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Protection Against CTL Escape and Clinical Disease in a Murine Model of Virus Persistence

Taeg S. Kim,* and Stanley Perlman2*†

CTL escape mutations have been identified in several chronic infections, including mice infected with mouse hepatitis virus strain JHM. One outstanding question in understanding CTL escape is whether a CD8 T cell response to two or more immunodominant CTL epitopes would prevent CTL escape. Although CTL escape at multiple epitopes seems intuitively unlikely, CTL escape at multiple CD8 T cell epitopes has been documented in some chronically infected individual animals. To resolve this apparent contradiction, we engineered a recombinant variant of JHM that expressed the well-characterized gp33 epitope of lymphocytic choriomeningitis virus, an epitope with high functional avidity. The results show that the presence of a host response to this second epitope protected mice against CTL escape at the immunodominant JHM-specific CD8 T cell epitope, the persistence of infectious virus, and the development of clinical disease. The Journal of Immunology, 2003, 171: 2006–2013.

Viruses have developed many mechanisms to evade the host immune response, thereby increasing the probability of persistence and of subsequent spread to uninfected hosts (reviewed in Ref. 1). One mechanism that has been documented in several infections is the selection of variants able to evade the CD8 CTL response. Most commonly, this involves mutations in immunodominant CD8 T cell epitopes, abrogating or significantly diminishing recognition by epitope-specific CTLs. Mutations in CD8 T cell epitopes have been identified in viral isolates from humans infected with HIV-1, hepatitis B or C virus, and from primates infected with SIV or hepatitis C virus. In several instances, variant viruses were identified at early times post inoculation (p.i.)1, in the presence of a normal or minimally compromised immune response, suggesting that the mutations contributed to disease progression. Mutations that diminish CTL recognition have also been detected at later times in the disease course, in humans or primates infected with HIV-1 or SIV, respectively. Under these circumstances, their role in pathogenesis is ambiguous because they may be selected only after other components of the immune response, such as CD4 T cells, fail to control the infection (2–6).

CTL escape is commonly identified in mice infected with the neurotropic JHM strain of mouse hepatitis virus (JHM) (3). Mice infected with JHM develop an acute, fatal encephalitis or acute or chronic demyelinating diseases, dependent upon the conditions of the infection (7). In one model, suckling C57BL/6 (B6) mice were infected with JHM and nursed by dams previously immunized to the virus (8). Nursing by immunized dams prevented acute encephalitis, but 40–90% of the suckling mice developed a demyelinating encephalomyelitis with clinical signs of hindlimb paralysis several weeks p.i. Infectious virus was isolated from asymptomatic mice, and in each case, the virus was mutated in the immunodominant CD8 T cell epitope recognized in this strain (9). This epitope encompasses residues 510–518 of the surface glycoprotein (epitope S510, H-2Db-restricted). The mutations diminished recognition in cytotoxicity assays, and infection of naive mice with CTL escape mutants resulted in greater mortality and morbidity when compared with mice infected with parental virus (10). Mutations in the epitope were detected as early as 10–12 days p.i. and were not identified in persistently infected SCID mice, confirming the role of immune pressure in their selection (11). Mutations were never detected in the second major CD8 T cell epitope recognized in B6 mice, spanning residues 598–605 of the S protein (epitope S598; H-2Kb-restricted), even when mice were infected with virus mutated in epitope S510 (10). CTL escape at epitope S598 most likely did not occur because this epitope has lower functional avidity (amount of peptide required to sensitize target cells for CD8 T cell lysis) than does epitope S510 (12), and epitope S598-specific CD8 T cells are not protective (10).

Several factors are likely to be important in the selection of CTL escape mutants. First, mutation in the epitope in question must not significantly attenuate the virus. Epitope S510 is in a region of the S protein that tolerates mutation and deletion (13, 14). Consistent with this, CTL escape mutations were not detected in maternal Ab-protected JHM-infected BALB/c or B10.A(18R) mice, in part because the immunodominant CD8 T cell epitope recognized in these strains is located within a conserved region of the nucleocapsid protein (3, 15, 16). Second, loss of CD8 T cell recognition must provide a selective advantage for the variant virus. A corollary of this is that the stronger the CD8 T cell response, the more likely CTL escape is to occur. Consistent with this, CTL escape was not observed in HIV-infected patients with rapid disease progression and a weak immune response to the virus (17). Third, the CD8 T cell response should be focused on a single epitope because a response to more than one strong epitope should prevent the outgrowth of virus with a single mutation (18). Fourth, because the CD8 T cell response is usually comprised of several different CD8 T cell clonotypes, it should be narrowly focused on only part of the MHC class I/peptide complex to facilitate escape. If different TCR clonotypes recognized different parts of the complex, single mutations would abrogate recognition by only a subset of epitope-specific CD8

