Cytotoxic T lymphocyte responses and CTL epitope escape mutation in HBsAg, anti-HBe positive individuals

S I Khakoo, R Ling, I Scott, A I Dodi, T J Harrison, G M Dusheiko, J A Madrigal

Abstract

Background/aims—Clearance of hepatitis B virus (HBV) infection, HLA class I restricted CTL responses are thought to mediate elimination of virus after acute self-limited infection from the host, and also to cause hepatocellular necrosis. Immunohistochemical studies have shown that display of HLA class I molecules is increased in active, compared with mild, chronic hepatitis and also during alpha interferon therapy. Using pulsed peptide protocols, HBV specific HLA class I restricted CTL have been demonstrated in the peripheral blood of patients with acute hepatitis B infection. This has led to the definition of a number of viral peptide epitopes derived from the nucleocapsid, surface, and polymerase proteins. In patients with acute hepatitis B infection, an epitope from the nucleocapsid region which can be presented by HLA-A2, and one which is dually restricted by HLA-A68.1 and HLA-A31, have been defined. Acid elution studies have been used to define a peptide presented by HLA-A11.

In contrast with acute hepatitis B, chronic hepatitis B typically is associated with a weak or undetectable specific CTL response in peripheral blood. However, HLA-A2 class I restricted CTL responses can be observed in patients with chronic hepatitis B infection, before and after alpha interferon therapy, and also persist after serological recovery from acute hepatitis B infection in patients who remain HBV DNA positive by polymerase chain reaction (PCR). These data suggest that an ongoing CTL response is important for controlling viral replication in all stages of hepatitis B infection. However, HLA class I immunogenetic studies failed to show a consistent pattern of protective or susceptibility alleles.

In some anti-hepatitis B e (HBe) positive individuals with chronic HBV infection, serum aminotransferases remain consistently normal, hepatic inflammatory activity is minimal, and HBV DNA in blood is detectable only by PCR and not by hybridisation or signal amplification assays. The infectivity of these patients is relatively low but they still represent a discernible transmission risk and disease reactivation may occur if the individual is immunocompromised.

Methods—An HLA/HBV dual transfectant model was used to demonstrate these CTL responses in individuals chronically infected with HBV. Subsequently, a known hepatitis B core (HBc) CTL epitope was sequenced in a family of five chronically infected individuals all sharing a HLA allele (HLA-A68.1).

Results—Low level HLA class I restricted cytotoxic T cell responses were detected in the peripheral blood of five of eight anti-HBe positive individuals. In the family of HLA-A68.1 positive chronically infected individuals, mutation of the HLA-A68.1 restricted hepatitis B core antigen (HBcAg) CTL epitope STLPETTVVRR was found in all four anti-HBe positive individuals but not in the sole hepatitis B e antigen (HBcAg) positive patient.

Conclusion—These data are consistent with a continued immune selection pressure on HBV in anti-HBe positive chronically infected individuals with low replicating HBV infection and suggest that mutation of a CTL epitope may be a consequence of the immune response, as opposed to the cause of viral persistence.

(Appendix)

Keywords: hepatitis B virus; HLA; hepatitis B core antigen; cytotoxicity; mutant

The cytotoxic T lymphocyte (CTL) response is an important mechanism for clearance of viruses. CTL that recognise peptides derived from viral antigens in the context of major histocompatibility complex (MHC) class I have been demonstrated in a number of viral infections. Diversification of the MHC class I region allows presentation of a broad spectrum of peptides to the host immune system derived from viral antigens, thereby allowing development of an antiviral CTL response.
negative. HBV, hepatitis B virus; HBe, hepatitis B e; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; pos, positive; Neg, negative.

Liver biopsy findings are shown where performed. HBV, hepatitis B virus; HBe, hepatitis B e; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; pos, positive; Neg, negative.

Table 1  Serological, histological, and HLA typing of the unrelated HBV positive chronically infected individuals

