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Comprehensive Analysis of Class I and Class II HLA Antigens and Chronic Hepatitis B Virus Infection

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Following an acute hepatitis B virus (HBV) infection, clearance or persistence is determined in part by the vigor and breadth of the host immune response. Since the human leukocyte antigen system (HLA) is an integral component of the immune response, we hypothesized that the highly polymorphic *HLA* genes are key determinants of viral clearance. *HLA* class I and II genes were molecularly typed in 194 Caucasian individuals with viral persistence and 342 matched controls who had cleared the virus. A single class I allele, *A*0301* (odds ratio [OR], 0.47; 95% confidence interval [CI], 0.30 to 0.72; $P = 0.0005$) was associated with viral clearance. The class II allele *DRB1*1302* was also associated with clearance (OR, 0.42; 95% CI, 0.19 to 0.93; $P = 0.03$), but its significance decreased in a multivariate model that included other alleles associated with disease outcome as covariates. *B*08* was associated with viral persistence both independently (OR, 1.59; 95% CI, 1.04 to 2.43; $P = 0.03$) and as part of the conserved Caucasian haplotype *A*01-B*08-DRB1*03*. The *B*44-Cw*1601* (OR, 2.23; 95% CI, 1.13 to 4.42; $P = 0.02$) and *B*44-Cw*0501* (OR, 1.99; 95% CI, 1.22 to 3.24; $P = 0.006$) haplotypes were also associated with viral persistence. Interestingly, both the *B*08* haplotype and *DR7*, which forms a haplotype with *B*44-Cw*1601*, have been associated with nonresponse to the HBV vaccine. The associations with class I alleles are consistent with a previously implicated role for CD8-mediated cytolytic-T-cell response in determining the outcome of an acute HBV infection.

Worldwide, chronic hepatitis B affects an estimated 350 million persons and is the leading cause of cirrhosis and hepatocellular carcinoma (20). Infection with hepatitis B virus (HBV) in adulthood results in viral persistence and development of chronic hepatitis in 5 to 10% of cases, but factors that determine viral persistence or clearance are not well understood. Certain groups of individuals are known to be at increased risk of developing chronic hepatitis B, including those who are human immunodeficiency virus (HIV) seropositive, male, immunosuppressed, and elderly at the time of infection (17). In addition, the host immune response plays a key role in determining the outcome, since a vigorous, polyclonal cytotoxic-T-lymphocyte (CTL) response correlates with viral clearance (9, 25). These immune responses may be genetically determined, since twin and family studies have suggested an inherited component in the development of chronic hepatitis B (3, 21).

The immune response is coordinated by the human leukocyte antigen (HLA) class I and class II molecules, which present foreign antigens to $CD8^+$ cytolytic T cells and $CD4^+$ helper T cells, respectively. The genes encoding these molecules are the most polymorphic in the human genome and are ideal candidates for the investigation of association with HBV outcomes.

HLA class II genes have previously been examined with respect to HBV outcomes. The largest study, which was from The Gambia, demonstrated an association between *DRB1*1302* and viral clearance (31). This finding was replicated in a small study of Caucasian subjects from Germany (16). We and others have noted a diverse array of additional class II associations (1, 28, 32, 33, 35), whose inconsistencies may be due to small, heterogeneous study populations and variability in typing methods (29). Surprisingly, *HLA* class I alleles have never been comprehensively examined despite their critical role in mediating CTL responses. Thus, using a large cohort of Caucasian individuals with well-defined viral clearance or persistence, we molecularly typed *HLA* class I and class II genes to determine associations of their polymorphisms with the outcome of HBV infection.

MATERIALS AND METHODS

Study subjects. Subjects in this study were participants in one of four studies: (i) AIDS Link to Intravenous Experience, which is an ongoing study of 2,921 injection drug users enrolled in Baltimore from February 1988 to March 1989 (34), (ii) the Multicenter Hemophilia Cohort Study (MHCS), which is a prospectively monitored cohort of 2,056 persons with hemophilia, von Willebrand's disease, or a related coagulation disorder from 16 comprehensive hemophilia treatment centers enrolled between 1982 and 1996 (14), (iii) the Hemophilia Growth and Development Study (HGDS) which is a continuing study of 333 children and adolescents with hemophilia enrolled between March 1989 and May

