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Mannose Binding Lectin Genotypes Influence Recovery from Hepatitis B Virus Infection

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Mannose binding lectin (MBL) is a central component of the innate immune response and thus may be important for determining hepatitis B virus (HBV) persistence. Since single-nucleotide polymorphisms (SNPs) in the gene encoding MBL (mbl2) alter the level of functional MBL, we hypothesized that mbl2 genotypes are a determinant of HBV persistence or recovery from viral infection. We tested this hypothesis by using a nested case control design with 189 persons with HBV persistence matched to 338 individuals who had naturally recovered from HBV infection. We determined genotypes of two promoter and three exon 1 SNPs in mbl2 and grouped these genotypes according to the amount of functional MBL production. We found that the promoter SNP -221C, which leads to deficient MBL production, was more common in those subjects with viral persistence (odds ratio [OR], 1.38; 95% confidence interval [CI], 1.01 to 1.89; \( P = 0.04 \)). Those subjects homozygous for the combination of promoter and exon 1 genotypes associated with the highest amount of functional MBL had significantly increased odds of recovery from infection (OR, 0.55; 95% CI, 0.37 to 0.84; \( P = 0.005 \)). Conversely, those homozygous for the combination of promoter and exon 1 genotypes which produce the lowest amount of functional MBL were more likely to have viral persistence (OR, 1.76; 95% CI, 1.02 to 3.01; \( P = 0.04 \)). These data are consistent with the hypothesis that functional MBL plays a central role in the pathogenesis of acute hepatitis B.

Chronic hepatitis B infection affects 400 million people and is the most common cause of cirrhosis and hepatocellular carcinoma worldwide. Although most adults recover from acute hepatitis B virus (HBV) infection, it is not fully understood why some develop chronic hepatitis B. The strength and breadth of the host immune response are important (3, 30), and recent data from chimpanzees and transgenic mice suggest that differences in innate immunity affect recovery from HBV infection (11, 16).

Mannose binding lectin (MBL) plays a central role in the innate immune response (32), which encodes MBL, is located on chromosome 10 and consists of four exons (Fig. 1). Several single-nucleotide polymorphisms (SNPs) in the gene’s promoter and in exon 1 that ultimately reduce the level of functional MBL have been described (8). Reduced MBL concentrations have been linked with diminished responses to several infectious diseases, including human immunodeficiency virus (HIV) infection and recurrent infections, and higher levels have been linked with inflammatory outcomes such as vascular complications of diabetes mellitus (5, 12, 24, 29). Although one exon 1 SNP has been associated with HBV persistence (31), this gene and its functionally different haplotypes have not been comprehensively examined in persons infected with HBV.

We hypothesized that the mbl2 SNPs that reduce functional MBL levels would be found more often in persons with HBV persistence and vice versa. To test this hypothesis, we determined genotypes of the promoter SNPs at -550 and -221 and the three exon 1 SNPs at codons 52, 54, and 57 by using a well-characterized cohort of individuals with either HBV persistence or recovery.

MATERIALS AND METHODS

Study participants. Subjects in this study were participants in one of two other studies: (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 (33), or (ii) the Multicenter AIDS Cohort Study (MACS), which is an ongoing study of 5,622 homosexual men enrolled in one of four U.S. cities between 1984 and 1985 and between 1987 and 1991 (4, 18).