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3 Abbreviations used in this paper: p.i., post inoculation; JHM, mouse hepatitis virus strain JHM; LCMV, lymphocytic choriomeningitis virus; RAG, recombination activation gene.
T cells, making their selection less likely. In support of this notion, we have shown that single mutations in amino acids 4 and 6 of epitope S510, located in the central region of the epitope and predicted to affect binding to the TCR (19, 20), diminished recognition by entire unfractionated populations of CNS-derived CD8 T cells in direct ex vivo cytotoxicity assays and by spleen-derived T cell lines (9, 21). A CD8 T cell response focused on the central region of the MHC class I/peptide complex has also been identified in mice infected with Sendai virus (22).

In apparent contradiction to the requirement for the factors previously listed, mutations in two or more CD8 T cell epitopes were identified in individual non-human primates infected with SIV or hepatitis C virus (5, 6, 23). However, only rarely was CTL escape demonstrated in the presence of a CD8 T cell response directed at two epitopes with high functional avidity (5). These results suggested that the presence of a high functional avidity peptide would protect against CTL escape, and by extension, disease progression.

To determine directly whether the presence of a second CTL epitope with high functional avidity prevents selection of CTL escape variants and therefore disease progression, a well-characterized, immunodominant epitope from lymphocytic choriomeningitis virus (LCMV) spanning residues 33–41 of gp33 (19) was introduced into the JHM genome using targeted recombination (24). Targeted recombination is based on the high rate of recombination exhibited by coronaviruses and has been used to introduce mutations into the 3′ region of the JHM genome in previous studies. This virus, and a matched control virus in which the primary mutations into the 3′ region of the JHM genome were not in epitope S510 or epitope S598 or in the regions flanking these epitopes. This plasmid contained an SbfI restriction enzyme site between gene 3 (encoding the S protein) and gene 4 to facilitate cloning. A consequence of the insertion of this restriction site was a change in gene 4 RNA expression (25). To revert levels of gene 4 to that observed in cells infected with nonrecombinant viruses, a second plasmid, in which the SbfI restriction site (Fig. 1) was removed by site-directed mutagenesis, was also developed. We then used overlapping extension PCR with two sets of primers to introduce epitope gp33 into both plasmids. The two inner primers used in these studies were 5′-GTG TAC ATT GCC ACC TGT GGC CTC ATC GGT CCC AAG ACT AC-3′ (sense) and 5′-GCC GAA ATT GTA CAC AGC TTT GGC CAT GGC TAC TTT CTG CCC-3′ (antisense) encompassing gp33-41 (underlined) and JHM sequences (see Fig. 1). The outer primers were: 5′-CAG CCT CGT CAG GAA AGA CAG AAA AT-3′ (sense) and 5′-CGG CCT CGT CCG CCG T-3′ (antisense). As a control, an additional construct was developed in which the H-2Db anchor (asn) residue of the gp33 epitope was mutated to ile using the same methodology. The inner primers for this construct were: 5′-GTG TAC ATT GCC ACC TGT GGC CTC ATC GGT CCC AAG ACT AC-3′ (sense) and 5′-GCC GAA ATT GTA CAC AGC TTT GGC CAT GGC TAC TTT CTG CCC-3′ (antisense). Donor RNAs were transcribed using T7 RNA polymerase and transfected into feline cells (AK-D cells) previously infected with a recombinant JHM encoding a feline surface glycoprotein (25, 26). In the absence of recombination, this virus grew only on feline cells. After recombination, virus containing the murine JHM S protein was able to infect murine, but not feline, cells allowing for efficient selection of recombinant virus. Recombinant virus was then propagated as described (25, 26). The resulting recombinant viruses were sequenced to confirm the inserted sequences before use in further studies. To control for

Materials and Methods

Virus and cells

Nonrecombinant and recombinant JHM were grown and titred as previously described (8). Virus was harvested from infected brains and spinal cords as previously described (8). 17Cl-1 BALB/c cells and HeLa cells expressing the cellular receptor for mouse hepatitis virus (HeLa-MHVR) were grown in DMEM supplemented with 10% FCS.