<table>
<thead>
<tr>
<th>Subject No</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HBV serology</th>
<th>ALT (iu/l)</th>
<th>HBV DNA</th>
<th>Liver biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2, 3</td>
<td>7, 44</td>
<td>HBsAg/anti-HBe pos</td>
<td>57</td>
<td>Neg</td>
<td>Chronic active hepatitis</td>
</tr>
<tr>
<td>2</td>
<td>2, 24</td>
<td>7, 51</td>
<td>HBsAg/anti-HBe pos</td>
<td>87</td>
<td>Neg</td>
<td>Chronic active hepatitis</td>
</tr>
<tr>
<td>3</td>
<td>2, 30</td>
<td>7, 18</td>
<td>HBsAg/anti-HBe pos</td>
<td>21</td>
<td>Neg</td>
<td>nd</td>
</tr>
<tr>
<td>4</td>
<td>1, 2</td>
<td>7, 13</td>
<td>HBsAg/HBeAg pos</td>
<td>230 1680 pg/ml</td>
<td>Chronic active hepatitis</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2, 11</td>
<td>35, 61</td>
<td>HBsAg/anti-HBe pos</td>
<td>15</td>
<td>Neg</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>2, 28</td>
<td>35, 51</td>
<td>HBsAg/anti-HBe pos</td>
<td>67</td>
<td>Neg</td>
<td>Inactive cirrhosis</td>
</tr>
<tr>
<td>7</td>
<td>2, 3</td>
<td>18, 51</td>
<td>HBsAg/anti-HBe pos</td>
<td>28</td>
<td>Neg</td>
<td>Mild hepatitis</td>
</tr>
</tbody>
</table>

Serum alanine transaminase (ALT, normal range 5–40 iu/l) and serum hepatitis B virus (HBV) DNA measurements (branched chain DNA assay; Chiron Corporation, Emeryville, California, USA) are shown. Liver biopsy findings are shown where performed (nd indicates liver biopsy not performed).

HBV, hepatitis B virus; HBe, hepatitis B e; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; pos, positive; Neg, negative.

positive immunostaining for HBsAg and hepatitis B core antigen (HBCAg). A patient with acute hepatitis B (HLA-A10, -A68, -B7, -B17) was used to establish the cytotoxicity assay. This patient was a 37 year old African male with multiple sexual partners who presented with a one month history of malaise and a two week history of jaundice. Hepatitis serology revealed that he was HBsAg positive, HBeAg positive, and HBC IgM positive, with an alanine aminotransferase (ALT) level of 2727 iu/l. Human immunodeficiency virus (HIV) and HCV testing were negative. He had no previous history of hepatitis. Loss of HBeAg was documented at six weeks following peak levels of ALT.

Uninfected controls (three HLA-B7 positive and one HLA-A2 positive) were derived from healthy HBsAg negative volunteers. They were used as HLA class I matched controls in the cytotoxicity experiments.

EXPERIMENTAL PROCEDURES

Transfectant cell lines

Dual transfectant cell lines expressing HBCAg and single HLA alleles were created from the HBV-A, -B, -C negative L721.221 or in the case of the HLA-A68.1 transfectants, the C1R cell line (HLA-A negative, HLA-B expressed at very low levels). Briefly, a hepatitis B core (HBC) gene of known sequence was cloned into the pREP8 vector (Invitrogen, Leek, Holland) or a modified pMEP4 vector (Invitrogen, Leek, Holland) in which the mouse metallothionin promoter had been exchanged for a respiratory syncytial virus promoter using the SalI sites flanking the promoter and the polyadenylation sites. The HBC-modified pMEP4 construct was transfected into the cells by electroporation and HBCAg expression verified by enzyme linked immunosorbent assay (ELISA).

Table 2  Serological, histological, and HLA typing of the family studied

<table>
<thead>
<tr>
<th>Subject No</th>
<th>Relationship</th>
<th>Age (y)</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HBV status</th>
<th>ALT (iu/l)</th>
<th>HBV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Father</td>
<td>53</td>
<td>11,11</td>
<td>7,15</td>
<td>HBsAg neg</td>
<td>36</td>
<td>Neg</td>
</tr>
<tr>
<td>9</td>
<td>Mother</td>
<td>49</td>
<td>68,68</td>
<td>51,50</td>
<td>HBsAg/anti-HBe pos</td>
<td>22</td>
<td>Neg</td>
</tr>
<tr>
<td>10</td>
<td>Daughter</td>
<td>28</td>
<td>11,68</td>
<td>5,7</td>
<td>HBsAg/anti-HBe pos</td>
<td>22</td>
<td>Neg</td>
</tr>
<tr>
<td>11</td>
<td>Daughter</td>
<td>27</td>
<td>11,68</td>
<td>5,7</td>
<td>HBsAg/anti-HBe pos</td>
<td>17</td>
<td>Neg</td>
</tr>
<tr>
<td>12</td>
<td>Daughter</td>
<td>26</td>
<td>11,68</td>
<td>15,50</td>
<td>HBsAg/HBeAg pos</td>
<td>45 &gt;16 000 pg/ml</td>
<td>Neg</td>
</tr>
<tr>
<td>13</td>
<td>Daughter</td>
<td>24</td>
<td>11,68</td>
<td>15,51</td>
<td>HBsAg/anti-HBe pos</td>
<td>20</td>
<td>Neg</td>
</tr>
<tr>
<td>14</td>
<td>Daughter</td>
<td>22</td>
<td>11,68</td>
<td>7,51</td>
<td>HBsAg neg</td>
<td>11</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Serum alanine transaminase (ALT, normal range 5–40 iu/l) and serum hepatitis B virus (HBV) DNA measurements (branched chain DNA assay; Chiron Corporation, Emeryville, California, USA) are shown. Liver biopsy findings are shown where performed (nd indicates liver biopsy not performed).

