

# 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/claims**—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 (HBcAg) 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. (*Gut* 2000;47:137–143)

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.<sup>1</sup>

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.<sup>2–4</sup> 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.<sup>5–8</sup> Acid elution studies have been used to define a peptide presented by HLA-A11.<sup>9</sup>

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).<sup>10–11</sup> 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.<sup>12–16</sup>

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.<sup>17–18</sup> 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-

**Abbreviations used in this paper:** HBV, hepatitis B virus; CTL, cytotoxic T lymphocyte; PCR, polymerase chain reaction; HBe, hepatitis B e; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; MHC, major histocompatibility complex; ALT, alanine aminotransferase; HIV, human immunodeficiency virus; HBc, hepatitis B core; HBcAg, hepatitis B core antigen; FACS, fluorescence activated cell sorter; ELISA, enzyme linked immunosorbent assay; PBMC, peripheral blood mononuclear cells.

Department of  
Medicine, Royal Free  
and University College  
School of Medicine,  
Royal Free Campus,  
Pond Street, London  
NW3 2QG, UK  
S I Khakoo  
R Ling  
T J Harrison  
G M Dusheiko

Department of  
Haematology, Royal  
Free and University  
College School of  
Medicine, Royal Free  
Campus, Pond Street,  
London, NW3 2QG,  
UK  
J A Madrigal

Anthony Nolan  
Research Institute,  
Pond Street, London,  
NW3 2QG, UK  
I Scott  
A I Dodi  
J A Madrigal

Correspondence to:  
Professor J A Madrigal,  
Anthony Nolan Research  
Institute, Royal Free  
Hospital, Pond Street,  
London NW3 2QG, UK.  
Email:  
madrigal@rfhsm.ac.uk

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

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

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.

suppressed.<sup>19</sup> 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.<sup>10</sup> 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.<sup>8</sup>

### Patients and methods

#### PATIENTS

Informed consent was obtained from each patient and ethical approval for the study of human subjects was granted by the ethics committee of the Royal Free Hospital, in accordance with the Declaration of Helsinki. Patients were divided into two groups. The first were drawn from a population of individuals chronically infected with HBV, six of whom were from the UK and one from Portugal (table 1). No patient had undergone recent (within the past two years) seroconversion to anti-HBe, and two patients (Nos 2 and 4) had received alpha interferon therapy between liver biopsy and inclusion in the study. The second 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

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 HBe 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 HLA-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).<sup>20–22</sup> Briefly, a hepatitis B core (HBc) gene of known sequence<sup>23</sup> 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

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

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.

(Wellcozyme HBeAg/anti-HBe, Murex Diagnostics, Dartford, UK) of cell lysates. Confirmation that this assay detected HBcAg had been performed using recombinant HBc (Glaxo-Wellcome, Greenford, UK). The transfected cells were then cloned by limiting dilution to generate cell lines expressing higher levels of HBcAg. These lines were transfected with the corresponding HLA allele of interest cloned into the expression vector pBJneo. 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 HBcAg by ELISA, where appropriate.

#### 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  $10^6$  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  $10^5$  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 ( $2 \times 10^6$ ) were harvested and resuspended in 500 µl of AB serum. Cells were

labelled for one hour with 100 µCi of  $^{51}\text{NaCrO}_4$  at 37°C, washed twice, counted, and then resuspended at  $10^5$ /ml. Non-radiolabelled ("cold") targets ( $5 \times 10^4$ ) were added to 100 µl effectors and then  $5 \times 10^3$  radiolabelled ("hot") targets added. Spontaneous  $^{51}\text{Cr}$  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:

Percent specific cytotoxicity =

$$\frac{\text{measured release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

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 at a concentration of 10 µg/ml and an isotype matched Ab were 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' GTTGTTA-GACGACGAGGCAGTC 3' (positions 2216 to 2237) and 5' CAGGGCATATT-GACAACAGTG 3' (2965 to 2985); and inner: 5' AGACGAAGGTCTAAATCGCCG 3' (2267 to 2287) and 5' GAAAAAAGGAGAT-TAAAATT 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).

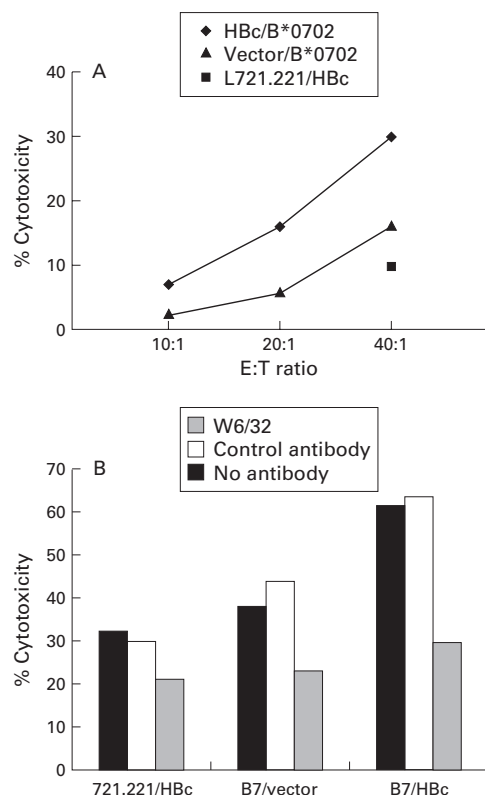
## Results

### CTL RESPONSES IN HBsAg 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-

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 antibody (W6/32) or an isotype control antibody (sheep anti-mouse IgG2a). The mean fluorescence intensity (MFI) of each of the cell lines is shown. Transfectant cell lines were >95% positive for HLA class I. Column three shows HBc expression, as determined by ELISA of cell lysates. The results for the positive and negative serum controls are also shown. The assay cut off was an optical density (OD) of 0.200

