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

Background/aims—Clearance of hepatitis B virus (HBV) is characterised by a strong cytotoxic T cell response. Persistence of HBV in chronic hepatitis B carriers may be related to failure of this response. The aim of this study was to determine whether HLA class I restricted cytotoxic T lymphocyte (CTL) responses persist in anti-Hepatitis B e (HBe) positive / HBV DNA negative individuals, and to correlate the presence of viral CTL epitope mutation with clinical outcome.

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 (HBeAg) CTL epitope STLPETTVRR was found in all four anti-HBe positive individuals but not in the sole hepatitis B e antigen (HBeAg) 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.

Key words: 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. In 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 immuno-
negative. (nd indicates liver biopsy not performed). 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.

One sibling was hepatitis B e antigen (HBeAg) positive; the remainder of the hepatitis B e antigen (HBeAg) positive carriers and then, in order to study viral factors, a family in which six of whom were chronically infected (table 2). Patients were divided into two groups. The first group comprised members of a family, all of whom had been exposed to hepatitis B, and five of whom were chronically infected (table 2). One sibling was hepatitis B e antigen (HBeAg) positive; the remainder of the hepatitis B surface antigen (HBsAg) positive individuals were anti-HBe positive. In the family, liver biopsy had been performed only on subject No 12 and this showed mild hepatitis with strongly suppressed.  CTL responses are likely to be important in controlling viral replication in these individuals but have been defined previously only in the context of HLA-A2. Our aim in this study was to demonstrate the presence of CTL restricted to other HLA antigens in these chronically infected individuals and then to see if virus adaptation might correlate with these responses. To perform these studies we first studied a group of randomly selected anti-HBe positive carriers and then, in order to study viral factors, a family in which six of seven members had the same HLA allele (HLA-A68.1) for which an immunodominant CTL epitope has previously been defined.

**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>Chronic active hepatitis</td>
</tr>
<tr>
<td>4</td>
<td>1, 2</td>
<td>7, 13</td>
<td>HBsAg/anti-HBe pos</td>
<td>230</td>
<td>1680 pg/ml</td>
<td>Chronic active hepatitis</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>Chronic active hepatitis</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).

**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>68, 68</td>
<td>51, 50</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>5, 7</td>
<td>HBsAg/anti-HBe pos</td>
<td>17</td>
<td>Neg</td>
</tr>
<tr>
<td>13</td>
<td>Daughter</td>
<td>24</td>
<td>11, 68</td>
<td>5, 7</td>
<td>HBsAg/anti-HBe pos</td>
<td>17</td>
<td>Neg</td>
</tr>
<tr>
<td>14</td>
<td>Daughter</td>
<td>22</td>
<td>11, 68</td>
<td>5, 7</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).
(Wellcozyme HBeAg/anti-HBe, Murex Diagnostics, Dartford, UK) of cell lysates. Confirmation that this assay detected HBeAg had been performed using recombinant HBe (Glaxo-Wellcome, Greenford, UK). The transfected cells were then cloned by limiting dilution to generate cell lines expressing higher levels of HBeAg. These lines were transfected with the corresponding HLA allele of interest cloned into the expression vector pBNeo. Cells expressing higher levels of the transfected HLA allele were stained with the anti-HLA class I monoclonal antibody W6/32 and positively sorted by a fluorescence activated cell sorter (FACS). Cell lines were maintained in selective medium (hygromycin 125 µg/ml; Calbiochem, Nottingham, UK) and G418 1 mg/ml (Gibco-BRL, Paisley, UK). Control lines consisting of vector plus HLA allele dual transfectant were created for each dual transfectant cell line. These lines were also positively sorted for high expression of the transfected HLA allele by FACS. All transfectant cell lines were checked for continued expression of HLA genes by FACS analysis and for HBeAg by ELISA, where appropriate.