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1990 (15), and (iv) the Multicenter AIDS Cohort Study (MACS), which is an ongoing study of 5,622 homosexual men enrolled in one of four cities in the United States between 1984 and 1985 and between 1987 and 1991, as previously described (10, 18). A nested case-control design was used such that case patients were subjects persistently infected with HBV and controls were subjects who were able to clear the virus. Each case was matched to two controls from the same cohort based on factors that have been associated with HBV outcomes, including age within 10 years, gender, and HIV status. Subjects were eligible to be tested for viral persistence (cases) if they had (i) a baseline positive hepatitis B surface antigen (HBsAg) test, (ii) a second specimen available for testing, and (iii) peripheral blood mononuclear cells available to be transformed into cell lines. Subjects were considered persistently infected with HBV if they tested positive for HBsAg at two visits separated by a minimum of 6 months. Testing for antibodies against hepatitis B core antigen (anti-HBc) and HBsAg (anti-HBs) was performed as needed to exclude primary HBV infection. Individuals with viral clearance (controls) were positive for anti-HBc and anti-HBs without the presence of HBsAg at two time points separated by a minimum of 6 months. HBV status of the HIV-positive subjects was defined before highly active antiretroviral therapy was available. We restricted our study to Caucasian subjects, since numbers representing other ethnic groups were too small to discriminate a significant effect by race (51 black subjects with viral persistence). Informed consent was obtained from all participants, and the study was approved by the institutional review boards at all participating institutions.

Serologic testing. All serum specimens were stored at −70°C until testing for HBV and HIV serology using commercially available kits and manufacturer's specifications.

HLA typing. An Epstein-Barr virus-transformed cell line was established for each subject, and DNA was isolated from these cell lines by phenol-chloroform extraction. High-resolution (allele level) *HLA* class I genotyping was carried out by using the standard sequence-specific oligonucleotide (SSO) probe typing protocols developed by the 13th International Histocompatibility Workshop (http://www.ihwg.org/protocols/protocol.htm). *HLA-A*, *-B*, and *-C* genes were amplified by using locus-specific PCR primers flanking exons 2 and 3, the polymorphic segments of the class I genes. The 1-kb PCR products were blotted onto nylon membranes and hybridized with a panel of SSO probes. *HLA* alleles were assigned by the reaction patterns of the SSO probes based on known *HLA* sequences. For samples with ambiguous SSO typing results, exons 2 and 3 were sequenced.

Class II molecular typing was done by single-strand conformational polymorphism (7) in combination with PCR-SSO. For PCR-SSO, the protocol was also based on the 13th International Histocompatibility Workshop and primers were designed to amplify exon 2.

Statistical analysis. All analyses were performed with SAS version 6.12 (SAS Institute, Cary, N.C.). In order to reduce the number of comparisons with inadequate power, low-frequency alleles (<3%) were not analyzed. Odds ratios (ORs), which reflect the likelihood of a subject's carrying a specific allele if that subject is persistently HBV infected, and *P* values were calculated by using conditional logistic regression. Known haplotypes composed of alleles that were significantly associated with disease outcome (i.e., $P \leq 0.05$) were also examined by using conditional logistic regression. We further examined all alleles associated with either persistence or clearance ($P \leq 0.05$) using a forward stepwise multivariate analysis. The allele with the lowest *P* value was placed in the model first, and subsequently each allele with a *P* value of ≤0.05 was manually added to the model. An allele was retained in the model if its *P* value remained ≤0.05.

Potential effects of heterozygosity on HBV outcomes were tested separately for the class I and II loci, using conditional logistic regression with the ORs reflecting the likelihood of persistence if heterozygous. Initially, heterozygosity was defined as having nonidentical alleles at all class I loci tested or at all class II loci tested. Subsequently, heterozygosity was examined at each class II locus individually in a univariate analysis. All of the class II loci were included as separate variables in a multivariate model to assess which locus had a stronger association.

RESULTS

A total of 194 individuals fulfilled our criteria for a persistent HBV infection and were matched to 342 individuals with viral clearance. Of the persistently HBV-infected individuals, 151 (77.8%) came from the MACS cohort, and the remaining 43 came from one of the other three cohorts. Of 46 individuals with viral persistence (30 from the MACS, 12 from the HGDS, 1 from the MHCS, and 3 from AIDS Link to Intravenous Experience), only one control subject met the selection criteria. Although matching criteria allowed a 10-year age difference, 95% of the controls matched cases within a 5-year range, and the mean ages of the cases and controls were similar, 30.7 and 30.5 years, respectively. In addition, the groups were similar with respect to the other matching criteria, with 99% being male and 69% being HIV seropositive.