Animals

Pathogen-free 5- to 6-wk-old B6 mice were purchased from the National Cancer Institute (Bethesda, MD). 5- to 6-wk-old suckling mice were intranasally inoculated with 6 × 10⁴ PFU JHM in 12 μl. Persistently infected animals were obtained as previously described (8). Briefly, 10-day-old suckling mice were inoculated intranasally with 2–6 × 10⁴ PFU of recombinant or nonrecombinant JHM and nursed by dams previously immunized with live wild-type JHM. All animal studies were approved by the University of Iowa Animal Care and Use Committee.

Recombinant virus

Targeted recombination was used to generate recombinant virus, as described elsewhere (25, 26). In brief, a plasmid containing genes 3–7 of JHM was used as the substrate for synthesis of donor RNA (Fig. 1). This plasmid was nearly identical to one used previously (25), except that five changes were introduced to match the amino acid sequence of the S protein of our laboratory strain of JHM (Ref. 9 and data not shown). These changes were not in epitope S510 or epitope S598 or in the regions flanking these epitopes. This plasmid contained an SbfI restriction enzyme site between gene 3 (encoding the S protein) and gene 4 to facilitate cloning. A consequence of the insertion of this restriction site was a change in gene 4 RNA expression (25). To revert levels of gene 4 to that observed in cells infected with nonrecombinant viruses, a second plasmid, in which the SbfI restriction site (Fig. 1) was removed by site-directed mutagenesis, was also developed. We then used overlapping extension PCR with two sets of primers to introduce epitope gp33 into both plasmids. The two inner primers used in these studies were 5′-GTG TAC ATT GCC ACC TGT GGC CTC ATC GGT CCC AAG ACT AC-3′ (sense) and 5′-GCC GAA ATT GTA CAC AGC TTT GGC CAT GGC TAC TTT CTG CCC-3′ (antisense) encompassing gp33-41 (underlined) and JHM sequences (see Fig. 1). The outer primers were: 5′-CAG CCT CGT CAG GAA AGA CAG AAA AT-3′ (sense) and 5′-CGG CCT CGT CCG CCG T-3′ (antisense). As a control, an additional construct was developed in which the H-2Db anchor (asn) residue of the gp33 epitope was mutated to ile using the same methodology. The inner primers for this construct were: 5′-GTG TAC ATT GCC ACC TGT GGC CTC ATC GGT CCC AAG ACT AC-3′ (sense) and 5′-GCC GAA ATT GTA CAC AGC TTT GGC CAT GGC TAC TTT CTG CCC-3′ (antisense). Donor RNAs were transcribed using T7 RNA polymerase and transfected into feline cells (AK-D cells) previously infected with a recombinant JHM encoding a feline surface glycoprotein (25, 26). In the absence of recombination, this virus grew only on feline cells. After recombination, virus containing the murine JHM S protein was able to infect murine, but not feline, cells allowing for efficient selection of recombinant virus. Recombinant virus was then propagated as described (25, 26). The resulting recombinant viruses were sequenced to confirm the inserted sequences before use in further studies. To control for

FIGURE 1. Strategy for site-directed mutagenesis of JHM. To create a recombinant virus that expressed epitope gp33, DNA encoding the epitope was inserted after the second amino acid of the gene 4 open reading frame (A) as described in Materials and Methods. Recombinant wild-type JHM was initially developed as a control (B). Epitope gp33 was inserted into plasmids either containing or lacking an SbfI restriction site, to modulate levels of expression of gene 4 (C). A second set of constructs, that encoded a variant epitope gp33 (asn to ile at anchor residue of the epitope) were also constructed (D). Donor RNA for targeted recombination was transcribed from these plasmids and transfected into cells infected with JMHV (a recombinant mouse hepatitis virus expressing the feline coronavirus S protein). Recombinant viruses were selected, and at least two independent isolates were propagated on 17 Cl-1 cells.
any unwanted mutations that might have occurred during the process of targeted recombination, at least two independent isolates of each recombinant virus were analyzed in these studies. The same results were obtained with both isolates in all experiments and only the results from one isolate of each recombinant virus are shown.