### Table 3 Expression testing of the transfectant cell lines

<table>
<thead>
<tr>
<th></th>
<th>Control Ab (MFI)</th>
<th>W6/32 (MFI)</th>
<th>ELISA (OD)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>721.221 untransfected</td>
<td>1</td>
<td>1.38</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>A2/vector</td>
<td>4.47</td>
<td>142.58</td>
<td>0.140</td>
<td></td>
</tr>
<tr>
<td>A2/ HBe</td>
<td>1.82</td>
<td>126.82</td>
<td>0.630</td>
<td></td>
</tr>
<tr>
<td>B7/ vector</td>
<td>2.06</td>
<td>63.52</td>
<td>0.104</td>
<td></td>
</tr>
<tr>
<td>B7/ HBe</td>
<td>1.18</td>
<td>75.83</td>
<td>0.815</td>
<td></td>
</tr>
<tr>
<td>C1R/A68/vector</td>
<td>1.87</td>
<td>41.45</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>C1R/ A68/ HBe</td>
<td>1.26</td>
<td>21.74</td>
<td>3.177</td>
<td></td>
</tr>
<tr>
<td>Positive serum control</td>
<td>—</td>
<td>—</td>
<td>1.566</td>
<td></td>
</tr>
<tr>
<td>Negative serum control</td>
<td>—</td>
<td>—</td>
<td>0.091</td>
<td></td>
</tr>
</tbody>
</table>

Tissue typing

HLA typing was performed using a standard microcytotoxicity assay and confirmation of the HLA-A68.1 allele in the family study was performed by heteroduplex analysis.

### Bulk lymphocyte culture

Peripheral blood mononuclear cells (PBMC) were prepared from the blood of patients by ficoll density gradient centrifugation and stored under liquid nitrogen until use. PBMCs were plated out at 10⁶ cells/ml in RPMI (Gibco-BRL, Paisley, UK) and 10% human AB serum. Stimulator cells (the HLA matched dual transfectants) were irradiated at 9000 rad and added at a concentration of 10⁷ cells/ml. Cells were restimulated on day 7 with the stimulator cells at a responder:stimulator ratio of 10:1 and interleukin 2 (Boehringer-Mannheim, Lewes, UK) at a concentration of 10 iu/ml. Cytotoxicity assays were performed on day 12. PBMC from HLA class I matched individuals were treated identically.

### Cytotoxicity assays

Target cells (2×10⁵) were harvested and resuspended in 500 µl of AB serum. Cells were labelled for one hour with 100 µCi of NaCrO₄ at 37°C, washed twice, counted, and then resuspended at 10⁶/ml. Non-radiolabelled ("cold") targets (5×10⁴) were added to 100 µl effectors and then 5×10⁴ radiolabelled ("hot") targets added. Spontaneous ⁵¹Cr release was calculated from wells containing 100 µl of medium and "hot" targets only, and total release from wells in which 100 µl of 1% Triton X-100 had been added to the "hot" targets. Percentage specific cytotoxicity was calculated 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 HBe/HLA dual transfectant, the vector/HLA dual transfectant cell lines, and an HBe 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 at a concentration of 10 µg/ml and an isotype matched Ab were used as controls.

Sequenceing of the HLA-A68.1 restricted CTL epitope of the HBe gene (HBe: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 HBe and polymerase regions (outer: 5' GTTGTTTAGAGCAAGCGGAGGAGCAGTC 3' (positions 2216 to 2237) and 5’ CAGGGCATATTGGACAACAGTG 3’ (2963 to 2985); and inner: 5’ AGACGAAGGTCATTAAATCGCCG 3’ (2267 to 2287) and 5’ GAAAAGAGATATATATATT 3’ (2487 to 2506)). The PCR product was then cloned using the TA cloning protocol (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 HBeAg POSITIVE PATIENTS

Before conducting the cytotoxicity assays, the transfectants were assayed for expression of MHC class I and HBeAg (table 3). Then, in order to detect the presence of HBeAg 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-
Specific cytotoxicity was greater against the HLA/HBc 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 HBe/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 HBc insert plus HLA-A*0201 transfectant) and the cytotoxicity assay repeated. In this experiment, lysis of the...
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 were sequenced from each individual. The sequences were compared with that of the adr subtype, and mutations were found in the region of the A68.1 restricted CTL epitope (Hbc 141-151) previously identified as immunodominant by Missale and colleagues 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 HBsAg 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, HBsAg 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 four anti-HBe positive individuals, the nucleotide substitution rate compared with the wild-type adr sequence was 0.076 per nucleotide in the

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 line 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-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-B*0702, but not HLA-A*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 and not HLA-A*0201. This 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. 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 STLPETTVVRCR 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.

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HBV CTL epitope mutation

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