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*J Immunol* 2000; 165:1146-1152; doi: 10.4049/jimmunol.165.2.1146

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Long Term Persistence of Herpes Simplex Virus-Specific CD8+ CTL in Persons with Frequently Recurring Genital Herpes

Christine M. Posavad,‡‡ Meei Li Huang,* Serge Barcy,* David M. Koelle,*†‡ and Lawrence Corey*†‡

Herpes simplex virus (HSV) establishes a lifelong infection in humans. Reactivation of latent virus occurs intermittently so that the immune system is frequently exposed to viral Ag, providing an opportunity to evaluate memory T cells to a persistent human pathogen. We studied the persistence of genital herpes lesion-derived HSV-specific CD8+ CTL from three immunocompetent individuals with frequently recurring genital HSV-2 infection. All CTL clones were HSV-2 type specific and only one to three unique clonotypes were identified from any single biopsy specimen. The TCRBV genes utilized by these clonotypes were sequenced, and clonotype-specific probes were used to longitudinally track these clonotypes in PBMC and genital lesions. CTL clonotypes were consistently detected in PBMC and lesions for at least 2 and up to 7 years, and identical clonotypes infiltrated herpes lesions spaced as long as 7.5 years apart. Moreover, these clones were functionally lytic in vivo over these time periods. Additionally, CTL clones killed target cells infected with autologous viral isolates obtained 6.5 years after CTL clones were established, suggesting that selective pressure by these CTL did not result in the mutation of CTL epitopes. Thus, HSV recurs in the face of persistent CD8+ CTL with no evidence of clonal exhaustion or mutation of CTL epitopes as mechanisms of viral persistence. The Journal of Immunology, 2000, 165: 1146–1152.

Herpes simplex virus type 2 is the major cause of genital herpes and one of the most prevalent sexually transmitted diseases worldwide. The epidemic of genital herpes continues to increase in the U.S.; ~22% of adults are infected with HSV-2, representing a 31% increase over the last decade (1). After primary infection at mucosal sites, HSV-2 remains latent in neuronal cells with intermittent HSV reactivations, resulting in the production of infectious virus with or without the onset of discernible disease. In immunocompetent persons infected with HSV-2, viral reactivation is high and occurs on an average of 20% of days (2). The high rate of HSV-2 shedding at mucosal sites suggests that the immune system is frequently or even chronically exposed to HSV Ag and likely results in the intermittent restimulation of HSV-specific T cells. Cellular immune defects are more closely associated with severe HSV disease than humoral immune defects, pointing to a critical role of HSV-specific T cells in the control and resolution of HSV disease. High frequencies of HSV-specific CD4+ and CD8+ T cell precursors are present in PBMC from immunocompetent HSV-seropositive individuals (3, 4), and both cell types infiltrate herpetic lesions (5, 6). In cross-sectional studies, immunosuppressed individuals with severe genital herpes infections (frequent and long-lasting lesions) had significantly lower frequencies of HSV-specific CD8+ CTL precursors than did individuals with mild disease (7). Clearance of HSV from lesions has been associated with the development of local HSV-specific cytotoxic activity; much of the cytolytic activity appears to be due to HSV-specific CD8+ CTL (6).

The high frequency of HSV reactivation despite robust CTL activity raises the question as to whether alterations in T cell specificity occur during the course of infection. Are there deletions of HSV-specific clonotypes and generation of new clonotypes over the course of infection? We sequenced the TCRBV genes utilized by HSV-specific CD8+ CTL clones that were isolated from lymphocytes infiltrating herpetic genital lesions (LIL). Clonotype-specific oligonucleotide probes were used to longitudinally track these clonotypes in PBMC and in LIL to determine their longevity and localization.

Materials and Methods

Subjects

Immunocompetent individuals with recurrent genital HSV infections were enrolled in an Institutional Review Board-approved protocol at the University of Washington Viral Disease Research Clinic. Subjects were part of our prospective cohort studies of the natural history of HSV-2 infection. We studied three HIV-seronegative individuals (two female, one male) with culture and serologically proven recurrent HSV-2. All three subjects had clinically symptomatic genital herpes for a mean of 19 years before the study and experienced symptomatic recurrences at a mean of 5 per year, a pattern typical of persons with frequent HSV-2 infection (8). Subjects were selected because their recurrences occurred on the buttocks (two individuals) and the thigh (one individual), sites where subjects would allow multiple biopsies.