**FACS analysis**

Lymphocytes were prepared from brains and used in intracellular IFN-γ cytokine assays as previously described (27). Peptides corresponding to CD8 T cell epitopes S510 or gp33 or irrelevant peptide (OVA 257–264) were used at a final concentration of 1 μM. Samples were washed and incubated in blocking buffer containing 10% rat serum and anti-FcRIII/II Ab (2.4G2). Cells were stained with FITC-conjugated anti-CD8 mAb (Ly2). For the detection of intracellular IFN-γ, cells were washed, fixed, permeabilized, and incubated with PE-conjugated anti-IFN-γ (XMG1.2) or PE-conjugated rat Ig (all purchased from BD PharMingen, San Diego, CA). After washing, cells were analyzed using a FACSscan Flow Cytometer (BD Biosciences, Mountain View, CA). The number of lymphocytes harvested from each infected brain ranged from \(8 \times 10^5\) to \(1.8 \times 10^6\). CNS-derived lymphocytes from individual animals were analyzed in these experiments.

**RNA sequence analysis**

RNA was extracted from infected tissues or tissue culture cells using Tri Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer’s instructions. cDNA was synthesized from \(2\) μg total RNA. For synthesis of PCR products, sets of primers specific for the S protein (nucleotides 1325–1343, GCG CCA TGG ACC CCT CGT CTT GGA ATA G and 2098–2118, GCC TGC AGT TAA CGA TAG AGC AGA GCC GGT TC) or the part of gene 4 encoding epitope gp33 (nucleotides 4060–4082 of the S gene, GTT GTT GTG ATG AGT ATG GAG G and nucleotides 260–278 of gene 4, CCT CTT GAA CTA CCA AGG) were used. The final PCR products were purified using a DNA Gel Extraction kit (Millipore, Bedford, MA) and cloned using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Plasmids were extracted using QIAprep Spin...
Miniprep kit (Qiagen, Valencia, CA) and sequenced at the DNA Sequencing Facility at the University of Iowa (Iowa City, IA).

Northern blot analysis

Total RNA was isolated at 13 h p.i. from BALB/c 17Cl-1 cells infected with JHM at 1 PFU/cell. Northern blot analysis was performed as previously described (25).

Results

Construction and characterization of recombinant JHM expressing the LCMV-derived gp33 epitope

To determine whether the presence of two CD8 T cell epitopes would prevent the development of CTL escape and clinical disease, recombinant JHM able to elicit a CD8 T cell response to the LCMV-specific gp33 epitope was developed. This epitope is both H-2D<sup>b</sup>- and H-2K<sup>b</sup>-restricted, although it binds with greater avidity to the H-2D<sup>b</sup> molecule (19). This epitope was introduced into the N-terminal part of gene 4 of JHM, a gene previously shown to be nonessential for growth in tissue culture or for virulence as measured by LD<sub>50</sub> of mice assays (25, 28) (Fig. 1). A recombinant wild-type virus lacking any inserted sequence was also developed.

We initially developed two recombinants, which differed only in the presence of an SbfI restriction site between genes 3 and 4 (rJ<sup>SbfI</sup>gp33 and rJ<sup>SbfI</sup>gp33, Fig. 1). The presence of this restriction site, initially added to expedite cloning, modified the level of transcription of gene 4, and consequently, the level of gp33 epitope expression. Infection of cells with virus containing this site (rJ<sup>SbfI</sup>gp33) resulted in a 2- to 3-fold increase in levels of RNA 4 when compared with levels observed in cells infected with non-recombinant JHM (Fig. 2). Replacement of the SbfI restriction enzyme site with the sequence naturally present in JHM (rJ<sup>SbfI</sup>gp33) resulted in a reduction in the levels of RNA 4 to those observed in nonrecombinant JHM-infected cells (Fig. 2).

Two additional recombinant viruses, in which the H-2D<sup>b</sup>-binding primary anchor residue of gp33 was mutated (KAVYIFATC), were also constructed (rJ<sup>SbfI</sup>gp33,N37I and rJ<sup>SbfI</sup>gp33,N37I, Fig. 1). Inclusion of the latter epitope served as a control for any untoward effects of the insertion on virus function. Insertion of epitope gp33 or epitope gp33,N37I did not significantly alter virus growth in tissue culture cells or virulence in mice, as measured by LD<sub>50</sub> assays (data not shown).

