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*J Immunol* 1998; 161:5842-5850; http://www.jimmunol.org/content/161/11/5842
Polyclonality and Multispecificity of the CTL Response to a Single Viral Epitope

T. Ishikawa,* † D. Kono,‡ J. Chung,‡ P. Fowler, † A. Theofilopoulos,‡ S. Kakumu,* and F. V. Chisari2†

The molecular anatomy of an immunodominant, Ld restricted CTL epitope located between residues 28–39 in hepatitis B surface Ag was defined to explore the immunologic constraints on mutational escape from the CTL response during a viral infection. Using a panel of hepatitis B surface Ag residue 28–39-specific CTL clones, the response to this epitope was found to be extremely diverse at the level of TCR fine specificity and β-chain usage. Although each clone recognized shared as well as unique residues within the epitope as TCR contact sites, even the shared residues were recognized differently by different TCRs. Despite these differences, all clones were comparably cytolytic following Ag stimulation and produced similar amounts of antiviral cytokines previously shown to inhibit HBV replication. These results demonstrate that the CTL response to individual viral epitopes can be markedly polyclonal and multispecific, such that mutational inactivation of a single TCR contact site will not usually lead to viral escape from all CTL clones of the same epitope specificity. Given these constraints and the fact that the CTL response is usually directed against several different epitopes during most viral infections, mutational inactivation of a single epitope is not likely to be sufficient to cause viral persistence. The Journal of Immunology, 1998, 161: 5842–5850.

The hepatitis B virus (HBV) is a noncytopathic, enveloped virus with a circular double stranded DNA genome that causes acute and chronic liver disease and hepatocellular carcinoma (1). Because of the variable disease spectrum associated with HBV, it is widely believed that the immune response to this virus mediates the pathogenesis of the associated liver disease (1). Based on extensive studies of HBV pathogenesis in man and animal models, there is considerable evidence that viral hepatitis is initiated by an Ag-specific antiviral cellular immune response that eventually eliminates the virus (1–4). Despite these advances, however, the mechanisms responsible for HBV persistence are not well understood.

For a noncytopathic virus to persist either it must not be able to induce an effective antiviral immune response or it must be able to overwhelm or evade it. Neonatal tolerance is probably responsible for both the lack of an antiviral immune response and for viral persistence following mother-infant transmission, which is the most common antecedent of persistent HBV infection worldwide (1). The immunologic basis for viral persistence during adult-onset infection, however, is not well understood. In view of the repeated observation that the CTL response to HBV is much less vigorous in chronically infected patients than it is during acute infection (5–8), reasonable candidates are the induction of peripheral tolerance or exhaustion of the T cell response, inhibition of Ag presentation, selective immune suppression, down-regulation of viral gene expression, and viral mutations that abrogate, anergize, or antagonize Ag recognition by virus specific T cells (reviewed in Ref. 1). While all of these alternatives are possible, direct evidence exists only for the escape mutation scenario (9).

We have argued against this hypothesis as a cause of HBV persistence because many conditions must be fulfilled that rarely occur during HBV infection for a mutant virus to be selected by CTL-mediated immune pressure (reviewed in Ref. 1). Perhaps the most important condition is the occurrence of a strong CTL response that is focused on a single viral epitope. This type of CTL response is unusual both during acute HBV infection, when the CTL response is typically directed against multiple epitopes, and during chronic hepatitis, when it is usually weak or undetectable (6, 7, 10, 11). Accordingly, we have found selection of CTL escape variants to be very uncommon during chronic HBV infection (12). Nonetheless, strong and narrowly focused CTL responses are seen occasionally in these patients, and in this setting viral escape mutations can occur (9). Vigorous oligoclonal expansions of T cells have also been described in other persistent viral infections, especially HIV (13–16). Even in these infections, however, viral mutations that affect recognition of an epitope by some CTL clones do not automatically affect all CTL clones specific for the same epitope, since different T cell clones can recognize different residues in the same epitope (17, 18).

The current study was performed to determine the extent to which epitope-inactivating mutations can lead to escape from the CTL response by defining the molecular anatomy of an immunodominant CTL epitope using a large panel of independently derived CTL clones. The data indicate that the CTL response to

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Received for publication May 4, 1998. Accepted for publication July 21, 1998. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grant R37CA40489 from the National Institutes of Health; by a Grant-in-Aid for International Scientific Research (Joint Research) from the Ministry of Education, Science, Sports, and Culture of Japan; and by funds from Toray Industries, Inc., Japan (to T.I.). This is manuscript 11483-MEM from The Scripps Research Institute.

