individuals using linear regression techniques. Variables representing locus-specific homozygosity status were created for each of the eight available loci. We first fit a separate model for each of the HLA loci. We then created a variable indicating whether a subject was homozygous for at least one of the loci and assessed its relationship with immune response. We then calculated a homozygosity count for each subject. Values of this count could range from 0 to 8, depending on the number of loci for which the subject was homozygous. However, due to the sparseness of the data, individuals homozygous for five or more loci were grouped with those homozygous for four loci. Using this count variable, we assessed the possible dose-response relationship between homozygosity and immune response by fitting the count as a simple ordinal variable.

All formal linear regression analyses described above were adjusted for the following set of potential confounding variables: age at enrollment in years (categorized as <30, 30 to 39, 40 to 49, or 60 to 62); gender; race (Caucasian or non-Caucasian); study site; and randomized treatment arm. Due to data skewness, all formal regression analyses were carried out using log-transformed SI values. All statistical tests were two sided, and all analyses were carried out using the SAS (SAS Institute, Inc., Cary, NC) and S-Plus (Insightful, Inc., Seattle, WA) software systems.

RESULTS

Subject sample demographics. Of the 331 subjects enrolled into the HLA and Immune Correlates of Protection AVA substudies, 275 subjects who received AVA by the SQ or IM route had both HLA typing and cellular immunity (LP) data available. These 275 subjects consisted of healthy individuals between 30 and 62 years of age and were the focus of this study. The majority of the subjects were Caucasian ($n = 218$), and 136 were male (Table 1). As described by Marano et al. (9), these subjects received AVA by the IM (4IM, 5IM, 7IM, and 8IM) or SQ (8SQ) route. There were statistically significant differences in LP across the age of study subjects and across the number of subjects enrolled at each clinical site. However, there were no differences in lymphoproliferative responses with regard to sex, race, or AVA vaccination regimen. The overall median SI for 275 study subjects, assessed by stimulation of PBMCs with PA, was 1.9 (IQR, 1.2 to 6.1). We did not find evidence of a significant association between age and HLA homozygosity.

Associations between HLA alleles and haplotypes and lymphocyte proliferative response. We did not identify significant relationships between either class I or class II alleles and lymphoproliferative responses (Table 2), as no single HLA locus demonstrated a significant ($P \leq 0.05$) global association. The locus with the smallest global P value was HLA class II DQB1 ($P = 0.058$). Two specific alleles, DQB1*06:04 (median SI, 5.03) and DQB1*05:02 (median SI, 4.15), demonstrated higher LP responses to PA. However, these potential allelic effects must be interpreted with caution due to the lack of a significant global effect and the relatively small sample size; the results from these analyses did not conclusively demonstrate that any HLA alleles were significantly associated with LP. Separate analyses were carried out to investigate whether associations between responses to PA and HLA haplotypes were present. The global tests of association for class I (P value $= 0.729$) or class II (P value $= 0.381$) haplotypes with LP responses to PA did not reach statistical significance.

HLA homozygosity and lymphocyte proliferative response. AVA-vaccinated subjects who were homozygous at any HLA locus demonstrated significantly lower PA-specific lymphoproliferation than those subjects who were heterozygous at all eight loci (median SI, 1.84 versus 2.95, $P = 0.009$) (Table 3). When we examined the association between homozygosity at increasing numbers of HLA loci and cell-mediated reactivity to PA, we discovered that increasing numbers of homozygous HLA loci were significantly associated with reductions in LP response ($P < 0.001$) in a dose-dependent manner. When we examined homozygosity effects for specific loci, we found that subjects who were homozygous at HLA-A, DQA1, and DQB1 loci (based on the four-digit allele types) had significantly lower PA-specific lymphoproliferation than subjects who were heterozygous at A (median SI, 1.48 versus 2.13, $P = 0.005$), DQA1 (median SI, 1.75 versus 2.11, $P = 0.007$), and DQB1 (median SI, 1.48 versus 2.13, $P = 0.002$) loci, respectively. In addition, LP responses to PA in subjects homozygous for the HLA-C and DPB1 loci were marginally significantly lower than in subjects who were heterozygous for HLA-C (median SI, 1.51 versus 2.08, $P = 0.067$) and DPB1 (me-

TABLE 2 HLA allelic associations with anthrax protective antigen-specific lymphoproliferative responses

