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Cytotoxic T-Lymphocyte Antigen 4 Gene and Recovery from Hepatitis B Virus Infection

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Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an inhibitory T-cell receptor expressed by activated and regulatory T cells. We hypothesized that single-nucleotide polymorphisms (SNPs) in the gene encoding CTLA-4 may affect the vigor of the T-cell response to hepatitis B virus (HBV) infection, thus influencing viral persistence. To test this hypothesis, we genotyped six *CTLA4* SNPs, from which all frequent haplotypes can be determined, using a large, matched panel of subjects with known HBV outcomes. Haplotypes with these SNPs were constructed for each subject using PHASE software. The haplotype distribution differed between those with viral persistence and those with clearance. Two haplotypes were associated with clearance of HBV infection, which was most likely due to associations with the SNPs −1722C (odds ratio [OR] = 0.60, $P = 0.06$) and +49G (OR = 0.73, $P = 0.02$). The wild-type haplotype, which contains an SNP leading to a decreased T-cell response (+6230A), was associated with viral persistence (OR = 1.32, $P = 0.04$). These data suggest that *CTLA4* influences recovery from HBV infection, which is consistent with the emerging role of T regulatory cells in the pathogenesis of disease.

Worldwide, over 300 million persons are chronically infected with hepatitis B virus (HBV), which can cause chronic liver disease and hepatocellular carcinoma (22, 35). In adults, recovery from HBV infection is associated with a broad, vigorous immune response (5, 27). Most research aimed at understanding the mechanisms underlying recovery from HBV infection has been focused on the factors that lead to the activation of antiviral T-cell responses. However, the intensity of the counterregulatory mechanisms that restrain the vigor of such T-cell responses may be just as important, as has been demonstrated in murine models of parasitic and viral infections (4, 30).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an inhibitory receptor expressed by T lymphocytes that acts largely as a negative regulator of T-cell responses (28). Mice deficient in CTLA-4 develop a lymphoproliferative disorder with multiorgan lymphocytic infiltration (32). In addition, antibody-mediated blockade of CTLA-4 in vivo has been shown to upregulate the strength of the immune response in murine models of infection (19), tumor immunity (21), vaccination (7), allergy (10), and autoimmune disease (16).

The gene encoding CTLA-4, which is located on the long arm of chromosome 2, has been extensively studied in a European population (14). Johnson et al. demonstrated that genotyping five *CTLA4* single-nucleotide polymorphisms (SNPs) discriminates all six known haplotypes with a frequency of >5% (haplotype-tagging SNPs) (Fig. 1) (14). Ueda et al. reported that an SNP at position +6230 (referred to as CT60 by Ueda et al.) in the 3′ untranslated region (UTR) of *CTLA4* determines levels of the soluble isoform of CTLA-4 (sCTLA-4), which has been shown in vitro to inhibit T-cell proliferation (33). Recently, two of these haplotype-tagging SNPs, at position +49 in exon 1 and position −318 in the promoter, were examined in persons treated for chronic hepatitis C with alpha interferon and ribavirin (36). The +49G allele alone and in a haplotype with −318C was associated with an increased frequency of sustained virologic response to treatment. However, this gene has not been previously examined with respect to HBV outcomes.

We hypothesized that *CTLA4* haplotypes and SNPs might explain some of the between-person differences in recovery from HBV infection. To test this hypothesis, we typed the five haplotype-tagging SNPs and the SNP at +6230 in a well-characterized cohort of individuals with HBV clearance and persistence. We restricted ourselves to these SNPs since the haplotype-tagging SNPs capture all major haplotypes of a gene and thus are the ones essential to examine in association studies (14).

MATERIALS AND METHODS

Study participants. Subjects in this study were participants in one of the following two cohorts: (i) the AIDS Link to Intravenous Experience (ALIVE) study, which is an ongoing study of 2,921 injection drug users enrolled in Baltimore, Md., from February 1988 to March 1989, as previously described (34), or (ii) the Multicenter AIDS Cohort Study (MACS), which is an ongoing study of 5,622 gay men enrolled in one of four United States cities between 1984 and 1985 and between 1987 and 1991 (6, 17).