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3 Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBs21–40, residues 21–40 of hepatitis B surface antigen; EHAA, Eagle Henk’s amino acid; MFI, mean fluorescence intensity.

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individual viral epitopes can be extremely diverse, supporting the idea that selection of TCR contact site escape variants is probably not responsible for the establishment of viral persistence during HBV infection.

### Materials and Methods

#### Cell lines and expression vectors

P815 cells (H-2b) were transfected with pMAMneo vector (Clontech, Palo Alto, CA) into which the HBV large envelope open reading frame (ayw subtype) had been introduced (19) to generate a cell line designated P815-preS1, and with an EBO vector (20) into which the HBV small open reading frame (ayw subtype) had been introduced, designated P815-S. Cell lines Dnuom, Knom, and W12.1 were produced by introducing the.Db, Kd, and Ld genes, respectively, into the KOL fibroblastoma cell line (H-2b) and were provided by Dr. James Forman (Southwestern Medical Center, Dallas, TX) (21, 22). RMA-S Ld cells are stable transformant of RMA-S cells and express the Ld molecule, they were provided by Dr. Herman N. Eisen (Massachusetts Institute of Technology, Cambridge, MA). All the cell lines except for RMA-S Ld were grown in DMEM (Life Technologies, Grand Island, NY) containing 10% FCS, L-glutamine (2 mM), streptomycin (50 μg/ml), penicillin (50 U/ml), HEPES buffer (15 mM), and anti-PPLO (HyClone, Logan, UT; 6 mg/ml). RMA-S Ld cells were maintained in RPMI 1640 (Life Technologies) supplemented as described above. Recombinant vaccinia viruses expressing the HBV large (HBVenv-vac) and major (HBS-vac) envelope polyepitopes (ayw subtype) were derived as previously described (7).

#### Synthetic peptides

Peptides were synthesized by Chiron Technologies (Clayton, Australia), Multiple Peptide Systems (San Diego, CA), and the Peptide Synthesis Core Facility at The Scripps Research Institute (La Jolla, CA). All the peptides were dissolved in 100% DMSO (Mallinckrodt, Paris, KY) at a concentration of 5 or 10 mg/ml and diluted to 1 mM with DMEM, after which they were stored at −20°C until use.

#### CTL induction

Murine HBsAg-specific CTLs were induced as previously described (22, 23). B10.D2 mice (H-2b) were immunized two or three times i.p. at 7-day intervals with 1 × 10⁹ plaque-forming units of HBs-vac. Spleen cells were harvested 7 days after the last injection, and spleenocytes (4 × 10⁶ cells/well) were cultured with irradiated (20,000 rad) P815-env transfectants (1 × 10⁷ cells/well; P815-preS1 or P815-S) or with 20 μg/ml of an antiviral peptide (HBs21–40), previously shown to contain an immunodominant Ld restricted CTL epitope (19), in complete Eagle Hank’s amino acid (EHA) culture medium (Life Technologies) containing streptomycin (50 μg/ml), penicillin (50 U/ml), anti-PPLO (HyClone, Logan, UT; 6 mg/ml), 10% FCS, 2-ME (5 × 10⁻⁷ M), and 2.5% EL-4 supernatant, as source of IL-2, IL-4, and IL-10 production, 1 × 10⁶ cloned CTL were grown in 24-well plates in the presence of 1 × 10⁵ P815-preS1 stimulator cells in complete EHA medium without IL-2 supernatant. Forty-eight hours after the stimulation, the culture supernatant was collected and stored at −20°C until ELISA analysis using commercially available reagents (Genzyme, Cambridge, MA) according to the manufacturer’s instruction.

#### CTL assay

The cytolytic activity of T cell lines and clones was assessed in a standard 4-h cytotoxic assay with 51 Cr-labeled target cells as previously described (17, 24). Clonal BV gene expansions were readily apparent without quantitation. RT-PCR products were analyzed by sequencing or cloned for sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA) or cloned into the pCRII plasmid (Invitrogen, Carlsbad, CA) and sequenced (Amersham, Arlington Heights, IL).