TABLE 1. *HLA* alleles associated with HBV clearance and persistence and their known haplotypes

Allele or haplotype	Frequency (%) in subjects with:		OR	95% CI	*P*
	Clearance (n = 342)	Persistence (n = 194)			
Alleles associated with clearance					
*A*0301*	15.7	8.1	0.47	0.30–0.72	0.0005
*DRB1*1302*	4.9	2.1	0.42	0.19–0.93	0.03
Alleles associated with persistence					
*B*08*	8.1	12.2	1.59	1.04–2.43	0.03
*B*44*	11.9	19.4	1.82	1.27–2.60	0.001
*Cw*0501*	6.8	12.1	1.81	1.16–2.82	0.009
*Cw*1601*	2.4	5.3	2.14	1.09–4.18	0.03
*DQB1*0201*	20.1	27.8	1.56	1.15–2.09	0.004
*DRB1*0301*	9.6	15.1	1.66	1.13–2.42	0.009
Haplotype from alleles associated with clearance					
*A*0301–B*07*	6.1	4.0	0.64	0.34–1.19	0.16
Haplotypes from alleles associated with persistence					
*A*0101–B*08-DQA1*0501-DQB1*0201-DRB1*0301*	5.3	8.0	1.58	0.93–2.68	0.09
*B*44–Cw*1601*	2.4	5.4	2.23	1.13–4.42	0.02
*B*44–Cw*0501*	5.3	10.4	1.99	1.22–3.24	0.006
*B*44–DRB1*0701*	4.6	7.8	1.83	1.06–3.15	0.03
*B*44–Cw*1601-DRB1*0701*	2.0	4.3	2.21	1.04–4.71	0.04

Associations with viral clearance. HBV clearance was associated with only one class I allele, *HLA-A*0301*, with an OR of 0.47 and a 95% confidence interval (95% CI) of 0.30 to 0.72 ($P = 0.0005$) (Table 1). The *A*0301* association appeared to be codominant, since the presence of two *A*0301* alleles was more protective than a single copy (Mantel-Haenszel test for trend, $P = 0.0007$). This allele appeared protective in each cohort, but protection was significant only in members of the MACS, the largest cohort in the study. Of the 13 individuals homozygous for *A*0301*, 11 experienced viral clearance. The known *A*0301-B*07* haplotype was not significantly associated with viral clearance ($P = 0.16$). In a multivariate model, which included the alleles associated with persistence (see below), the OR for *A*0301* was unchanged ($P = 0.0008$) (Table 2).

An analysis of the class II loci showed an association of the allele *DRB1*1302* with viral clearance (OR, 0.42; 95% CI, 0.19 to 0.93; $P = 0.03$). However, when *DRB1*1302* was added to

TABLE 2. Final multivariate model combining alleles associated with clearance and persistence with a P value of <0.05 from univariate analysis[a]

Allele	OR	95% CI	P
*B*08*	1.66	1.08–2.55	0.02
*B*44*	1.81	1.25–2.60	0.002
*A*0301*	0.47	0.30–0.73	0.0008
*DRB1*1302*	0.46	0.21–1.04	0.06

[a] *DRB1*1302* was borderline significant but has previously been associated with HBV clearance; thus, it is presented here in the final model. Alleles considered in the multivariate model include all alleles significant in univariate analysis, specifically, *A*0301*, *DRB1*1302*, *B*08*, *B*44*, *Cw*0501*, *Cw*1601*, *DQB1*0201*, *DRB1*0301.*

the multivariate model the OR was stable (0.46), but it was borderline significant ($P = 0.06$) (Table 2).

Associations with viral persistence. Four class I alleles were associated with viral persistence: *B*08* (OR, 1.59; 95% CI, 1.04 to 2.43; $P = 0.03$), *B*44* (includes *HLA-B*4402* and *HLA-B*4403*) (OR, 1.82; 95% CI, 1.27 to 2.60; $P = 0.001$), *Cw*0501* (1.81; 95% CI, 1.16 to 2.82; $P = 0.009$), and *Cw*1601* (OR, 2.14; 95% CI, 1.09 to 4.18; $P = 0.03$) (Table 1). In addition, two class II alleles, *DQB1*0201* (OR, 1.56; 95% CI, 1.15 to 2.09; $P = 0.004$) and *DRB1*0301* (OR, 1.66; 95% CI, 1.13 to 2.42; $P = 0.009$), were associated with viral persistence. In the multivariate model, which included alleles associated with viral clearance, only *B*08* ($P = 0.02$) and *B*44* ($P = 0.002$) were retained (Table 2).