To investigate the hypothesis that *CTLA4* SNPs might be associated with recovery from acute hepatitis B, a nested case-control design was used in which one person with viral persistence was matched to two persons from the same cohort who had viral clearance but were otherwise similar with regard to nongenetic factors. Matching criteria included age within 10 years, gender, human immunodeficiency virus (HIV) type 1 status, ethnicity, and geographic location,

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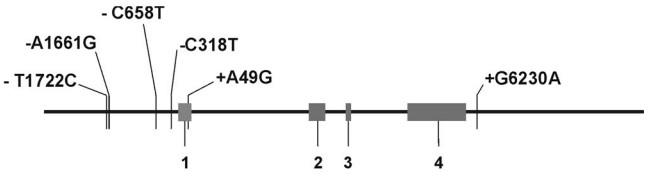

FIG. 1. Schematic representation of the *CTLA4* gene drawn to scale. The four exons are represented as boxes. The SNPs used in this investigation are labeled with their positions and the corresponding polymorphic bases. The SNPs at −1772, −1661, −658, and −318 are located in the promoter region. The SNP at +49 is in exon 1, and the SNP at +6230 is in the 3′ UTR.

factors that have been associated with HBV recovery (8, 12). Subjects were considered persistently infected with HBV if their serum or plasma tested positive for hepatitis B surface antigen (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 HBV recovery were positive for anti-HBc and anti-HBs without the presence of HBsAg at two time points separated by a minimum of 6 months. The HBV status of HIV-positive subjects was determined before any antiretroviral therapy was available.

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. HIV type 1 antibody testing was done by enzyme immunoassay with reactive results confirmed as positive by Western blotting as previously reported (6, 9, 17, 34). HBsAg, anti-HBs, and anti-HBc testing was done using commercially available kits according to the manufacturer's (Abbott Laboratories, Abbott Park, Ill.) specifications.

DNA extraction and *CTLA4* genotyping. For each individual, Epstein-Barr virus-transformed cell lines were established, and genomic DNA was extracted from these cell lines using phenol-chloroform extraction.

Six SNPs in *CTLA4* were genotyped by using the AcycloPrime-FP SNP detection assay (Perkin-Elmer, Boston, Mass.), a single-base extension method, according to the manufacturer's specifications (11). In this method, a 200-base-pair fragment containing the SNP of interest is amplified (primers are listed in Table 1) with cycling conditions of 95°C for 10 min; 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 60 s; and then a final extension step of 72°C for 10 min. After amplification, the excess primer and deoxynucleoside triphosphates are degraded with shrimp alkaline phosphatase and exonuclease I used according to the manufacturer's specifications. These enzymes are heat inactivated allowing for the final step, which adds one of two fluorescent terminators, representing the alleles present at the SNP of interest, to a primer ending immediately upstream of the SNP site (extension primer). The cycling protocol for the single-base extension step is 95°C for 2 min followed by 15 cycles of 95°C for 15 s and 55°C for 30 s. The results are identified by the amount of fluorescence polarization of each allele as determined by the Victor^{2}V instrument (Perkin-Elmer). In order to determine the accuracy of this method, we verified the results with direct sequencing for 25 samples from each SNP and found no errors.

Statistical analysis. Allele frequencies in those with viral persistence and clearance were calculated from the genotypes of the subjects. The Hardy-Weinberg equilibrium was assessed for each SNP by using the chi-square test with 1 degree of freedom.

A haplotype is a combination of SNPs in a gene or a locus region that occurs more often than expected by chance, indicating linkage disequilibrium in the region. In association studies, haplotypes are inferred from the population in the study. In order to reconstruct haplotypes, we used PHASE version 2.0 (https://depts.washington.edu/ventures/clickthru/ReleaseAgreement.php?raf=PHASEV2) (29). We also used PHASE to perform a permutation test to determine if there was a difference in the distribution of haplotypes between those with viral persistence and those with clearance. The permutation test examines the null hypothesis that the haplotypes in those with viral clearance and those with persistence are derived from the same population versus the alternative hypothesis that those with viral clearance are more similar to each other than to those with viral persistence. The test was run five separate times at 100 permutations to check for consistency. Additional runs with up to 10,000 permutations gave similar results and goodness of fit parameters. For each haplotype, we determined odds ratios (ORs), which reflect the likelihood of carrying a specific haplotype if persistently HBV infected, and *P* values by using conditional logistic regression software (SAS, version 10; SAS Institute, Cary, N.C.). A *P* value of <0.05 was considered significant for this analysis.