HLA locus	Allele	*n*	Median SI	Q1	Q3	Allele *P* value	Global *P* value[a]
A	*6801	15	1.28	1.09	2.23	0.004	0.223
B	*3503	8	1.43	1.16	3.33	0.029	0.553
	*4403	39	2.58	1.45	7.36	0.046	
C	*0102	16	7.01	1.80	13.21	0.005	0.206
DPA1	None						0.809
DPB1	*0201	81	2.71	1.34	7.11	0.085	0.258
	*1001	9	4.53	2.33	10.04	0.063	
	*1501	5	8.49	1.66	17.08	0.024	
DQA1	None						0.565
DQB1	*0502	10	4.15	2.02	8.59	0.047	0.058
	*0604	19	5.03	1.42	11.94	0.011	
DRB1	*0102	21	1.80	1.31	2.58	0.092	0.133
	*0407	6	0.89	0.62	1.25	0.059	
	*1101	39	4.05	1.46	7.84	0.009	
	*1363	5	17.08	1.42	26.09	0.086	

[a] Data represent the results of linear regression analysis. *P* values for SI levels were determined on the basis of log transformation. Analyses were adjusted for gender, age groups, study site, route of immunization, time between vaccinations, time between blood draw procedures, and number of AVA doses. Alleles corresponding to $P < 0.10$ are included.

dian SI, 1.62 versus 2.11, $P = 0.097$). Similar results were achieved based on the two-digit allele types.

We also evaluated the association between HLA homozygosity and humoral PA-specific antibody responses to anthrax vaccination. While those who are homozygous appear to have slightly lower AbPA responses than those who are heterozygous, these differences are small and we do not have adequate statistical power to confirm or refute the presence of a significant association (see Table S1 in the supplemental material).

DISCUSSION

It is well recognized that interindividual variations exist in immune responses to vaccines and that these variations are in part due to host genetic polymorphisms in HLA and other genes (24). Each HLA molecule binds a distinct set of self- and antigenic peptides that can trigger CMI responses and influence humoral responses through T helper cells. HLA-homozygous individuals logically present a more limited range of peptides than heterozygous individuals at the same HLA loci. Presentation of a narrower range of peptides may lead to diminished $CD4^+$ and $CD8^+$ T cell recognition and in turn may elicit weaker antigen-specific T cell responses. However, diminished T cell response can also be a result of T cell-mediated defects or allelic polymorphisms in genes other than HLA (25, 26).

The goal of this study was to examine associations between LP responses to PA following AVA vaccination and HLA genotypes, haplotypes, and homozygosity. We did not find significant associations of individual HLA alleles or haplotypes with interindividual variations in cellular immune responses to AVA. We did, however, find statistically significant associations with overall homozygosity and homozygosity at one class I locus and at two class II loci whose alleles are in tight linkage disequilibrium (LD). We found that class I (A) and class II (DQA1 and DQB1) homozygosity (as determined by four-digit molecular HLA typing) was significantly associated with an overall decrease in LP response compared with class I (A) and class II (DQA1 and DQB1) heterozygosity. Furthermore, according to the results of the two-digit molecular HLA typing, class I (A and B) and class II (DRB1, DQA1, and DPB1) homozygosity was associated with decreased PA-specific lymphoproliferation. The linkage between decreased lymphocyte proliferation and HLA homozygosity suggests that HLA-heterozygous individuals generate stronger CMI responses to anthrax PA than homozygous subjects who carry these specific alleles. It is possible that differences in the repertoire of PA-derived epitope presentation are likely the basis for these associations between HLA homozygosity and decreased LP response. However, the biological significance of these results and whether these findings can be generalized to other *B. anthracis* virulence factors (LF and EF) are unclear. Further, based on comparisons with a subset of European-American subjects in this study with available genome-wide genotyping on the Affymetrix 6.0 array (12), we did not find any association between heterozygosity at the HLA loci and genome-wide heterozygosity (data not shown).

HLA class II alleles are encoded by DR, DQ, and DP polymorphic genes, and many class II alleles have been found to be important immunogenetic markers for both vaccine-induced viral and bacterial immune responses (14, 27). A relationship between specific DRB1-DQA1-DQB1 haplotypes and a significantly lower AbPA humoral response to AVA has been previously demonstrated (14). In particular, that study found an association between the DRB1*01:02-DQA1*01:01-DQB1*05:01 haplotype and significantly lower AbPA levels following AVA (14). Our study found that A, DQA1, and DQB1 homozygosity (at four-digit molecular resolution) and A, B, DRB1, and DQA1 homozygosity (at two-digit molecular resolution) are significantly associated with diminished PA-specific lymphoproliferation. This suggests that AVA vaccination induces a dominant HLA-restricted immune response to PA antigens and that HLA homozygosity at both class I