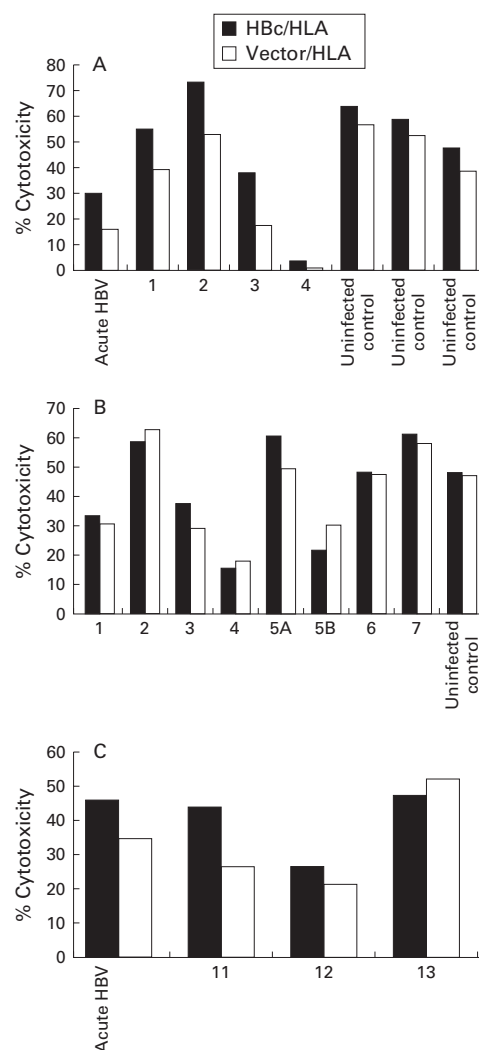
	Control Ab (MFI)	W6/32 (MFI)	ELISA (OD)
721.221 untransfected	1	1.38	nt
A2/vector	4.47	142.58	0.140
A2/ HBc	1.82	126.82	0.630
B7/ vector	2.06	63.52	0.104
B7/ HBc	1.18	75.83	0.815
C1R/A68/vector	1.87	41.45	0.100
C1R/ A68/ HBc	1.26	21.74	3.177
Positive serum control	—	—	1.566
Negative serum control	—	—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



**Figure 1** Cytotoxicity assay of an individual with acute hepatitis B. (A) Peripheral blood mononuclear cells were stimulated with the HBc/HLA B\*0702 dual transfectant and then tested against HBc/B\*0702 dual transfectant, vector/B\*0702 dual transfectant, or L721.221/HBc single transfectant. Cold targets were used at a ratio of 10:1 to hot targets. (B) To confirm the presence of a major histocompatibility complex (MHC) class I restricted cytotoxic response, the culture was restimulated with the dual transfectant line and retested in the presence of the anti-MHC class I antibody W6/32, an isotype matched control antibody, or no antibody.

control and the L721.221/B\*0702/HBc dual transfectant (fig 1A). 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



**Figure 2** Summary of the cytotoxicity results of the HLA-B7 positive (A), HLA-A2 positive (B), and HLA-A68.1 (C) individuals. Subject No 4 was HBeAg positive, and the remainder were anti-HBe positive, except where indicated. Results are expressed as percentage specific cytotoxicity against the HBc/HLA transfectant cell line compared with that of the vector/HLA dual transfectant. In (B), 5A indicates the result when peripheral blood mononuclear cells (PBMC) from subject No 5 were stimulated with the HBc/HLA dual transfectant and 5B the result when PBMC from the same individual were stimulated with the vector/HLA dual transfectant as a control for an allospecific response. In (C), subject No 12 was HBeAg positive, and subject Nos 11 and 13 anti-HBe positive. Assays were performed at effector to target ratios of 40:1 using cold targets at a ratio of 10:1 to hot targets.

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 HBc/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



dual transfectant line compared with the control lines was considered to represent HBe specific responses. Although expression of the control lines was similar to the HLA/HBe 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 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.<sup>13</sup> 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.<sup>25-27</sup> Previous studies have demonstrated a mutation in the HLA-A2 HBe epitope (HBe18-27) 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.<sup>28, 29</sup> 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.<sup>30, 31</sup> 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,<sup>32</sup> 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 T<sub>H</sub> and B cell epitopes or the pre-core region have not been excluded.<sup>33, 34</sup> Interestingly, the mother had the most dis-

rupted 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.<sup>8</sup> 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.<sup>35, 36</sup> In the studies of Missale *et al*, CTL reactivity was found against peptides up to five amino acids longer than the minimally defined HBe 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.<sup>9, 16</sup> This may allow binding of peptides such as STLPEITTVVRCR 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 HBe gene have been suggested to disrupt critical functions of the core and polymerase proteins. Although this has not been formally tested, mutagenesis studies of this region would suggest that this is unlikely to be the case for the mutations described in this study.<sup>37-39</sup>

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 HBe viral mutants specific to the HLA background of the individual.

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