To investigate the hypothesis that mbl2 SNPs may be associated with recovery from acute hepatitis B, a nested case control design was used. When possible, participants from the cohorts with persistent HBV infections were matched to two persons from the same cohort who had recovered from HBV infection but who were otherwise similar with regard to nongenetic factors. If two matched controls were not available, then one control was matched. Matching criteria included geographic location and factors that have been associated with recovery from hepatitis B, including age within 10 years, gender, ethnicity, and human immunodeficiency virus type 1 (HIV-1) infection status (9, 15). Subjects were considered persistently infected with HBV if their sera or plasma tested positive for HBV surface antigen (HBsAg) at two visits separated by a minimum of 6 months. Testing for antibodies against HBV core antigen (anti-HBc) and HBsAg (anti-HBs) was performed as needed to exclude primary HBV infection. Individuals recovered from hepatitis B 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 infection statuses of HIV-positive subjects were determined before 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-1 antibody testing was done by enzyme immunoassay, with reactive results confirmed as positive by Western blotting as previously reported (4, 18, 33). HBsAg, anti-HBs, and anti-HBc testing was done using commercially available kits according to the specifications of the manufacturer (AUSZYME, AUSAB, and CORZYME, respectively; Abbott Laboratories, Abbott Park, IL).

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DNA extraction and mbl2 genotyping. For each individual, cell lines transformed with Epstein-Barr virus were established, and genomic DNA was extracted from these cell lines by using phenol-chloroform. The mbl2 SNPs at /H11002 550, /H11002 221, codon 52, and codon 57 were genotyped using the AcycloPrime-FP SNP detection assay (Perkin Elmer, Boston, MA), a single-base extension method performed according to the manufacturer’s specifications (14). In this method, a 200-bp 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 according to the manufacturer’s specifications. In the final step, one of two fluorescent terminators representing the alleles present at the SNP of interest is added 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 (FP) of each allele as determined by the Victor2V instrument (Perkin Elmer, Boston, MA). We verified the FP results with direct sequencing for 10 samples from each SNP and found no errors.

The SNP at codon 54 was genotyped by PCR sequence-specific primers with which two complementary reactions were run for each SNP. The 2 reactions shared a common primer, but the second primers differed at their 3-terminal ends by one base, which represents the bases at the polymorphic alleles. The PCR products were run on a 1% agarose gel, and an allele was assigned as present if a band was detected. The procedure was verified by sequencing the SNPs from 10 individuals with no errors. Ambiguous genotypes from either method were assigned based on direct sequencing.

Statistical analysis. The first analysis consisted of examining the individual SNPs. Allele frequencies for each SNP were calculated from the genotypes and compared with respect to recovery from infection and viral persistence by using conditional logistic regression (SAS version 10; SAS, Cary, NC). An odds ratio (OR) of 1 was associated with viral persistence, and an OR of 1 was associated with recovery. Hardy-Weinberg equilibrium was assessed for each SNP by using the chi-square test with 1 degree of freedom.

Since examining individual SNPs does not fully account for differences in the amounts of functional MBL produced, we analyzed subjects grouped by genotypes that have been found to correlate with the amount of functional MBL (8). The group designation system follows previously established nomenclature for the gene. Groups were established by determining haplotypes consisting of exon 1 and /H11002 221 genotypes. Haplotypes were constructed using software designed for population-based studies, PHASE version 2.0 (http://www.stat.washington.edu/stephens/software.html) (28). The exon 1 genotype with the C allele at codon 52, the G allele at codon 54, and the G allele at codon 57 was designated A since this produces normal levels of functional MBL (Fig. 1 table). If the genotype included one or more of the alternate alleles, T allele at codon 52, A allele at codon 54, or A allele at codon 57, it was designated O since any one of these significantly reduces the amount of functional MBL. The analysis was then expanded to include the /H11002 221 SNP. The genotype with the G allele at /H11002 221, which produces normal levels of functional MBL, was designated Y, whereas that with the C allele, which profoundly down-regulates the amount of functional MBL, was designated X. If the exon 1 genotype was O, the /H11002 221 SNP was not included.
TABLE 1. Primers used for FP and sequence-specific primers (SSP)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP primers</td>
<td></td>
</tr>
<tr>
<td>Exon 1 forward</td>
<td>TGGTGGCAGCGTCTTACCTCA</td>
</tr>
<tr>
<td>Exon 1 reverse</td>
<td>CCCAGGCTTTCCTCTCTGGA</td>
</tr>
<tr>
<td>52D extension (CTT)</td>
<td>CAACGGCTTTCCTCAGGCC</td>
</tr>
<tr>
<td>57C extension (C/T)</td>
<td>CAACACGTACCTGGTTCCCCCTT</td>
</tr>
<tr>
<td>−550 forward</td>
<td>ACTCTGCAAGGGCCAAGCTTA</td>
</tr>
<tr>
<td>−550 reverse</td>
<td>CAGCTGATCCCTCCAGGAC</td>
</tr>
<tr>
<td>−550 extension (G/C)</td>
<td>GAAATGGCTTACCAGGCAAGCTTG</td>
</tr>
<tr>
<td>−221 forward</td>
<td>GGTATCAGGTGCGAGATGG</td>
</tr>
<tr>
<td>−221 extension (G/C)</td>
<td>CGTCCCCATTTGTTCTCAGC</td>
</tr>
</tbody>
</table>