TABLE 3 HLA homozygosity associations with lymphocyte proliferation to anthrax protective antigen

HLA locus parameter	Category or level	*n*	Median SI	Q1	Q3	*P* value[a]
4 digit						
A	Heterozygous	231	2.13	1.24	6.78	**0.005**
	Homozygous	44	1.48	1.03	2.28	
B	Heterozygous	258	2.08	1.24	6.40	0.105
	Homozygous	17	1.17	0.98	1.65	
C	Heterozygous	250	2.08	1.24	6.40	0.067
	Homozygous	25	1.51	1.07	2.28	
DRB1	Heterozygous	249	1.94	1.22	6.23	0.295
	Homozygous	26	1.84	1.03	4.53	
DPA1	Heterozygous	102	2.03	1.17	7.38	0.342
	Homozygous	173	1.90	1.19	4.80	
DQA1	Heterozygous	225	2.11	1.25	6.78	**0.007**
	Homozygous	50	1.75	1.04	2.64	
DQB1	Heterozygous	233	2.13	1.25	6.92	**0.002**
	Homozygous	42	1.48	1.01	2.23	
DPB1	Heterozygous	213	2.11	1.21	6.76	0.097
	Homozygous	62	1.62	1.12	3.78	
Any locus	Heterozygous	70	2.95	1.30	9.56	**0.009**
	Homozygous	205	1.84	1.16	4.49	
No. homozygous	0	70	2.95	1.30	9.56	**<0.001**
	1	81	1.89	1.22	5.05	
	2	69	2.13	1.25	6.40	
	3	27	1.69	1.02	3.62	
	4+	28	1.40	1.02	2.08	
2 digit						
A	Heterozygous	230	2.13	1.24	6.78	**0.006**
	Homozygous	45	1.51	1.04	2.33	
B	Heterozygous	253	2.11	1.25	6.68	**0.017**
	Homozygous	22	1.20	0.96	1.65	
C	Heterozygous	237	2.02	1.22	6.28	0.343
	Homozygous	38	1.67	1.14	4.69	
DRB1	Heterozygous	232	2.11	1.25	6.74	**0.020**
	Homozygous	43	1.57	1.04	2.64	
DPA1	Heterozygous	90	1.86	1.13	6.92	0.669
	Homozygous	185	1.94	1.22	5.05	
DQA1	Heterozygous	188	2.29	1.27	7.20	**0.017**
	Homozygous	87	1.78	1.06	3.62	
DPB1	Heterozygous	187	2.12	1.22	7.29	0.059
	Homozygous	88	1.69	1.11	3.60	
DQB1	Heterozygous	184	1.97	1.24	5.89	0.456
	Homozygous	91	1.86	1.12	6.06	
Any locus	Heterozygous	44	2.34	1.15	6.73	0.901
	Homozygous	231	1.90	1.19	5.87	
No. homozygous	0	44	2.34	1.15	6.73	**0.006**
	1	57	3.10	1.28	11.23	
	2	80	2.03	1.38	4.53	
	3	40	1.89	1.25	7.07	
	4+	54	1.38	1.02	2.36	

[a] *P* values for SI levels were determined on the basis of log transformation. Statistically significant *P* values are in bold type ($P < 0.05$). IQR, interquartile range. Linear regression analysis was adjusted for gender, age groups, study site, route of immunization, time between vaccinations, time between blood draw procedures, and number of AVA doses.

and class II antigen presentation pathways potentially diminishes an individual's ability to generate strong cellular immune responses to PA antigens. The possible use of PA-derived epitopes for DRB1*04:01 and DRB1*07:01 and class II tetramers as tools to examine PA-specific Th2 $CD4^+$ cellular immune responses in AVA vaccinees was recently illustrated (28). This is of great interest in light of the potential use of HLA tetramers as tools to monitor T cell responses in vaccinated individuals and for design of subunit and peptide vaccine candidates against *B. anthracis* and other pathogens (29, 30).

Narrower restriction of immune responses in HLA-homozygous individuals (the long recognized "homozygote disadvantage") has been reported for hepatitis C virus (31), human immunodeficiency virus (HIV) (32, 33), hepatitis B virus (HBV) (34), and herpes simplex virus type 1 (HSV-1) (35) infections. These studies clearly demonstrated that HLA homozygosity may be a susceptibility factor for infection (36). Studies correlating HLA homozygosity with the immune responses to HBV, measles virus, and mumps virus vaccines have demonstrated similar results (22, 33, 37). For example, HLA homozygosity has been correlated with

nonresponse to hepatitis B (HBsAg) vaccine in individuals homozygous for the haplotype HLA-B8, SC01, DR3 (38). These observations, together with our current findings, indicate that HLA homozygosity may adversely influence immune responses to bacterial as well as viral vaccines.