The epitope-specific primary and memory T cell responses are proportional to the level of epitope expression, within a range of values (29–31). To assess the effect of the level of epitope gp33 expression on CTL escape and clinical disease, we first determined the effect of different levels of RNA 4 on the CD8 T cell response. Six-week-old B6 mice were infected intranasally with 6 x 10<sup>4</sup> PFU recombinant virus, a dosage that resulted in a uniformly fatal encephalitis at 7–8 day p.i. Lymphocytes were harvested from the brains of moribund mice and analyzed directly ex vivo in intracellular IFN-γ assays as described in Materials and Methods. A robust CD8 T cell response to epitope S510 was detected in all mice infected with recombinant virus (Fig. 3 and Table I). We detected epitope gp33-specific CD8 T cells in mice infected with rJ<sup>SbfI</sup>gp33 or rJ<sup>SbfI</sup>gp33,N37I and a background level of responding cells when mice were infected with virus encoding epitope gp33,N37I. Consistent with the different levels of RNA 4 shown in Fig. 2, ~2-fold more epitope gp33-specific CD8 T cells were detected in the CNS of mice infected with virus containing the SbfI restriction site (Table I).

Epitope gp33, but not epitope gp33,N37I, protected mice from clinical disease and CTL escape

Forty to 90% of suckling B6 mice infected with wild-type JHM and nursed by dams immunized to the virus developed a clinically apparent demyelinating encephalomyelitis several weeks after inoculation (3). CTL escape mutants were detected in these mice (9). In the present experiments, suckling mice were infected with recombinant wild-type virus and nursed by dams immunized to JHM to determine whether infection with recombinant virus also resulted in the selection of CTL escape mutants, with the development of clinical disease several weeks after infection. As shown in Table II, 5 of 13 mice developed hindlimb paralysis, a fraction similar to that observed after infection with nonrecombinant parental virus. Next, we determined whether the presence of the gp33 epitope affected the course of this disease. One half of each litter was inoculated with virus expressing epitope gp33 and the other half was inoculated with virus expressing epitope gp33,N37I, to control for variability in the amount of protective Ab transmitted to the suckling mice. Infection with virus expressing wild-type epitope gp33 (rJ<sup>SbfI</sup>gp33 or rJ<sup>SbfI</sup>gp33,N37I) prevented the development of clinical disease in 25 of 26 mice (Table II). Thus, the CD8 T cell response to epitope gp33 after infection with either rJ<sup>SbfI</sup>gp33 or rJ<sup>SbfI</sup>gp33,N37I, although differing in magnitude (Fig. 3), both protected mice from clinical disease. By contrast, the frequency of development of clinical disease in mice infected with rJ<sup>SbfI</sup>gp33,N37I or rJ<sup>SbfI</sup>gp33,N37I was similar to that previously reported for wild-type JHM-infected mice, with 43% of mice developing evidence of hindlimb paralysis at days 19–67 p.i. (Table II). Because the absence of the SbfI restriction site and consequent 2-fold decrease in epitope gp33-specific CD8 T cell response did not affect clinical outcome, results obtained with rJ<sup>SbfI</sup>gp33 and rJ<sup>SbfI</sup>gp33 (dJgp33) or rJ<sup>SbfI</sup>gp33,N37I and rJ<sup>SbfI</sup>gp33,N37I (dJgp33,N37I) were combined in the analyses described below.

To evaluate the effect of epitope gp33 on CTL escape at epitope S510, RNA was harvested from the CNS of infected mice and the regions encompassing each epitope were sequenced. Analysis of symptomatic mice infected with recombinant wild-type JHM revealed the presence of mutations in epitope S510 in all samples.

Table 1. Antigen specificity of CD8 T lymphocytes harvested from CNS of mice with acute encephalitis

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of Mice</th>
<th>S510 (%) ± SE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of S510 (×10&lt;sup&gt;6&lt;/sup&gt;) ± SE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>gp33 (%)</th>
<th>No. of gp33 (×10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>OVA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JHM</td>
<td>8</td>
<td>26.6 ± 4.9</td>
<td>6.0 ± 3.1</td>
<td>2.0 ± 1.1</td>
<td>ND</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>rJ&lt;sup&gt;SbfI&lt;/sup&gt;wt</td>
<td>5</td>
<td>26.2 ± 5.9</td>
<td>6.0 ± 2.4</td>
<td>1.8 ± 0.9</td>
<td>ND</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>rJ&lt;sup&gt;SbfI&lt;/sup&gt;gp33</td>
<td>7</td>
<td>21.1 ± 6.7</td>
<td>6.4 ± 3.3</td>
<td>21.9 ± 4.7</td>
<td>6.7 ± 2.7</td>
<td>3.0 ± 2.0</td>
</tr>
<tr>
<td>rJ&lt;sup&gt;SbfI&lt;/sup&gt;gp33,N37I</td>
<td>7</td>
<td>28.8 ± 7.5</td>
<td>6.5 ± 5.4</td>
<td>11.7 ± 2.9</td>
<td>2.4 ± 1.7</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>rJ&lt;sup&gt;SbfI&lt;/sup&gt;gp33,N37I</td>
<td>6</td>
<td>22.6 ± 6.3</td>
<td>5.2 ± 1.8</td>
<td>1.4 ± 0.6</td>
<td>ND</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td>rJ&lt;sup&gt;SbfI&lt;/sup&gt;gp33,N37I</td>
<td>3</td>
<td>24.9 ± 3.1</td>
<td>3.8 ± 1.2</td>
<td>2.4 ± 1.9</td>
<td>ND</td>
<td>2.3 ± 1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean percentage of CD8 T cells specific for epitopes S510 or gp33, or for irrelevant (OVA 257–264) epitope.