The two class II alleles significant in the univariate analysis along with *B*08* are often inherited as the conserved haplotype *A*01-B*08-DRB1*0301-DQA1*0501-DQB1*0201*. The association of this haplotype with viral persistence was similar in magnitude to the individual associations of *B*08*, *DQB1*0201*, and *DRB1*0301* (OR, 1.58; 95% CI, 0.93 to 2.68; $P = 0.09$). In order to determine whether *B*08* or either of these class II alleles had the stronger association, we excluded people with the haplotype and tested the associations of *B*08*, *DQB1*0201*, and *DRB1*0301* individually. *B*08* alone was the most predictive (OR, 2.03) of persistence compared to *DQB1*0201* (OR, 1.42) and *DRB1*0301* (OR, 1.40), concurring with the results of the multivariate analysis.

*B*44* forms a haplotype with two of the other alleles associated with viral persistence, *Cw*0501* and *Cw*1601*. The association of *B*44-Cw*0501* (OR, 1.99; $P = 0.006$) and *B*44-Cw*1601* (OR, 2.23; $P = 0.02$) with viral persistence was similar in magnitude to that of the individual alleles. The extended haplotype *B*44-Cw*1601-DRB1*0701* was also associated with viral persistence (OR, 2.21; $P = 0.04$). *Cw*1601* was observed only in the presence of *B*44*, so it was not possible to examine its effects independent of *B*44*. However, the strength of the *B*44* association among individuals without either *C* allele was weaker (OR, 1.10). The same was also true of the association of *Cw*0501* among individuals without *B*44* (OR, 1.72).

HLA heterozygosity and HBV outcomes. We tested the hypothesis that being fully heterozygous at the *HLA* class I or II loci is advantageous for viral clearance since, on average, heterozygotes are expected to present a wider array of antigenic epitopes to the T cells (13). There was no protection due to complete class I heterozygosity defined either by the two-digit (serological equivalent) (OR, 0.76; 95% CI, 0.50 to 1.14; $P = 0.18$) or the four-digit (high-resolution) nomenclature (OR, 0.69; 95% CI, 0.45 to 1.07; $P = 0.10$) (Table 3). On the other hand, viral clearance was more common among individuals who were heterozygous at all class II loci (OR, 0.62; 95% CI, 0.40 to 0.95; $P = 0.03$). Next, potential confounding effects of *HLA-DQB1*0201* and *HLA-DRB1*0301*, class II alleles associated with viral persistence, were excluded by removing individuals with these alleles from the zygosity analysis. The protective effect of heterozygosity on viral clearance was independently significant (OR, 0.41; 95% CI, 0.19 to 0.85; $P = 0.02$). Heterozygosity at *DQB1* was most strongly associated with viral clearance in a univariate analysis of individual class II loci (OR, 0.50; 95% CI, 0.31 to 0.83; $P = 0.008$) and in a multivariate model of all class II loci (Table 3).

TABLE 3. Association of HLA class I and class II heterozygosity and hepatitis B virus outcomes

	Frequency (%) of all heterozygous subjects with[a]:		OR	95% CI	P
	Clearance ($n = 342$)	Persistence ($n = 194$)			
Class I					
2-digit nomenclature	74.9	70.0	0.76	0.50–1.14	0.18
4-digit nomenclature	79.5	73.8	0.69	0.45–1.07	0.10
Class II	77.8	69.7	0.62	0.40–0.95	0.03
Univariate					
DQA1	81.6	76.2	0.70	0.44–1.01	0.12
DQB1	86.9	78.8	0.50	0.31–0.83	0.008
DRB1	91.4	86.9	0.56	0.32–1.00	0.05
Multivariate					
DQA1	NA	NA	1.12	0.58–2.14	0.74
DQB1	NA	NA	0.50	0.26–2.14	0.05
DRB1	NA	NA	0.80	0.34–1.89	0.61

[a] NA, not applicable.

DISCUSSION

In this comprehensive study of *HLA* class I and class II genetic effects on the outcome of HBV infection in Caucasians, *A*0301* and *DRB1*1302* were associated with a 2-fold increase in HBV clearance, whereas *B*08* and two *B*44* haplotypes were associated with a >1.5-fold increase in viral persistence. It is notable that overall, the class I alleles have the strongest associations, suggesting that the $CD8^+$ CTLs are important in determining viral clearance or persistence. These findings concur with the recent demonstration that chimpanzees acutely infected with HBV and then depleted of $CD8^+$ T cells were unable to eliminate the virus (27).