#### Antagonism assay

After labeling with 3²Cr and extensive washing, target cells (P815) were cultured, plated (5 × 10⁵/cells) in 96-well flat-bottom plates. They were incubated with various concentrations of the variant peptides for 1 h, followed by a suboptimal concentration (0.001 μM) of wild-type peptide for another hour before addition of the effector CTL clones.

#### TCR β-chain analysis

TCR BV gene usage of CTL clones was first determined by a BV multiprobe RNase protection assay, and then the appropriate BV chains were obtained by RT-PCR. The details of the RNase protection assay encompassing 18 BV genes have been previously described (24). Clonal BV gene expansions were readily apparent without quantitation. RT-PCR products were used for sequencing from 20 ng of total cellular RNA, specific sense BV and antisense BC primers (25) at 2 ng/μl, and standard reagents (Boehringer Mannheim, Indianapolis, IN). PCR conditions were 30 cycles of 94°C for 30 s, 48–50°C for 30 s depending on the primer, and 72°C for 40 s. PCR products were either sequenced directly by fluorescent cycle sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA) or cloned into the pCRII plasmid (Invitrogen, Carlsbad, CA) and then sequenced (Amersham, Arlington Heights, IL).

#### Cytokine production

For measurements of TNF-α, IFN-γ, IL-2, IL-4, and IL-10 production, 1 × 10³ cloned CTL were grown in 24-well plates in the presence of 1 × 10⁵ P815-preS1 stimulator cells in complete EHA medium without IL-2 supernatant. Forty-eight hours after the stimulation, the culture supernatant was collected and stored at −20°C until ELISA analysis using commercially available reagents (Genzyme, Cambridge, MA) according to the manufacturer’s instruction.

#### Ld binding assay

The RMA-S peptide binding assay was performed as described previously (26, 27). In brief, RMA-S Ld cells were cultured overnight at 25°C to increase the cell surface expression of empty class I molecules. Cells (1 × 10⁶/well) were plated (96-well plates) in 100 μl of RPMI containing 2% FCS plus peptide (final concentration, 0.3 μM). After overnight incubation at 25°C, the cells were washed and suspended in HBSS supplemented with 2% horse serum. mAb against Ld (30-5-7S) was added, and incubation took place on ice for 30 min followed by washing. Subsequently, the cells were incubated with FITC-labeled goat anti-mouse F(ab)² for 30 min on ice, after which they were washed and analyzed in a FACS scan flow cytometer (Becton Dickinson, Mountain View, CA). Ten thousand cells were gated and analyzed for fluorescence intensity. The fluorescence index was calculated as follows: (MFI in the presence of peptide − MFI in the absence of peptide)/MFI in the absence of peptide). The mean fluorescence intensity (MFI) is the mean channel number of 1 × 10⁴ gated cells.

#### Results

##### Characteristics of HBsAg-specific CTLs

Eleven HBsAg-specific CTL clones established from three immunized B10.D2 mice were used for this study. The immunization and in vitro expansion protocols used before cloning by limiting dilution are shown in Table I. Clones that displayed good growth characteristics and the ability to specifically lyse >90% of the P815-preS1 target cells at an E:T cell ratio of 5 were selected for this study. All CTL clones recognized P815-preS1 target cells with approximately the same efficiency (Fig. 1A). As displayed for clone 6C2 in Fig. 1B, we showed that all the CTL clones displayed the same fine specificity, using target cells pulsed with a panel of truncated synthetic peptides corresponding to an immunodominant CTL epitope located between residues 28–39 of HBsAg (HBs28–39) that we have previously described (22). Next, using peptide pulsed (HBs28–39, 1 μM) H-2Kd-positive mouse fibroblasts that express Kd, Dd, or Ld as target cells, we showed that all the CTL clones recognized the epitope in the context of the Ld molecule (Fig. 1C). Peptide dose titration experiments demonstrated that the CTL clones displayed comparable epitope binding affinity (Fig. 2A).
Importantly, as shown in Table II, all the clones produced comparable amounts of IFN-γ and TNF-α following Ag stimulation. Finally, as shown in Fig. 2, TCR β-chain analysis revealed six distinct clonal populations among the 11 CTL clones studied, three derived from mouse 1, two derived from mouse 2, and one derived from mouse 3, with no specific β-chain preference. Not surprisingly, CTLs were identified that used the same TCR BV chain but different BD and BJ segments. Importantly, none of the populations dominated the others. Collectively, these results indicate that CTL clones that display widely varying TCR β-chain usage can recognize the same CTL epitope with similar affinity in the context of the same MHC restriction element, and that their functional responses are comparable upon recognition of this epitope. The results also demonstrate that the immunization and in vitro stimulation protocols used to produce the CTL clones can influence the response at the level of TCR repertoire diversity.