<sup>b</sup> Absolute number of epitope-specific cells (fraction of epitope-specific cells × fraction of CD8 T lymphocytes × total cells/brain).

<sup>c</sup> Not detected (= irrelevant peptide).
(data not shown), as observed in mice infected with nonrecombinant virus (9). Similarly, epitope S510 was mutated in all symptomatic rJ.gp33.N37I-infected mice, with low levels of wild-type sequence still detectable in 3 of 12 animals (Table III). Most of the mutations that we detected in mice infected with rJ.wt or rJ.gp33.N37I were shown previously to significantly reduce recognition by epitope S510-specific CD8 T cells (9, 21). Of note, only sporadic changes consistent with Taq polymerase errors during PCR amplification were detected in the 600 nucleotides flanking epitope S510, and no changes were detected in epitope S598. Large deletions, comprising >100 nucleotides and encompassing epitope S510, were detected in 5 of 13 animals. Virus containing large deletions, which always included epitope S510, were previously detected in B6 mice persistently infected with JHM (11, 32, 33). However, they also occur in persistently infected strains of JHM and may not result from evasion of the CD8 T cell response (12, 14). Whatever their etiology, a consequence of these deletions was elimination of epitope S510 and subsequent CTL response.

Only one of 26 mice infected with rJ.gp33 developed clinical disease. A high titer of infectious virus was cultured from the CNS of this animal and mutations in epitope S510 were detected in the RNA harvested from its spinal cord (Table IV). To investigate the extent to which the response to epitope gp33 prevented mutations at epitope S510, we analyzed 8 asymptomatic rJ.gp33-infected mice at 60–80 day p.i. Infectious virus could not be cultured from the CNS of any of these animals. Wild-type epitope S510 was detected in all mice, but mutations in the epitope were also identified in viral RNA prepared from two mice (Table IV). As before, no consistent changes were noted in the regions flanking the epitope or in epitope S598, showing that the mutations in epitope S510 were not random, but rather driven by the epitope S510-specific CD8 T cell response.

Deletions in epitope gp33 occurred during virus persistence

One explanation for the low level selection of virus mutated in epitope S510 in mice infected with rJ.gp33 was that the sequence encoding epitope gp33 was deleted during the course of persistence. Inactivation of epitope gp33 would make rJ.gp33 effectively equivalent to wild-type virus and might facilitate mutations in the S510 epitope. To examine this possibility, we initially analyzed CNS-derived RNA from the single symptomatic and eight asymptomatic rJ.gp33-infected mice described in Table IV for changes in

### Table III. Sequence analysis of epitope S510 in RNA harvested from spinal cords of symptomatic mice chronically infected with rJ.gp33.N37I

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day p.i.</th>
<th>Titer</th>
<th>Clones</th>
<th>Mutations</th>
<th>Amino Acid Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>107</td>
<td>41</td>
<td>5.2</td>
<td>2/6</td>
<td>1544 (G to A)</td>
</tr>
<tr>
<td>111</td>
<td>48</td>
<td>5.3</td>
<td>Bulk</td>
<td>1517–1631 deleted</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>112</td>
<td>65</td>
<td>n/d</td>
<td>5/6</td>
<td>1534 (C to T)</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>114</td>
<td>35</td>
<td>4.9</td>
<td>Bulk</td>
<td>1447–1657 deleted</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>116</td>
<td>32</td>
<td>3.8</td>
<td>5/5</td>
<td>1541 (A to G)</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>118</td>
<td>47</td>
<td>4.3</td>
<td>Bulk</td>
<td>1498–1653 deleted</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>120</td>
<td>56</td>
<td>2.5</td>
<td>4/4</td>
<td>1537 (T to C)</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>122</td>
<td>67</td>
<td>1.8</td>
<td>8/8</td>
<td>1537 (T to A)</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>211</td>
<td>30</td>
<td>4.7</td>
<td>2/10</td>
<td>1542 (T to A)</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>218</td>
<td>19</td>
<td>4</td>
<td>Bulk</td>
<td>1516–1629 deleted</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>219</td>
<td>39</td>
<td>4.7</td>
<td>Bulk</td>
<td>1530–1617 deleted</td>
<td>CSLWNGPHL</td>
</tr>
<tr>
<td>220</td>
<td>32</td>
<td>3.6</td>
<td>6/8</td>
<td>1543 (G to A)</td>
<td>CSLWNGPHL</td>
</tr>
</tbody>
</table>