Viruses

HSV-1 strain E115 and HSV-2 strain 333 were used throughout unless otherwise noted. HSV-1 × HSV-2 intertypic recombinant viruses (IRV) RH1G7, RS1G25, RS1G31, and RH07 (9, 10) containing 0.30–0.46, 0.59–0.73, 0.67–0.73, and 0.82–1.0 HSV-2 map units, respectively, were the kind gift of Bernard Roizman. HSV-1 × HSV-2 IRV Ds1(32),

‡‡ Address correspondence and reprint requests to Dr. Christine Posavad, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, P.O. Box 19024, Room D3-100, Seattle, WA 98109. E-mail address: posavad@u.washington.edu
The Journal of Immunology

Table I. Characterization of lesion-infiltrating CD8\(^+\) CTL clones\(^a\)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Biopsy Date</th>
<th>Clone</th>
<th>% Specific Lysis Mock</th>
<th>HSV-1</th>
<th>HSV-2</th>
<th>Allo</th>
<th>HLA Restriction(^b)</th>
<th>HSV-2 Map Unit(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/95(^*)</td>
<td>1B.4</td>
<td>7</td>
<td>2</td>
<td>24</td>
<td>1</td>
<td>A24</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1B.5</td>
<td>6</td>
<td>9</td>
<td>27</td>
<td>1</td>
<td>A24</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1B.13</td>
<td>1</td>
<td>0</td>
<td>33</td>
<td>2</td>
<td>A24</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1B.14</td>
<td>4</td>
<td>3</td>
<td>32</td>
<td>2</td>
<td>A24</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1B.18</td>
<td>5</td>
<td>3</td>
<td>21</td>
<td>3</td>
<td>A24</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td>2</td>
<td>10/94</td>
<td>2B.1</td>
<td>–3</td>
<td>–1</td>
<td>46</td>
<td>2</td>
<td>A2</td>
<td>0.67–0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2B.11</td>
<td>0</td>
<td>–1</td>
<td>36</td>
<td>0</td>
<td>A2</td>
<td>0.67–0.73</td>
</tr>
<tr>
<td>3</td>
<td>6/91</td>
<td>3B.4</td>
<td>0</td>
<td>–1</td>
<td>47</td>
<td>–2</td>
<td>B45</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3B.8</td>
<td>3</td>
<td>–1</td>
<td>36</td>
<td>–3</td>
<td>B45</td>
<td>0.0–0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3B.11</td>
<td>–3</td>
<td>1</td>
<td>47</td>
<td>0</td>
<td>A2 or B8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3B.22</td>
<td>–3</td>
<td>0</td>
<td>48</td>
<td>2</td>
<td>A2</td>
<td>0.68–0.70</td>
</tr>
</tbody>
</table>

\(^{a}\) CD8\(^+\) cells were positively selected from PHA-stimulated lesion-derived cells and cloned with PHA, IL-2, and irradiated allogenic PBMC. Cells were screened for HSV-specific CTL activity, and positive clones were expanded with PHA, CTL clones were tested in a \(^{51}\)Cr release assay with autologous or allogenic (HLA class I mismatched) LCL that were mock infected or infected overnight with HSV-1 or HSV-2 using an E:T ratio of 10:1.

\(^{b}\) CTL clones were tested for lytic activity against HSV-2 infected autologous LCL or HSV-2-infected allogenic LCL that were HLA mismatched at one or more HLA class I alleles.

\(^{c}\) HSV-1 × HSV-2 IRV were used to determine the approximate region on the HSV-2 genome recognized by CD8\(^+\) CTL clones.

\(^{d}\) Only 5 of the 14 clones from Pt. 1 are displayed; the additional 9 clones not displayed had killing patterns identical with those of the 5 shown in the table.