*B*08* and its extended haplotype (*A1-B8-DR3-DQ2*) have been associated with other immune-mediated diseases, including autoimmune hepatitis (22, 24). In 57 to 88% of those instances, *B*08* is observed on this haplotype (24). Here, *B*08* appeared to be more predictive of outcome than the other alleles in the haplotype. Functional studies of individuals with the *A*01-B*08-DR3* haplotype have suggested a defect in early T-cell activation (5) and decreased production of type 1 cytokines, including interleukin 2, gamma interferon, and interleukin 12 (4). Decreased natural killer cell activity has also been

noted in people with this haplotype (8). However, neither the mechanism nor the precise gene responsible for these findings has been elucidated, thus retaining the possibility that *B*08* may be at least partly responsible for these phenotypes.

Although *B*44* on haplotypes with both *Cw*1601* and *Cw*0501* was associated with viral persistence, *B*44* in the absence of *Cw*1601* and *Cw*0501* was not. The stronger association with the haplotypes may indicate that these alleles are simply markers of the true disease allele through linkage disequilibrium. The *B*44-Cw*1601* haplotype often includes the class II allele *DRB1*0701*, whose serological equivalent (*DR7*) has been associated with viral persistence in a small study from Qatar (1). However, in our study, *DRB1*0701* alone was not significantly associated with HBV persistence (OR, 1.23; 95% CI, 0.87 to 1.75; $P = 0.25$).

Poor response to the HBsAg recombinant form of HBV vaccine has been associated with some of the same alleles associated with viral persistence in the present study, suggesting similarities in the immune defect leading to viral persistence and vaccine nonresponsiveness. Several studies have demonstrated nonresponse to HBV vaccine among individuals with the extended *A*01-B*08* haplotype (2, 11, 19). *DR7* has also been reported to be associated with nonresponse to hepatitis B vaccine (11, 12, 23). In the one study that examined *HLA-A* and *-B* genes, *B*44* was more common in nonresponders (40.7%) than in responders (23.9%) (23).

*A*0301* was significantly associated with viral clearance in a multivariate model. Examination of the A3 supertype, which includes *A*0301* and other *A* alleles with cross-reactive epitopes (26), indicated that it could not account for the *A*0301* association. The molecule encoded by *A*0301* may be particularly efficient at presenting HBV epitopes to the $CD8^+$ T cells, but functional studies are necessary to confirm this hypothesis.

*DRB1*1302*, an allele that has been associated with HBV clearance in The Gambia (31), was the only other allele associated with viral clearance here. The borderline significance of this allelic effect in the multivariate analysis may have been a consequence of its low frequency (3.8%). Alternatively, the prominence of this allele in the Gambian study may indicate greater importance in certain ethnic groups or in those who acquire infections in infancy or early childhood.

The association of class II heterozygote advantage confirmed findings for the Gambian cohort (30). The multivariate analysis suggested that the effect of class II heterozygosity is due primarily to the *DQB1* locus. Class I zygosity may also be predictive of outcome to HBV infection, and our failure to detect such an association may reflect insufficient statistical power.

There are three primary limitations to this study. First, Bonferroni correction for multiple comparisons would have excluded all but the association of *A*0301* with viral clearance. However, that statistical maneuver for genetic association studies is controversial; its conservative overcorrection likely leads to type II error (6). Our multivariate analysis, not performed in previous HLA studies, highlighted the alleles that are independently associated with either viral clearance or persistence and strengthens our univariate findings. Furthermore, the biologic plausibility of those associations linked to vaccine nonresponsiveness makes them worthy of further investigation. Second, despite the large sample size, we had limited power to detect associations with very-low-frequency alleles. Third, we combined several cohorts in this study and acknowledge that we did not have the power to test each allele individually in each cohort. Thus, the associations we detected may not apply to every population and we may also have missed associations that are operative only in certain populations. Further research, such as by replication in another cohort, is needed to confirm our findings.

The data reported herein support a role for *HLA* molecules, particularly class I, in determining the outcome of a HBV infection and may provide clues regarding the pathogenesis of HBV infection. Functional studies are needed to understand the basis for these associations and the link between the genes associated both with viral persistence and with nonresponse to the HBV vaccine.

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