Likewise, our previous work with measles virus vaccine demonstrated significant associations of overall and specific homozygosity at HLA loci with lower levels of measles virus IgG antibodies after one dose of vaccine (37). Homozygosity at an increased number of HLA loci, as well as homozygosity at the class I A locus, has been correlated with both decreased mumps virus vaccine-specific antibody levels and lymphoproliferation (22). However, two doses of measles virus vaccination appear to diminish this "homozygote disadvantage" despite HLA homozygosity status at least for measles virus humoral Ab responses (22), suggesting that additional vaccine doses may overcome this genetic disadvantage (39). Similarly, homozygosity within the DPB1 locus showed no disadvantage for both rubella virus-induced IgG antibody levels after two doses of rubella vaccine (40). This dampening phenomenon may imply that additional anthrax immunizations may be necessary to induce higher levels of immunity in individuals who are homozygous for specific HLA alleles.

This is the first report of the effect of HLA genotypes and HLA homozygosity on cell-mediated (lymphoproliferative) immune responses following AVA vaccination. The strengths of our study included the use of subjects selected from a multicenter, randomized clinical trial that tested multiple schedules of the licensed AVA vaccine. Our study also used high-resolution HLA class I and class II genotyping using PCR-based technologies, including automated RSCA and sequence-based typing. Limitations of the study included a somewhat small sample size and limited racial diversity of our study sample, since 80% of the study subjects were Caucasian. Replication of our findings in African-Americans and other racial groups will be required to clarify the role of homozygosity of HLA alleles in AVA-induced immunity in different races and ethnicities. Additionally, lymphocyte proliferation testing was used as a proxy for PA-specific CMI. Due to the long (43-month) follow-up of the AVA000 study, a traditional, accurate, and sensitive *in vitro* assay was utilized to assess the functional capacity of T lymphocytes to respond to the major component of the AVA vaccine. However, these data on recall lymphoproliferative response to rPA in our AVA-vaccinated subjects do not represent a true "CMI" correlate of protection induced by vaccination (41).

The central role of cellular immune responses in postvaccination protection from anthrax was recently demonstrated in a study of AVA-induced long-term protection in rhesus macaques (15). A striking aspect of those data was that a three-dose intramuscular AVA priming series elicited persistent production of functional PA-specific gamma interferon (IFN-γ)- and interleukin-4 (IL-4)-producing T cells and of memory B cells as long as 50.5 months postvaccination when serum AbPA titers were low or undetectable. Irrespective of the humoral antibody titers at the time of infection, nonhuman primates were able to mount a robust and protective anamnestic response after aerosol exposure to *B. anthracis*. Analogous CMI profiles and anamnestic anti-PA IgG responses were also evident in human AVA vaccinees (42).

While we found a strong recall lymphoproliferative response to rPA in our AVA-vaccinated subjects, Ingram et al. found significant elevations of T cell IFN-γ release in response to *B. anthracis* LF (domain IV) but not PA as measured by enzyme-linked immunosorbent spot (ELISpot) assays in United Kingdom-licensed Anthrax Vaccine Precipitated (AVP)-vaccinated subjects (29). Naturally infected subjects demonstrate strong $CD4^+$ T cell responses to both PA and LF (29). Those authors theorized that vaccination skewed the immune response toward a Th2 response, an idea that was supported by the limited response in IL-5 and IL-13 and contrasted with the response seen in the cutaneous anthrax patients (29). It is not known if AVA vaccination similarly biases toward a Th2 response or what the lymphoproliferative response would be to LF.

In conclusion, this report illustrates HLA gene contribution to host immunity associated with variable cellular immune responses to AVA. This information is likely to help to identify antigenic protective peptides within the PA of *B. anthracis* and influence future anthrax vaccine design. New anthrax vaccine candidates that offer protection and result in long-lasting immunity are needed. Additional studies are also necessary to replicate these findings and determine whether HLA-heterozygous individuals generate a stronger cellular immune response to other virulence factors (*B. anthracis* LF and EF) than HLA-homozygous subjects.

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These activities have been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic Conflict of Interest policies.

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