Based on the haplotype analysis, we determined whether selected individual SNPs were associated with viral clearance or persistence by using conditional logistic regression to determine the ORs and *P* values. The SNPs were examined in all subjects and then stratified by ethnicity (i.e., black versus white) to evaluate ethnic differences. Since those in the ethnicity category "other" were heterogeneous and few in number, they were excluded from the ethnicity analysis. A *P* value of <0.05 was considered significant. SNPs with *P* values of <0.05 were also stratified by HIV status.

RESULTS

Study subjects. The study group was composed of 189 persons with chronic hepatitis B and 338 persons who had recovered from HBV infection (40 chronically infected persons had only one match), for a total of 378 and 676 alleles, respectively. No significant differences were detected between those with HBV recovery and those with HBV persistence with respect to the matching criteria: 64% were HIV positive, the mean age was 34 years, and 98% were male. The majority of the study group (76%) was white, and out of the remainder, 22% were black and 2% were in the category "other."

Haplotype analysis and HBV recovery. All of the haplotype-tagging SNPs met the Hardy-Weinberg equilibrium and defined seven unique haplotypes. These haplotypes had frequencies similar to those previously published (14), and six of them had frequencies of >5% (Table 2). The overall distribution of these haplotypes in those with viral recovery and those with persistence appeared to be different (*P* values from the five-permutation runs ranged from 0.01 to 0.10). Analysis of individual haplotypes revealed that the wild-type haplotype (haplotype 3) was associated with viral persistence (OR = 1.33; 95% confidence interval [CI], 1.01 to 1.73; *P* = 0.04). Haplotype 2, which differs from the wild type at the haplotype-tagging

TABLE 1. Primers used for genotyping *CTLA4* SNPs using fluorescence polarization

SNP (NCBI reference no.)	Amplification primers (5′ to 3′)		Extension primer (5′ to 3′)[a]
	Forward	Reverse	
−T1722C (rs733618)	AAAAGTGAAAAACAAATGTTCC	ACTTTAGCCCATGTTATTCTTCT	GAACACACAGCAGTGGCAGGGACAG (R)
−A1661G (rs4553808)	TCCTCTTGAGGGCAGGAACA	TGTGCCATGTTGGTGTGATG	CAGACTGGGCAACAGAGGTTTTT (R)
−C658T[b]	TTGGGTTGGCTTTTCTTTGGA	CACCATCCTTCTAATGGTCCCTTG	ATCACAAGAAATAAACTGAAAATAGGC (R)
−C318T (rs5742909)	GGATGGTTAAGGATGCCCAGAA	GGAAGCCGTGGGTTTAGCTG	CTCCAAGTCTCCACTTAGTTATCCAGATCCT (F)
+A49G (rs231775)	GACCTGAACACCGCTCCCATA	TGACTGCCCTTGACTGCTGAA	GCACAAGGCTCAGCTGAACCTGGCT (F)
+G6230A (rs3087243)	CTGCAAGTCATTCTTGGAAGG	AGATCAAAATGGCTGCAAGG	GATTTCTTCACCACTATTTGGGATATAAC (F)

[a] F, forward; R, reverse.
[b] The rs number is not available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp. The flanking sequence for this SNP is GCTTCTTTTC(C/T)GCCTATTTTC.

TABLE 2. *CTLA4* haplotypes in persons with HBV clearance and persistence

Haplotype	Haplotype-tagging SNP at position:					Proportion of indicated haplotype (%)		OR	95% CI	*P*
	−1722	−1661	−658	−319	+49	Recovery (n = 676)[a]	Persistence (n = 378)[a]			
1	T	A	C	C	G	30.9	27.2	0.86	0.65–1.15	0.31
2	C	A	C	C	G	9.5	6.0	0.58	0.34–0.97	0.04
1 and 2 combined	T or C	A	C	C	G	40.3	33	0.74	0.57–0.98	0.03
3	T	A	C	C	A	36.9	44.3	1.33	1.01–1.73	0.04
4	T	A	T	C	A	6.8	7.6	1.18	0.72–1.95	0.51
5	T	G	C	C	A	8.9	7.6	0.83	0.51–1.34	0.44
6	T	G	C	T	A	7.0	7.3	1.11	0.67–1.83	0.67

[a] Values for n indicate two times the number of individuals since each person carries two haplotypes.