Table I. Protocols used to induce HBsAg-specific CTLs in B10.D2 mice

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Clone</th>
<th>Immunization</th>
<th>Stimulation</th>
<th>Time (wk)</th>
<th>Cloning (cell/well)</th>
</tr>
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<tr>
<td>1</td>
<td>6C2</td>
<td>S-vac (i.p.) × 2</td>
<td>Tx (P815-preS1)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>11B5</td>
<td>S-vac (i.p.) × 2</td>
<td>Tx (P815-preS1)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>11B10</td>
<td>S-vac (i.p.) × 2</td>
<td>Tx (P815-preS1)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>13F6</td>
<td>S-vac (i.p.) × 2</td>
<td>Tx (P815-preS1)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>14F7</td>
<td>S-vac (i.p.) × 2</td>
<td>Tx (P815-preS1)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>SWB1</td>
<td>S-vac (i.p.) × 3</td>
<td>HBs21-40</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>SWB9</td>
<td>S-vac (i.p.) × 3</td>
<td>HBs21-40</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>SWD10</td>
<td>S-vac (i.p.) × 3</td>
<td>HBs21-40</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>B6D</td>
<td>S-vac (i.p.) × 3</td>
<td>Tx (P15-S)</td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>D11B</td>
<td>S-vac (i.p.) × 3</td>
<td>Tx (P815-S)</td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>E7C</td>
<td>S-vac (i.p.) × 3</td>
<td>Tx (P815-S)</td>
<td>4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* s-vac; recombinant vaccinia virus that expresses HBsAg (ayw subtype). Mice were immunized i.p. with 1 × 10⁸ PFU of S-vac weekly the indicated number of times. Tx; tumor cell lines (P815) transfected with HBs gene (ayw subtype).

A. Relative Cytolytic Activity

B. Fine Specificity

C. MHC Restriction

D. TCR Affinity

FIGURE 1. Characteristics of HBsAg28-39-specific CTL clones. A. Relative cytolytic activity. All the HBsAg-specific CTL clones were tested for their ability to kill ⁵¹Cr-labeled P815-preS1 target cells in a 4-h ⁵¹Cr release assay at the E:T cell ratios (0.1, 0.3, 1, 3, and 10) shown on the x-axis. The results are expressed as the mean percent specific lysis as described in Materials and Methods. B. Fine specificity. All the CTL clones were incubated with ⁵¹Cr-labeled P815 target cells and various concentrations of N- and C-terminal truncations of HBs28–39 at an E:T cell ratio of 10. As shown for clone 6C2, all the CTL clones recognized HBs28–39 as the minimal optimal epitope. C. MHC restriction. H-2k-positive mouse fibroblasts that had been stably transfected with the K¹, D¹, and L¹ alleles were labeled with ⁵¹Cr, washed, pulsed with 1 μM HBs28–39, and incubated for 4 h with CTL clone 6C2 at an E:T cell ratio of 10. D. TCR affinity. The relative ability of the CTL clones to recognize HBs28–39 was assessed in a 4-h ⁵¹Cr release assay using varying peptide concentrations and an E:T cell ratio of 10.
A set of 48 peptides containing alanine (A), tyrosine (Y), glutamic acid (E), and arginine (R) substitutions at each position (Table III) was used to define the MHC and TCR contact sites in the wild-type epitope (IPQSLDSWWTSL) and to analyze the fine specificity of the 11 CTL clones. The binding affinity of these peptides to Ld was examined with an Ld-transfected, TAP-deficient RMA-S cell line by FACS quantitation of cell surface Ld expression after addition of the peptides. As shown in Fig. 3 (lower panel), all four substitutions at positions 2 and 12 strongly reduced the Ld binding affinity of the peptides, implying that these are dominant anchor residues for this epitope. Other positions (especially positions 5 and 10) also contribute to Ld binding, but to a lesser degree, since Ld expression was less profoundly reduced by substitutions at these positions and some of the substitutions at these positions did not reduce binding. In parallel experiments, we analyzed the ability of each of the 11 CTL clones to kill 51 Cr-labeled target cells that had been pulsed with a series of concentrations of the wild-type peptide and each of the 48 substituted peptides. As shown in the upper panel of Fig. 3 for clone 6C2 at an E:T cell ratio of 10 and a peptide concentration of 0.1 mM, substitutions at several positions, including the presumptive anchor positions 2 and 12, reduced or abolished CTL recognition. Similar experiments were performed for each clone. In each instance, several substitutions were identified that inhibited CTL recognition without affecting Ld binding affinity (e.g., positions 1, 4, 6, and 7 in the case of clone 6C2), suggesting that the corresponding wild-type residues probably serve as TCR contact sites for that clone. Peptide competition experiments were performed to test this hypothesis.