HBV, hepatitis B virus; HBe, hepatitis B e; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; pos, positive; Neg, negative.
Table 3 Expression testing of the transfectant cell lines. The transfectant cell lines were tested for HLA class I expression by FACS analysis. Cells were stained with an anti-class I specific antibody W6/32 from mean chromium release of triplicate wells using the following formula:

\[
\text{Percent specific cytotoxicity} = \frac{\text{measured release-spontaneous release}}{\text{total release-spontaneous release}} \times 100
\]

All experiments were performed twice and spontaneous release was always less than 30% of total release. Cytotoxicity of each bulk culture was tested against the HBC/HLA dual transfectant, the vector/HLA dual transfectant cell lines, and an HBC single transfectant. The blocking experiment was performed using lymphocytes derived from the patient with acute hepatitis B. These experiments were performed following a single restimulation with the dual transfectant line. In these experiments the anti-HLA class I specific antibody W6/32 was used as controls.

Sequencing of the HLA-A68.1 restricted CTL epitope of the HBC gene (HBC: 141-151).

HBV DNA was prepared from 50 µl of serum by proteinase K digestion using standard conditions, followed by phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in 20 µl of double distilled water, and 5 µl of the final product was used in a nested PCR reaction using primers to the HBc and polymerase regions (outer: 5' GTTGTGTTAGACGGACGGACGTGC 3' (positions 2216 to 2237) and 5' CAGGGCATATTGACAACAGTG 3' (2965 to 2985); and inner: 5' AGACGAGAGGTCTAAATCGCCG 3' (2267 to 2287) and 5' GAAAAAGGAGATTTAATT 3' (2487 to 2506)). The PCR product was then cloned using the TA cloning vector (Invitrogen, Leek, Netherlands) and sequenced using the Sequenase version 2 protocol (Amersham, Buckinghamshire, UK). Confirmation of the sequence was obtained by direct sequencing of a fresh PCR product on an ABI automated sequencer following concentration using a QIAEX II protocol (Qiagen, Crawley, UK).

Results

CTL RESPONSES IN HBcAg POSITIVE PATIENTS

Before conducting the cytotoxicity assays, the transfectants were assayed for expression of MHC class I and HBcAg (table 3). Then, in order to detect the presence of HBcAg specific HLA class I restricted CTL in the HBV carriers, the conditions for the cytotoxicity assay were established using lymphocytes from a patient with acute hepatitis B as a positive con-
control and the L721.221/B*0702/Hb dual transfectant (fig 1A). Specific cytotoxicity was greater against the HLA/Hb dual transfectant cell line than the controls at the three effector to target ratios tested. Following one restimulation with the dual transfectant line, blocking with the anti-HLA class I specific antibody W6/32 (10 µg/ml) caused a twofold reduction (from 62% to 30%) in specific lysis against the dual transfectant line; no change was observed with an isotype matched control antibody (fig 1B).

Cytotoxicity assays were then performed in seven HBsAg positive patients (six HBeAg negative, anti-HBe positive and one HBeAg positive, anti-HBe negative). All experiments were performed at effector to target ratios of 40:1. All three anti-HBe positive HLA B7 positive patients had stronger responses against the dual transfectant than the control cell lines. The differences between the CTL responses against the dual transfectant line and those against the control lines were significantly greater for the anti-HBe positive patients than for the uninfected controls. These differences exceeded the mean+3 SD of the differences observed for the uninfected controls. These responses were therefore considered positive (fig 2A). Furthermore, they were of similar magnitude to those found in the patient with acute hepatitis B. The response of the HBeAg positive individual was less than those of the uninfected controls and was therefore considered negative.