Bx1(13), Bx1(24), and RE6 containing 0.68–0.72, 0.0–0.57, and 0.78–1.0, and 0.0–0.18 and 0.72–0.84 HSV-2 map units, respectively, were the kind gift of Howard Marsden (11). Clinical isolates 9349 and 9434 from patient (Pt.) 3 were isolated from a swab of an HSV-2 buttck lesion on December 15, 1997, and December 18, 1997, respectively. All viruses were grown and titered on Vero cells as previously described (12).

Cells

PBMC were isolated by Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ) density centrifugation. EBV-transformed B cell lines (LCL) were established and maintained as previously described (13).

Lesion-derived HSV-specific CD8\(^+\) CTL clones

Lesion-infiltrating T cells were expanded in bulk from vesicle fluid or punch biopsy of HSV-2 culture-positive lesions with 0.8 \(\mu\)g/ml PHA-P (Murex Diagnostics, Dartford, U.K.), 32 U/ml IL-2 (Schlierpeher Biosystems, Columbia, MD), allogenic irradiated PBMC, and 50 \(\mu\)M acyclovir (5). CD8\(^+\) T cells were positively selected using CD8 microbeads and MiniMACS columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. The TCRBV, TCRBD, and TCRBI sequence of each clone was cloned with PHA, IL-2, and irradiated autologous PBMC, screened for HSV-specific CTL activity; and expanded (4).

Cytotoxicity assays

Standard \(^{51}\)Cr release assays were performed (4). Briefly, target LCL (autologous or partially/completely mismatched at HLA class I) were mock infected or infected for 18 h with HSV-1, HSV-2, or a clinical HSV-2 IRV, or a clinical HSV-2 isolate at a multiplicity of infection of 10 in the presence of 100 \(\mu\)Ci \(^{51}\)Cr; washed; and counted. CD8\(^+\) T cells were cloned at 1 cell/well with PHA, IL-2, and irradiated allogenic PBMC, screened for HSV-specific CTL activity; and expanded (4).

DNA extraction, PCR, and sequencing

Total DNA was extracted from CTL clones (1–2 × 10\(^8\)), PBMC (5–10 \(^9\)), or bulk lesion cultures (3–5 × 10\(^9\)) by the single-step guanidinium method (14), and cDNA was synthesized using random hexamer primers, Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD), and dNTPs (Boehringer Mannheim, Indianapolis, IN). To determine the TCRBV usage of individual clones, cDNA was amplified with 1 of 26 TCRBV family-specific 5’-primers, a 3’-primer from the TCRBC region, dNTPs, and Amplitaq (Perkin-Elmer, Norwalk, CT), Primers (Fred Hutchinson Cancer Research Center, Biotechnology Center, Seattle, WA) were designed using primer sequence data from Ref. 15. Products were separated on 2% agarose gels, soaked in ethidium bromide, and visualized by UV illumination. TCRAC 5’- and 3’-primers were used as a positive control. Negative controls included no cDNA and cDNA synthesis mixture with no RNA. PCR conditions consisted of 30 s denaturation at 95°C, 30 s annealing at 55°C, and 1 min extension at 72°C for 35 cycles.

Liquid hybridization

PCR product (7 \(\mu\)l) was mixed with 10 mM Tris (pH 8.0), 280 mM NaCl, 100 \(\mu\)M dNTPs, formamide, and 10\(^6\) cpm \(^{32}\)P-labeled oligonucleotide in a total volume of 25 \(\mu\)l as previously described (16, 17). The amount of formamide used for each probe was adjusted to \(T_m\) 34°C. Mixtures were heated to 97°C for 5 min and cooled to room temperature during 15 min; and 10 \(\mu\)l were electrophoresed in 6% acrylamide gels, dried, and exposed to x-ray film.

Southern blotting

PCR products were separated on a 6% acrylamide gel and transferred to a nitrocellulose membrane. Specific DNA sequences were identified by hybridization with \(^{32}\)P-labeled clone-specific oligonucleotide in a mixture containing 5% SSC, 5% Denhardt’s solution, 1% SDS, and 100 \(\mu\)g/ml salmon sperm DNA. After hybridization at 68°C overnight, membranes were washed using high stringency conditions and exposed to x-ray film.