SSP
54B mutant forward | CCCCCCTTTTCTCTCTTGGTG |
54B wild type forward | CCCCCCTTTTCTCTCTTGGTG |
54B reverse | CGTCCCCATTTGTTCTCAGCAGAG |

a Letters in parentheses indicate bases at polymorphic alleles.

since the amount of functional MBL was already so low that it was not significantly altered by the −221 genotype. Thus, the possible haplotypes were YA, XA, and O. The frequencies of these haplotypes were similar in whites and blacks (Fig. 1 table), so the analyses were done with the entire cohort and the results were then stratified by ethnicity if significant. Since each person has two haplotypes, ultimately, six mbl2 genotypes were possible for each subject: YA/YA, YA/XA, XA/XA, YA/O, XA/O, and O/O. For the analysis, we divided patients into high-MBL (YA/YA) and low-MBL or MBL-deficient (XA/XA and O/O) groups based on published data regarding MBL concentrations measured in persons with these genotypes (8). All other genotypes were considered intermediate, and these groups were compared with respect to recovery from infection and viral persistence by using conditional logistic regression. The haplotype analyses were also extended to include the −550 SNP to determine the effect of the −550 promoter SNP.

A P of <0.05 was considered significant in all analyses, and results for any genotype that met this criterion were stratified by the individuals’ HIV infection statuses to exclude HIV association. Genotypes were also stratified by subjects’ ethnicity (i.e., black versus white) to evaluate ethnic differences. Since those subjects in the other-ethnicity category were heterogeneous and small in number, they were excluded from the ethnicity analysis.

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 in each group, respectively. No significant differences were detected between those recovered from hepatitis B 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 was white (76%), with 22% black and 2% of other ethnicity.

Individual SNPs and haplotypes. In univariate analysis of the −550, −221, codon 52, codon 54, and codon 57 SNPs, only −221C (X allele) was associated with viral persistence (OR, 1.38; 95% confidence interval [CI], 1.01 to 1.89; P = 0.04) (Table 2). Homozygosity for the X allele was infrequent (3.7%) and did not strengthen this relationship. Homozygosity for the Y allele was associated with viral clearance, but the association was not stronger than that for Y alone (OR, 0.65; P = 0.03). None of the other SNPs in either the homozygous or heterozygous state were associated with outcome. The association with the X allele was the same when results were stratified by black and white ethnicities and when they were stratified by HIV infection statuses (Table 4).

Haplotype analysis demonstrated that those with the YA haplotype were more likely to recover from an HBV infection (OR, 0.73; 95% CI, 0.56 to 0.94; P = 0.02). Since the X allele at −221 occurred only with the A haplotype, the XA haplotype did not differ from X alone. The O haplotype was not associated with either recovery or viral persistence.