**Notes:**
- a Number with hindlimb paralysis.
- b Titters are expressed as log_{10} PFU/g brain.
- c Number of cDNA clones with mutations/total analyzed.
- d Nucleotide change and location within epitope S510 (nucleotides 1528–1554 of S gene).
- e Sequences of bulk PCR products.
- f These deletions encompass epitope S510–518.
- g Not detected (<100 PFU/g brain).
The deletions encompassing epitope gp33 or gp33.N37I were not detected in four mice de
dected in three of 20 mice infected with rJ.gp33.N37I (data not shown). To deter-
tions encompassing epitope gp33.N37I were also detected in 13 of 15 mice infected with rJ.gp33.N37I and harvested at day 20 p.i. or after 10 passages
through tissue culture cells (data not shown).

### Table V. Sequence analysis of epitope gp33 in RNA harvested from mice infected with rJ.gp33 or rJ.gp33.N37I

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day p.i.</th>
<th>Clones</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>rJ.gp33</td>
<td>20</td>
<td>4/5</td>
<td>MAKAVYNFATCALIGP</td>
</tr>
<tr>
<td>335</td>
<td>20</td>
<td>2/5</td>
<td>MAKAVYNFATCALIGP</td>
</tr>
<tr>
<td>336</td>
<td>20</td>
<td>3/5</td>
<td>MAKAVYNFATCALIGP</td>
</tr>
<tr>
<td>337</td>
<td>22</td>
<td>3/4</td>
<td>MAKAVYNFATCALIGP</td>
</tr>
<tr>
<td>rJ.gp33.N37I</td>
<td>22</td>
<td>4/4</td>
<td>MAKAVYNFATCALIGP</td>
</tr>
<tr>
<td>338</td>
<td>22</td>
<td>2/5</td>
<td>MAKAYV f/s</td>
</tr>
<tr>
<td>339</td>
<td>22</td>
<td>1/4</td>
<td>MAKAYV f/s</td>
</tr>
<tr>
<td>340</td>
<td>1/5</td>
<td>MAKAYV f/s</td>
<td></td>
</tr>
</tbody>
</table>

* Amino acid changes within sequence encompassing epitope gp33. Residues comprising epitope gp33 are bolded.

Discussion

Cytotoxic T cell escape mutants have been identified in many chronic viral infections, at both early and late times p.i. Mutations selected at early times p.i. generally occur in the presence of a relatively normal immune response and are likely to be critical in establishing persistence (2–6). In the present study, we manipulated virus-host interactions to examine the mechanism of selection of CTL escape variants and their role in virus persistence as well as disease progression. Our results show that the inclusion of epitope gp33, a CD8 T cell epitope with high functional avidity (19, 34, 35), within the JHM genome prevented the emergence of CTL escape at epitope S510, the persistence of infectious virus, and the development of clinical disease in most mice. The high functional avidity of epitope gp33 was an important factor in its ability to protect mice. The other CD8 T cell epitope, S598, recognized in B6 mice infected with JHM (12) or with rJ.gp33 or rJ.gp33.N37I (data not shown), has much lower functional avidity (12). Consistent with this lower functional avidity, the host response to this epitope did not protect against the selection of CTL escape at epitope S510 and was inefficient in protecting mice from clinical disease or in clearing infectious virus (10). Additionally, mutations in this epitope have never been detected in mice with chronic demyelination, possibly also as a consequence of this lower functional avidity. Alternatively, mutations in epitope S598 may not be selected because they adversely affect virus fitness, thereby minimizing any selective advantage.