For the HLA A-2 positive individuals, only one individual showed a higher response against the Hb/Hla dual transfectant than against both of the control targets. All others had a stronger response against the control cell lines. To ensure that this response did not represent purely a non-specific response against the dual transfectant line, PBMCs from this patient were stimulated with a control cell line (vector without Hb insert plus HLA-A*0201 transfectant) and the cytotoxicity assay repeated. In this experiment, lysis of the
Hepatitis B virus (HBV) A68.1 cytotoxic T lymphocyte (CTL) epitope mutation

**Figure 3** Deduced amino acid sequences of the hepatitis B virus (HBV) A68.1 cytotoxic T lymphocyte (CTL) epitope from the HLA-A68.1 positive family compared with that of the HBV adr subtype.\(^1\) Numbering is from the start of the core gene and the A68.1 CTL epitope is boxed.

<table>
<thead>
<tr>
<th>adr subtype</th>
<th>Subject 9</th>
<th>Subject 10</th>
<th>Subject 11</th>
<th>Subject 12</th>
<th>Subject 13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E W I R</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>T P S R</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>130</td>
<td>140</td>
<td>150</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>- - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
</tbody>
</table>

HLA-A*0201/HBc dual transfectant was lower than that against the control cell line, demonstrating that this individual did indeed make a HBc specific CTL response (fig 2B).

Lymphocytes were available from three of the chronically infected family members, subject Nos 11–13 (two anti-HBe positive and one HBeAg positive). These were tested against the HLA-A68.1/HBc and HLA-A68.1/vector dual transfectants. Only one individual, subject No 13 (anti-HBe positive), made a significantly greater CTL response (17% specific cytotoxicity) against the HLA-A68.1/HBc dual transfectant than the HLA-A68.1/vector control transfectant. CTL responses against the two transfectants were not significantly different for the other anti-HBe positive subject and the HBeAg positive subject (fig 2C).

**MUTATIONS IN THE A68.1 CTL EPITOPE IN THE A68.1 POSITIVE HBV CHRONIC CARRIERS**

To determine whether mutation of a defined HBV CTL epitope might be related to viral persistence or the result of immune selection pressure, the region of the HBc ORF encoding the HLA-A68.1 restricted CTL epitope was sequenced from a family of HLA-A68.1 positive individuals. HBV DNA could not be amplified from two individuals (subject Nos 8 and 14, both HBsAg negative) and therefore results are shown only for the other five family members. In total 180 nucleotides of the HBc ORF of each individual were sequenced. When the deduced amino acid sequence was compared with that of the adr subtype,\(^6\) mutations were found in the region of the A68.1 restricted CTL epitope (HBc 141-151) previously identified as immunodominant by Missale and colleagues,\(^8\) in comparison with the other regions of the core gene (fig 3). These mutations occurred in all four anti-HBe positive family members but not in the sole HBeAg positive daughter. The same coding mutation (substitution of CGA (arginine) by TGT (cysteine)) was found in the HBV genomes of the three anti-HBe positive, HBV DNA negative siblings. In subject No 13, one clone was found with the wild-type CTL epitope sequence but this was not confirmed by direct sequencing of the PCR product, suggesting that although it may persist it is not the dominant quasispecies in this individual. In the mother, subject No 9, two different nucleotide substitutions (A for C at position 2339 and A for G at position 2444) were found, resulting in the following derived amino acid substitutions: asparagine for threonine (HBc 146) and isoleucine for leucine (HBc 148). In the four anti-HBe positive individuals, the nucleotide substitution rate compared with the wild-type adr sequence was 0.076 per nucleotide in the A68.1 CTL epitope, whereas it was 0.034 outside this region. This suggests significant clustering of nucleotide substitutions within the CTL epitope (\(\chi^2=33.4, p<0.0001\)). Mutations in the CTL epitope thus correlated with loss of HBeAg and low serum levels of HBV DNA, as opposed to persistence at high levels of viremia. As the mutations were different between mother and anti-HBe positive daughters and were not found in the HBeAg positive individual, they are unlikely to represent the dominant viral quasispecies of the inoculating viral population but are more likely to be a consequence of selection by CTL responses.