SNPs −1722 and +49, was associated with viral clearance (OR = 0.58; 95% CI, 0.34 to 0.97; P = 0.04). Haplotype 1, which only differs from the wild type at position +49, also trended toward an association with viral clearance (OR = 0.86; 95% CI, 0.65 to 1.15). Since the SNP +49G was found in both haplotypes 1 and 2, we combined persons with these haplotypes and detected an association with viral clearance (OR = 0.74; 95% CI, 0.57 to 0.98; P = 0.03). None of the other haplotypes were associated with outcome.

Single-locus analysis and HBV recovery. Since the SNPs at −1722 and +49 were at the positions at which haplotypes 1 and 2 differed from the wild type, we examined them in order to determine whether one or both were individually associated with outcome. The SNP +49G was detected more often in persons who recovered from HBV infection (OR = 0.73; 95% CI, 0.56 to 0.95; P = 0.02), and the SNP −1722C also trended in that direction (OR = 0.60; 95% CI, 0.36 to 1.01; P = 0.06) (Table 3). In addition, although the +6230 SNP was not a haplotype-tagging SNP, we examined it given its reported importance in CTLA-4 function (33). The mutant base +6230A, which was present with the wild-type haplotype 82% of the time, was detected more often in persons with viral persistence (OR = 1.32; 95% CI, 1.01 to 1.72; P = 0.04). Stratification by ethnicity did not alter these associations, with the exception of −1722C having a stronger association with HBV recovery in whites than in blacks. The associations were not significantly different between HIV-positive and -negative subjects, since the 95% confidence intervals overlapped. The ORs for the HIV-negative and -positive individuals, respectively, were as follows: for −1722C, 0.86 (95% CI, 0.37 to 1.99) and 0.52 (95% CI, 0.26 to 1.05); for +49G, 0.94 (95% CI, 0.59 to 1.49) and 0.71 (95% CI, 0.50 to 1.00); and for +6230A, 1.11 (95% CI, 0.69 to 1.80) and 1.33 (95% CI, 0.95 to 1.87).

For the +49 SNP, the association was stronger in homozygous persons than in heterozygous ones for the allele (test for trend, P = 0.04). In particular, the OR for +49G/G was 0.48, whereas for +49G/A it was 0.78. For the +6230 SNP, the associations were equivalent in the homozygous and heterozygous states with odds ratios of 1.64 and 1.52, respectively. For the −1722 SNP, too few subjects (<1%) were homozygous for the mutation to be assessed for codominance.

DISCUSSION

The outcome of an HBV infection varies according to the vigor of the immune response, a process that is regulated by a number of molecules, including the cell surface receptor CTLA-4. We found that *CTLA4* haplotypes are important determinants of HBV recovery. Haplotypes containing the +49G allele either alone or with −1722C were associated with clearance of HBV infection. Since these haplotypes may alter the ability of CTLA-4 to downregulate the immune response, these data suggest that the vigor of counterregulatory mechanisms contributes to the clearance of an HBV infection.

Prior studies suggest that the +49G SNP may be a major determinant of this association. The +49G allele has been associated with increased risk for diseases resulting from an overly vigorous immune response, including insulin-dependent diabetes mellitus, Grave's disease, autoimmune hepatitis, and systemic lupus erythematosus (1, 2, 13, 20). Likewise, in an earlier study this allele was also associated with improved clearance of HCV infection resulting from alpha interferon-based therapy (36). These studies could not rule out the role of other SNPs linked to +49 since the other haplotype-tagging SNPs were not studied. However, further support for the role of this mutation exists since the A→G mutation at position +49 leads to a nonsynonymous amino acid change from threonine to alanine, thus changing the polarity of the amino acid

TABLE 3. Proportions of selected *CTLA4* SNPs in participants with HBV recovery and persistence, combined and stratified by ethnicity