Our results also showed that the ability of epitope gp33 to prevent clinical disease largely resulted from enhanced CTL-mediated immune surveillance during the early phase of the infection, since this epitope was usually deleted by 20 day p.i. Previously, we showed that mutations in epitope S510 were detected by 10–12 day p.i., suggesting that this is the critical time period for the selection of CTL escape variants in JHM-infected mice (11). Our data also indicated that the 2-fold lower epitope gp33-specific CD8 T cell response that occurred in response to infection with
rJ−SbfI-gp33, as compared with infection with rJ+SbfI-gp33, was sufficient to protect mice from CTL escape and clinical disease.

CTL escape in epitope S510 still occurred at low levels in the presence of epitope gp33, and in the case of one mouse, was clinically significant (Table IV). In previous studies, 10–60% of suckling mice infected with wild-type JHM cleared infectious virus and did not develop clinical disease at 60 day p.i. When spinal cord RNA from these asymptomatic, wild-type virus-infected mice was analyzed for mutations in epitope S510, point mutations were detected in cDNA clones in two of 14 mice (9, 11). The results from the present study are similar, in that CTL escape was infrequent in mice that remained asymptomatic. These results collectively suggest that if virus is effectively cleared, whether it be by the CD8 T cell response to a second epitope or by other mechanisms in mice infected with wild-type virus, the rate of CTL escape is greatly diminished.

Exactly how CD8 T cell escape occurred in the presence of an immune response directed at two epitopes with high functional avidity is not clear. One possible explanation for these results is that the response to epitopes S510 and gp33 was temporally staggered, as occurs in HIV-infected patients (4, 36). Temporal changes in the fraction of CD8 T cells responding to two different CD8 T cell epitopes were identified in mice persistently infected with JHM (37), although both epitopes were recognized at all times p.i. We have not analyzed the CD8 T cell response in suckling mice at early times after inoculation. However, a large fraction of lymphocytes harvested from the CNS of 5-wk-old-infected B6 mice responded to both epitopes S510 and gp33 at early times p.i. (Table I) making a temporal difference in response unlikely to be a major factor in CTL escape. A second possibility is that the epitope gp33- and S510-specific CD8 T cells were not uniformly distributed throughout the CNS. In this case, virus mutated in a single epitope would have a selective advantage in a microdomain within the CNS. This possibility has not yet been evaluated experimentally.

An alternative explanation for CTL escape in the presence of responses to two CD8 T cell epitopes may relate to a feature observed in mice infected with recombinant viruses, including JHM. In our experiments, sequence encoding the gp33 epitope was introduced into the JHM genome by targeted recombination. The epitope was introduced into gene 4, a gene previously shown not to be essential for growth in tissue culture cells or for the capability to cause acute encephalitis (25, 28). The part of gene 4 containing epitope gp33 was deleted in nearly all virus isolated from B6 mice infected with rJ.gp33 or rJ.gp33,N371. The deletion was driven by a requirement for optimal virus replication in the presence of an intact host immune response because it did not occur in infected RAG1−/− mice. The deletion process was not, however, only mediated by the CD8 T cell-mediated immune response to epitope gp33, because it also occurred in mice infected with rJ.gp33,N371. Epitope gp33 was not recognized in these animals. Another possibility is that the deletion of epitope gp33 or gp33,N371 represented escape from JHM-specific Ab surveillance. Although this would be consistent with deletion of the epitope in infected B6, but not RAG1−/− mice, Ab escape variants were not detected in mice chronically infected with JHM in a previous study (38).

In most mice, all inserted sequence was deleted resulting in reversion to a wild-type gene 4 product. This reversion to wild-type sequence is consistent with a role for the gene 4 product in virus persistence. In some cases, however, the deletions in gene 4 that occurred during persistence resulted in an altered reading frame, suggesting that the gain in virus fitness was partly at the level of RNA structure. Deletion of a foreign epitope is not unique to JHM because an inserted CD8 T cell epitope was also deleted from recombinant Coxackie B virus by day 4 p.i. (39) and from recombinant polioviruses after passage through tissue culture cells (40).

In summary, our results show that a CD8 T cell response directed at more than one epitope with high functional avidity diminishes persistent infection, clinical disease, and CTL escape and provide a strategy for more effective vaccine design. Although epitope gp33 was usually deleted by 20 day p.i., its presence before that time facilitated the development of a protective anti-JHM immune response. Consequently, inclusion of a second CD8 T cell epitope that was not prone to deletion might completely prevent the selection of any CTL escape mutants.

Acknowledgments

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