**Competition assay**

Once the substitutions that abrogated peptide recognition were identified for each CTL clone, the corresponding substituted peptides were incorporated into a competition assay to monitor their ability to block the binding of the wild-type peptide to the Ld molecule. Briefly, 51Cr-labeled P815 target cells were preincubated with the substituted peptides at several concentrations that did not sensitize the target cells to killing by the corresponding CTL clone. The wild-type peptide was then added at a concentration (in most cases 0.001 mM) that had been previously determined to sensitize the target cells to approximately half-maximal lysis by the corresponding CTL clone. After 1 h, the CTL clones were added and a 4-h 51Cr release assay was performed. If the substituted peptide binds to Ld and if it is used in sufficient (at least 100-fold) molar excess, it will saturate the available Ld binding sites and block binding by the wild-type peptide such that the percent specific 51Cr release

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**Table II. Cytokine profiles of HBsAg 28–39 specific clones**

<table>
<thead>
<tr>
<th>Clone</th>
<th>BV/BD/BJ</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6C2</td>
<td>1S1/2/2S5</td>
<td>581</td>
<td>8,930</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>9,380</td>
</tr>
<tr>
<td>11B5</td>
<td>1S1/2/2S5</td>
<td>147</td>
<td>16,940</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;400</td>
</tr>
<tr>
<td>11B10</td>
<td>8S2/2/2S2</td>
<td>817</td>
<td>26,280</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>15,100</td>
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<tr>
<td>13F6</td>
<td>8S2/1/2S6</td>
<td>454</td>
<td>18,280</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>1,720</td>
</tr>
<tr>
<td>14F7</td>
<td>8S2/1/2S6</td>
<td>371</td>
<td>8,430</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;400</td>
</tr>
<tr>
<td>SWB1</td>
<td>16S1/2/2S6</td>
<td>63</td>
<td>910</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;400</td>
</tr>
<tr>
<td>SWB9</td>
<td>16S1/2/2S6</td>
<td>437</td>
<td>11,800</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>4,250</td>
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<tr>
<td>SWD10</td>
<td>6S1/1/2S1</td>
<td>187</td>
<td>15,670</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;400</td>
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<tr>
<td>B6D</td>
<td>2S1/1/1S1</td>
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<td>11,560</td>
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<td>D11B</td>
<td>2S1/1/1S1</td>
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<td>1,860</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<tr>
<td>E7C</td>
<td>2S1/1/1S1</td>
<td>161</td>
<td>10,230</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>4,480</td>
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</table>

*CTL clones 1 × 10^6 were incubated with 1 × 10^6 stimulator cells (P815-preS1) in complete medium without EL-4 supernatant. Forty-eight hours after stimulation, the culture supernatant was collected and analyzed for cytokine concentration. All the cytokines were measured with commercially available ELISA kits, and the cytokine values were expressed as pg/ml.