**Discussion**

A dual transfectant system was chosen to study CTL responses in individuals chronically infected with HBV. This system was chosen to present endogenously processed antigens and avoid the bias of pulsed peptide protocols based on HLA class I binding motifs. Using this experimental system, we have demonstrated the presence of CTL responses in chronically infected HBsAg positive anti-HBe positive carriers in the context of HLA-B*0702 and HLA-A*0201. Specific lysis was present against both types of control cell lines. The CTL response against the dual transfectant in these experiments could be considered to consist of natural killer cell responses, allospecific CTL responses, and anti-HBc specific responses. Use of a cell line which differed from the dual transfectant only in the HBc gene allowed the natural killer cell responses and allospecific CTL responses to be controlled for. Thus the increases in lysis against the
dual transfectant line compared with the control lines was considered to represent HBc specific responses. Although expression of the control lines was similar to the HLA-Hbc dual transfectant cell lines, increased lysis was seen in the HLA-A*B*0702 positive controls. This may be related to the slightly higher HLA expression of this transfectant compared with the control cell lines. We noted that all three HLA-A2, -B7 positive anti-HBe positive patients had detectable cytolytic responses in the context of HLA-A*B*0702, but not HLA-A*B*0201. Although this may be related to the experimental design, it could also indicate focusing of the CTL response on peptides presented in the context of HLA-B*0702 in these individuals. Interestingly, Sampliner et al have demonstrated a protective effect of the allele HLA-B7 in an immunogenetic study of HBV infection in a large family. Overall these data suggest that an HLA-B7 restricted epitope might be usefully included in peptide based immunotherapy for chronic HBV infection. Selection pressure by HLA class I restricted CTL has been demonstrated to lead to viral escape mutation in a number of viral infections, including lymphocytic choriomeningitis virus, HIV, and Epstein-Barr virus. Previous studies have demonstrated a mutation in the HLA-A2 HBc epitope (HBC18–27) but, consistent with our data, this was not found to be widespread in HLA-A2 positive chronic HBV carriers who did not have demonstrable CTL responses. Our finding of persistent CTL responses in anti-HBe positive chronic HBV carriers would suggest that CTL epitope mutation may also occur in HBV infection, under selection pressure.

Analysis of an HLA-A68.1 positive family allowed the study of viral adaptation in the context of a restricted immunogenetic background. Viral transmission is likely to have been “vertical” from the mother at a time when she had a higher level of HBV replication. As this patient had unique substitutions in the A68.1 CTL epitope, these are likely to have accumulated after transmission to the daughters, or have been represented as a minor quasispecies. In either case, in the anti-HBe positive siblings these mutations appeared to have been selected for. In a study of HBV infected Chinese individuals, higher mutation rates of HBV were found during clearance of infection than in the highly replicative immunotolerant phase of HBV infection, which is consistent with these data.

Analysis of the HLA-A68.1 restricted CTL epitope in the various family members revealed coding mutations in the CTL epitope in four of the family members. Sequencing studies of this epitope in a population of HLA-A68.1 positive and negative individuals would give further insight into the role of mutation of this region in HBV infected individuals. Also, as the sequencing studies were directed specifically at a region of HBV, known to be associated with HLA-A68.1 restricted CTL responses, coexistent mutation in Tt and B cell epitopes or the pre-core region have not been excluded. Interestingly, the mother had the most disrupted CTL epitope, with a substitution of asparagine for threonine at position six and isoleucine for valine at position eight of the epitope. Previous work has suggested that mutation of amino acid residues six, seven, or 11 of this epitope may abrogate the CTL response to this peptide. In the mother at position six, and in the daughters at position 11, the amino acid substitutions were not conservative, and so are likely to disrupt either the MHC peptide TCR interaction or peptide binding to the HLA molecule in the negatively charged F pocket. In the studies of Missale et al, CTL reactivity was found against peptides up to five amino acids longer than the minimally defined HBc 141–151 epitope and it has also been confirmed that HLA-Aw68 can accept peptides anchored at amino acid residue two and at the carboxyl terminal residue by the peptide bulging out of the peptide binding groove. This may allow binding of peptides such as STLPETTVVCR to the HLA molecule, as the anchor residues (threonine at position two and a carboxyl terminal arginine) are still present. Mutations in this region of the HBc gene have been suggested to disrupt critical functions of the core and polymerase proteins. Although this has not been formally tested, mutation of this region would suggest that this is unlikely to be the case for the mutations described in this study. Our data suggest that HLA class I restricted CTL are detectable in chronic anti-HBe positive/DNA negative HBV carriers, demonstrating an ongoing immune response in these patients with low level viral replication. We suggest that a consequence of this response could be the emergence of HBc viral mutants specific to the HLA background of the individual.

This work was supported by the Anthony Nolan Bone Marrow Trust. We thank Dr P Parham for the HLA class I cDNA clones.

HBV CTL epitope mutation