Genotypic groups based on expected functional MBL levels. Several studies have demonstrated a consistent relationship between MBL levels and mbl2 genotypes (8, 27). Individuals homozygous for YA (YA/YA) have the highest levels, whereas persons with an X allele at −221 on one chromosome and an O on the other chromosome (XA/O) or who are homozygous for O (O/O) have the lowest levels (8). Using these previously established data, we divided our genotypes into one of three groups: (i) YA/YA, (ii) XA/O or O/O, and (iii) all other combinations, which included those with MBL levels intermediate between those of the two former groups. Those subjects with the YA/YA genotype had significantly increased odds of recovery from infection (OR, 0.55; 95% CI, 0.37 to 0.84; P = 0.005), whereas those with XA/O or O/O were more likely to have viral persistence (OR, 1.76; 95% CI, 1.02 to 3.01; P = 0.04) (Table 3). A clear trend between mbl2 genotype and HBV infection outcome was noted in these three groups (P of 0.002 for Maentel-Haenszel test for trend). These relationships were not altered with stratification by HIV infection status or ethnicity (Table 4).

Since a small effect of the −550 promoter on MBL levels has been demonstrated (8), we divided those with the Y4 genotype into two groups based on the −550 SNP. There was no difference in the frequencies of recovery from infection or viral persistence between those with either C or G at −550 (OR, 0.81 for both groups). Homozygosity for Y4 with a C at −550 and for Y4 with a G at −550 also led to similar outcomes (OR, 0.66 and 0.60, respectively).

DISCUSSION

In this study, we find that mbl2 genotypes correlating with high MBL levels are associated with recovery from an HBV infection whereas those correlating with lower levels are asso-
Table 3. Proportion of individuals with genotypes yielding high, intermediate, and low MBL levels among those recovered from hepatitis B and those with viral persistence

<table>
<thead>
<tr>
<th>MBL level (haplotypes)*</th>
<th>% Of subjects&lt;sup&gt;a&lt;/sup&gt; with the indicated MBL level among:</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered subjects (n = 301)</td>
<td>Subjects with viral persistence (n = 179)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (YA/YA)</td>
<td>37.9</td>
<td>24.6</td>
<td>0.55</td>
<td>0.37–0.84</td>
</tr>
<tr>
<td>Intermediate (YA/XA, YA/O, or XA/XA)</td>
<td>49.2</td>
<td>56.4</td>
<td>1.29</td>
<td>0.88–1.87</td>
</tr>
<tr>
<td>Low or none (XA/O or O/O)</td>
<td>13.0</td>
<td>19.0</td>
<td>1.76</td>
<td>1.02–3.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> The MBL haplotypes are as defined in Materials and Methods.
<sup>b</sup> n, number of study subjects.

Table 4. ORs for significant SNPs and haplotypes stratified by ethnicity and HIV infection status

<table>
<thead>
<tr>
<th>Genotype(s)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OR (95% CI) for subjects with the following characteristic&lt;sup&gt;b&lt;/sup&gt;:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>black (n = 110)</td>
</tr>
<tr>
<td>−221C</td>
<td>1.53 (0.71–3.30)</td>
</tr>
<tr>
<td>YA/YA</td>
<td>0.61 (0.27–1.37)</td>
</tr>
<tr>
<td>XA/O or O/O</td>
<td>1.36 (0.42–4.41)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Genotypes are defined in Materials and Methods.
<sup>b</sup> n, number of study subjects with the indicated characteristic.
with chronic hepatitis B have an ongoing infection would serve as a strong confounder in a posthoc evaluation of MBL levels. A meaningful measurement of MBL levels in our cohort would require a sample taken prior to HBV infection, which is not available. Fortunately, there is a well-established relationship between genotypes and MBL levels, as has been documented for mbl2 in infection status did not significantly alter the results.

In summary, this is the first study to clearly demonstrate that genetically determined differences in mbl2 are important determinants of recovery from HBV infection. These data support the importance of the innate immune response in this process, but further study is needed to explain the precise role of MBL in HBV pathogenesis.

ACKNOWLEDGMENTS

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