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**Figure 2.** TCR analysis. The nucleotide and predicted amino acid sequences for TCR β-chain CDR3 junctional regions of CTL clone are shown. The BD gene segments are underlined.
induced by the corresponding CTL clone will be reduced. On the contrary, if the substituted peptide does not bind to L<sup>d</sup>, it will not inhibit binding by the wild-type peptide, and the percent specific <sup>51</sup>Cr release will not be reduced. Sample results for clone 6C2 and two substitutions, i.e., Pro<sup>2</sup>→Tyr at position 2 and Ser<sup>4</sup>→Ala at position 4, are shown in Fig. 4. In the position 2 substitution (Fig. 4, upper panel), increasing concentrations of the substituted peptide did not recognize the wild-type peptide, suggesting that the Pro<sup>2</sup>→Tyr substitution at position 2 abrogated L<sup>d</sup> binding. This is compatible with the L<sup>d</sup> binding assay results shown in Fig. 3 (lower panel), indicating that position 2 is an MHC contact site. In the position 4 substitution (Fig. 4, lower panel), increasing concentrations of the substituted peptide inhibited recognition of the wild-type peptide, suggesting that the Ser<sup>4</sup>→Ala substitution at position 4 did not reduce the L<sup>d</sup> binding capacity ability of the peptide and indicating that this position is a TCR contact site for clone 6C2.

The foregoing analysis was performed at all the pertinent positions for all the CTL clones, yielding the epitope maps shown in Fig. 5. In every instance, positions 2 and 12 were shown to be MHC binding sites, confirming the results of the binding assay (Fig. 3, lower panel). In contrast, several different positions were served as TCR contact sites for the various clones, the specificity of which was clearly determined at the level of the TCR, since each β-chain displayed its own unique specificity pattern and because there were no discrepancies between clones that used the same β-chain. For example, threonine at position 10 was uniquely recognized as a TCR contact site by 2 clones (13F6 and 14F7) that shared the same β-chain, which is not used by any of the other

![Table III. Substituted peptides used for epitope mapping](image)

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Ala substitution</th>
<th>Tyr substitution</th>
<th>Glu substitution</th>
<th>Arg substitution</th>
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<tr>
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<tr>
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<td>PQLSDSWTS</td>
<td>Y</td>
<td>PQLSDSWTS</td>
<td>R</td>
</tr>
<tr>
<td>T</td>
<td>PQLSDSWTS</td>
<td>Y</td>
<td>PQLSDSWTS</td>
<td>R</td>
</tr>
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</table>

* Each position of antigenic peptide, HBs28-39, was substituted with alanine, tyrosine, glutamic acid, or arginine. These 48 peptides were used for epitope mapping of the CTL clones.
clones. Other positions were recognized by multiple TCRs. For example, isoleucine at position 1 was recognized by five clones representing two different \( \beta \)-chains, serine at position 4 was recognized by nine clones representing four different \( \beta \)-chains, etc. Importantly, aspartate at position 6 was the only TCR contact site that was recognized by all the clones. Interestingly, as shown in Fig. 5, leucine and threonine at positions 5 and 10, respectively, appear to be able to function as both MHC binding sites and TCR contact sites depending on the clone.

The same residue is recognized differently by different TCRs

To further examine the diversity of the TCR repertoire, a set of peptides containing every possible substitution at positions 4 and 6 (Fig. 6) was tested for recognition by three separate clones, two of which (6C2 and 11B5) used the same \( \beta \)-chain. In a preliminary experiment we established that the \( L^d \) binding affinity of these peptides was comparable to that of the wild-type peptide (data not shown). As shown in Fig. 7, hydrophobic and charged substitutions at both positions were generally incompatible with recognition by clones 6C2 and 11B5. In contrast, many of these substitutions were recognized by clone 13F6, while certain polar substitutions (e.g., Y and H) that were tolerated by clones 6C2 and 11B5 abrogated recognition by clone 13F6, reflecting its different TCR.

Search for CTL antagonists

There are several reports that substitutions of TCR contact sites of the antigenic peptide can yield analogue peptides that can still interact with the TCR but be unable to deliver a full stimulatory signal, thus acting as an antagonist for the TCR (28–34). To examine whether the substitutions at the dominant TCR contact sites (positions 4 and 6) may result in variant sequences with an antagonistic effect on TCR recognition of the wild-type peptide (HBs28–39), antagonism assays were performed with clone 6C2 using the same set of peptides containing all possible substitutions at positions 4 and 6 (Fig. 6). Briefly, \(^{51}\)Cr-labeled P815 target cells were preincubated with the wild-type or substituted peptide, and a 4-h \(^{51}\)Cr release assay was performed at an E:T cell ratio of 10 using CTL clones 6C2 and 11B5 that express the same \( \beta \)-chain (1S1/2/2S5) and clone 13F6 that expresses a different \( \beta \)-chain (8S2/1/2S6). The residues shown on the \( x \)-axis represent substitutions at positions 4 (left) or 6 (right). The amino acid indicated at the far left on the \( x \)-axis (S at position 4; D at position 6) is the wild-type residue at each position. The underlined residues identify the variant peptides that were not recognized by any of the three clones.