Ethnicity group and allele	Proportion (%) of alleles with the indicated variant		OR	95% CI	*P*
	Recovery[a]	Persistence[b]			
Whites and blacks					
−1722C	9.2	6.0	0.60	0.36–1.01	0.06
+49G	40.3	32.7	0.73	0.56–0.95	0.02
+6230A	37.9	45.0	1.32	1.01–1.72	0.04
Whites					
−1722C	8.5	4.2	0.48	0.25–0.92	0.03
+49G	39.2	32.0	0.74	0.54–1.00	0.05
+6230A	43.8	50.7	1.28	0.92–1.72	0.10
Blacks					
−1722C	11.4	10.5	0.90	0.37–2.21	0.82
+49G	41.8	33.3	0.73	0.41–1.30	0.29
+6230A	18.4	26.2	1.63	0.84–3.18	0.15

[a] The numbers of alleles associated with recovery, by ethnicity group, were as follows: whites and blacks, 676; whites, 512; blacks, 152.

[b] The numbers of alleles associated with persistence, by ethnicity group, were as follows: whites and blacks, 378; whites, 290; blacks, 80.

and potentially altering the function of the protein. Lymphocytes from donors carrying +49G appear to express less CTLA-4 on their surfaces, proliferate more under conditions of suboptimal activation, and exhibit less CTLA-4-mediated inhibition of T-cell responses (25, 26).

Our data also suggest a role for the promoter SNP −1722C in HBV recovery. Its strong association may be due to its linkage with the SNP at position +49, since it is only on a haplotype with +49G. Alternatively, since it is in the promoter region of the gene, it may alter the transcriptional regulation of CTLA-4. If −1722 has an independent association, our data would support decreased CTLA-4 production with −1722C leading to an increased chance of recovery from HBV infection.

We also found that those with the allele +6230A in the 3′ UTR were 1.3 times more likely to have viral persistence. A recent study demonstrated that this mutant allele was associated with a decreased T-cell response and is associated with protection from autoimmune disease (33). This allele's being found on the wild-type haplotype 82% of the time may partially account for the association of this haplotype with viral persistence.

CTLA-4 was initially thought to act largely in *cis*, restraining the activation of the cells that express it. More recently, it has become clear that CTLA-4 also acts in *trans (*3). Notably, CTLA-4 is constitutively expressed on $CD4^+$ $CD25^+$ regulatory T cells (Tregs), and such expression is important for Treg-mediated suppression of T-cell proliferation (4, 31). Tregs have been shown to play an essential role in restraining the vigor of the immune response to both herpes simplex virus and *Leishmania major* in mouse models (4, 30). Since some studies have not demonstrated an effect of CTLA-4 blockade on Treg-mediated suppression (15, 24), further functional work is needed to demonstrate that *CTLA4* polymorphisms alter the vigor of the T-cell response by affecting Treg development and/or function.

Approximately half of our subjects were HIV infected, but this should not have affected the results of our study for several reasons. First, HBV infection occurs prior to HIV infection in injection drug users, so the outcome of the viral hepatitis infection is determined prior to acquiring HIV (23). Likewise, when HIV and HBV are acquired via sexual transmission, HBV infection usually occurs first (18). Second, those with viral recovery and those with persistence are matched with respect to HIV status. Third, stratification of the analysis by HIV status did not significantly alter the results.

There are a few limitations to our study. First, we did not genotype all known *CTLA4* SNPs but limited our study to the haplotype-tagging SNPs identified in a European population. Since *CTLA4* has not been previously studied in blacks, it is possible that novel haplotypes exist in this population. We may have missed such haplotypes if they did not include SNPs genotyped in this study. Second, there may be associations with SNPs or haplotypes that are infrequent (<5%), but we did not test those since we had limited power to study SNPs with low frequencies.

In summary, this study is the first to study a gene important for the counterregulatory mechanisms of the immune system in the context of natural recovery from a potentially chronic viral infection. These findings build on the emerging literature on the importance of regulatory T cells in recovery from chronic viral illnesses and are the first to suggest that CTLA-4 plays an important role in the outcome of natural HBV infection. Further work is needed to further understand the contributions of the *CTLA4* SNPs and haplotypes to the outcome of acute HBV infection.

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