![Epitope Map](image1)

**FIGURE 5.** Epitope maps. Competition assays were performed for all 11 CTL clones as described in Fig. 4 using alanine-, tyrosine-, glutamic acid-, and arginine-substituted peptides. The positions that scored as TCR and MHC contact sites are shown as black and striped boxes, respectively. Positions that did not score as either TCR or MHC contact sites are designated spacers and are indicated by gray boxes.

![Substitutions TCR Recognition Sites](image2)

**FIGURE 6.** TCR contact site substitutions. All possible substitutions at the dominant TCR contact sites (positions 4 and 6) were used to define the extent to which HBsAg28–39-specific CTLs can recognize or be antagonized by variant residues at these positions.

![Wild Type: IPQSLDSWWTSL](image3)

**FIGURE 7.** Diversity of TCR Ag recognition within a single epitope. \(^{51}\)Cr-labeled P815 target cells were incubated for 1 h with 0.1 \( \mu \)M of the wild-type or substituted peptide, and a 4-h \(^{51}\)Cr release assay was performed at an E:T cell ratio of 10 using CTL clones 6C2 and 11B5 that express the same \( \beta \)-chain (1S1/2/2S5) and clone 13F6 that expresses a different \( \beta \)-chain (8S2/1/2S6). The residues shown on the \( x \)-axis represent substitutions at positions 4 (left) or 6 (right). The amino acid indicated at the far left on the \( x \)-axis (S at position 4; D at position 6) is the wild-type residue at each position. The underlined residues identify the variant peptides that were not recognized by any of the three clones.
None of the substituted peptides blocked the ability of clone 6C2 to kill target cells pulsed with up to 5 μM of the wild-type peptide (not shown), indicating that none of the position 4 or 6 substitutions created an antagonistic variant of this epitope with respect to clone 6C2.

Discussion

Experiments in lymphocytic choriomeningitis-infected TCR transgenic mice have proven that immune selection of CTL escape variants can occur during viral infections (35, 36). The extremely high CTL precursor frequency and the extremely narrow TCR repertoire in the transgenic mouse studies, however, do not occur in most natural viral infections, where lower precursor frequencies and broader repertoires are the rule. Nonetheless, mutational escape from the CTL response has been detected in a number of persistent human virus infections, including those due to HIV (37–41), human T lymphotropic virus-1 (42), EBV (43–45), hepatitis C virus (34, 46), and HBV (9, 15). Documentation that mutational inactivation of a single CTL epitope can occur during an infection, however, does not necessarily translate into escape from the CTL response in general, nor does it establish that CTL escape is a common occurrence or prove that CTL escape is responsible for viral persistence in these infections. We have argued that these examples of CTL escape during HBV and HCV infection are special cases because when CTL responsiveness and viral nucleotide sequence analysis have been studied simultaneously in populations of chronically infected patients, CTL escape has been shown to be uncommon (12, 34).

Nonetheless, the role of CTL escape in viral persistence remains controversial. The current study was undertaken, therefore, to examine the requirements for an epitope to escape recognition by the CTL response, since the same requirements should apply to all the epitopes normally recognized during a viral infection. To simplify the analysis, a single strain of inbred mice (B10.D2) was primed to produce a CTL response to a single viral protein, HBsAg. We and others have previously demonstrated (22, 47) that the CTL response to this protein in these mice is primarily directed against a single, unusually long (12-mer), immunodominant, Ld-restricted epitope located between residues 28–39 of the major envelope protein of HBV (IPQSLDSWWTSSL). As summarized in Fig. 5, during this study we demonstrated that positions 2 and 12 are dominant anchor residues responsible for the ability of the Ld molecule to bind the epitope, and that positions 5 and 10 can function as minor anchor residues. We also demonstrated that residue 1 and 4–10 can serve as TCR contact sites depending on the CTL clone being studied. Importantly, we showed that the CTL response to this epitope is remarkably polyclonal (involving several different TCR β-chains) and multispecific (each β-chain displaying a unique fine specificity profile), emphasizing the impressive diversity of the CTL response at the level of a single epitope, as previously reported (48–52).

As shown in Fig. 7, several distinct chemical interactions account for TCR recognition of the MHC-peptide complex. For example, in the case of position 4, amino acid polarity is important for TCR recognition by clones 6C2 and 11B5 (S to polar group amino acid substitution is acceptable for TCR recognition), while in the case of position 6, the size or the shape of the side chain seems to be important (D to N substitution is most acceptable). Recognition of both of these positions by clone 13F6, however, does not follow these rules, suggesting that the shape of the exposed peptide surface or the framework of the peptide binding groove is recognized by this clone’s TCR. These observations and other results reported herein are pertinent to the question of viral escape from the CTL response. For example, Fig. 5 demonstrates that nonconservative substitutions at nine of the 12 positions in this epitope either had no effect on recognition by any of the CTL clones, or they affected recognition by some of the clones but not others. Thus, mutations involving 75% of the epitope would not be expected to abolish recognition by all the CTL clones. Furthermore, although certain substitutions at positions 2, 6, and 12 abrogated recognition of the epitope by all the CTL clones, many substitutions either did not or should not affect recognition by any of them. Since the Ld binding motif includes P and Q at position 2 and hydrophobic residues at the C-terminus (in this case position 12), the corresponding substitutions at these positions should be tolerated. Likewise, examination of Fig. 7 reveals that only seven of the possible 19 substitutions at TCR contact position 6 abrogated recognition by the three CTL clones tested, and only six substitutions at TCR contact position 4 (which is not recognized by all of the clones) had this effect. It is important to note that even this number of tolerable substitutions at these TCR contact sites is probably an overestimate, since only three CTL clones were studied in this manner and also because the CTL clones studied certainly do not represent the entire TCR repertoire for this epitope. Finally, the considerations described herein for epitope-inactivating mutations also apply to mutations that generate TCR antagonists (not observed in this study). Specifically, because of the TCR diversity of the CTL response to any given epitope, mutations that function as antagonists for one CTL clone need not, and usually do not, have the same effect on others.

Interestingly, the competition studies summarized in Fig. 5 indicate that leucine and threonine at positions 5 and 10, respectively, can function as TCR contact sites for certain clones, although the Ld binding assay clearly demonstrated that they also function as minor MHC binding sites for this epitope (see Fig. 3, lower panel). Perhaps the simplest explanation for this seeming paradox is that because of its unusual length, this epitope can display more than one stable orientation in the Ld-binding groove, one of which causes these residues to bind Ld, while they are free to interact with the TCR in the other orientation. According to this scenario, the position(s) would score as a TCR contact site for CTL clones that require interaction with this residue(s) for activation. Alternatively, the position(s) would score as an MHC binding site(s) for clones that do not recognize this residue(s) as long as the substitution reduced the amount of target cell-associated peptide below the threshold needed for recognition by the CTL clone being tested. If not, the position would score as neutral, as shown for some of the clones in Fig. 5. Resolution of this interesting paradox will require structural analysis of the peptide-MHC complex.

In conclusion, the current results demonstrate that the CTL response to a single viral epitope can be extremely diverse. Since all the CTL clones displayed comparable cytolytic activity (Fig. 1) and cytokine expression profiles (Table II), and since no single TCR was dominant (Figs. 2 and 5), most of the mutations that might occur in this epitope are not likely to abolish all CTL effector activity. In the rare instance in which a mutation leads to escape from all CTL clones of a single specificity, the concomitant CTL response to other viral epitopes, each of which could be as diverse as the response described in this report, make it very unlikely that mutational inactivation of a single CTL epitope will lead to viral persistence in our opinion. Some have argued that the CTL response can be narrowly focused early during viral infections (41, 53). If this is correct, CTL escape mutations could contribute to viral persistence by retarding the rate of viral clearance. However, the diversity of the CTL response to individual epitopes illustrated herein suggests that even under these conditions, escape
from all CTL clonal specificities would not be guaranteed. Furthermore, the procedures used to monitor the CTL response during infection should be as unbiased as possible to avoid artificially restricting the CTL repertoire and inappropriately viewing the response as narrow when, in fact, it might be quite diverse.

Acknowledgments
We thank Drs. Luca G. Guidotti, Kazuki Ando, and Yasunari Nakamoto for helpful discussion, and Ms. Jennifer Newmann for assistance with manuscript preparation.

References