Results

Characterization of lesion-infiltrating HSV-specific CD8\(^+\) CTL clones

HSV-specific CD8\(^+\) CTL clones were isolated from LIL expanded from three immunocompetent HSV-2 infected individuals. Of the 960 cloning wells, 10, 17, and 20% were positive for growth from Pts. 1, 2, and 3, respectively; of these, 21, 5, and 10%, respectively, demonstrated cytotoxic activity to HSV-2. All of the clones that reacted to HSV-2 were subsequently restimulated and tested for HLA-restricted CTL activity. Table I displays all the lesion-derived CD8\(^+\) CTL clones from each of the three subjects that grew and maintained HSV-specific lytic activity after restimulation. All
of the CD8\(^+\) CTL clones specifically lysed autologous HSV-2-infected LCL and not autologous mock infected LCL or HSV-2-infected allogeneic LCL completely mismatched at the HLA class I locus (Table I). Interestingly, all the clones were HSV-2 type specific in that they lysed autologous HSV-2-infected LCL and not HSV-1-infected LCL (Table I). None of the clones killed the NK-sensitive K562 cells (data not shown), confirming the specificity of these clones.

The HLA class I-restricting element of each clone was determined using a panel of LCL that were completely mismatched or partially matched at one or more class I alleles. Each clone was tested for lytic activity against HSV-2-infected autologous or mismatched LCL (data not shown). The HLA-restricting element of clones 1B.4 and 1B.5 was HLA A24, clone 2B.1, 2B.11 and 3B.22 was HLA A2 and clone 3B.4 and 3B.8 was HLA B45 (Table I). Clone 3B.11 lysed LCL matched at A2 and B8 but not LCL matched at A1 or B45. Clone 3B.11 lost killing activity after two rounds of restimulation; thus, the HLA-restricting allele could not be confirmed to either A2 or B8.

Because all lesion-derived clones were HSV-2 type specific, HSV-1 \(\times\) HSV-2 IRV were utilized to define the antigenic specificity more closely. Clones 1B.4, 1B.5, 3B.4, and 3B.8 recognized an HSV-2 epitope contained within 0.0–0.18 map unit (Table I). Clones 2B.1 and 2B.11 recognized an epitope contained within 0.67–0.73 map unit, and clone 3B.22 recognized an epitope contained within 0.68–0.70 map unit. The region on the HSV-2 genome recognized by 3B.11 could not be determined because the clone lost killing activity after two rounds of restimulation. These data indicate that the lesion-derived CD8\(^+\) T cell response among these subjects with frequently recurring HSV-2 appears, at least by these methods, to be narrowly focused.

**TCRBV usage of lesion-derived HSV-specific CD8\(^+\) CTL clones**

RT-PCR using 26 TCRBV family-specific primers was performed on the lesion-derived CD8\(^+\) CTL clones followed by DNA sequencing of the TCRBV, TCRBD, and TCRBJ regions. For each of the clones, a single dominant band was visualized on ethidium bromide-stained agarose gels with only 1 of the 26 TCRBV family-specific primers, a pattern confirming their clonality. All 14 of the clones isolated from Pt.1 utilized TCRBV12J1S1I2 genes, and the TCRBV, TCRBD, and TCRBJ regions were identical at the nucleotide level (Table II). This suggests that these clones were the progeny of a single progenitor clone, although whether this clone was expanded within the lesion in vivo or by in vitro stimulation is unknown. Lesion-derived clones from Pt.3 (3B.4 and 3B.8) were also identical at the nucleotide level, and each utilized TCRBV17J1S5 genes. Clone 2B.1 and 2B.11 from Pt.2 were sister clones and utilized TCRBV10J2S4 genes, whereas clones 3B.11 and 3B.22 utilized TCRBV5S2J2S2 and TCRBV21J2S3, respectively (Table II). Thus, only Pt.3 demonstrated evidence of more than one unique lesion-derived CD8\(^+\) T cell clonotype from their original biopsy specimen.

**Detection of HSV-specific CD8\(^+\) CTL clones by RT-PCR**

Liquid hybridization was used to detect the presence of CTL clones in PBMC and LIL. \(^{35}\)P-labeled clonotype-specific oligonucleotide probes (15-mers) spanning the CRD3 region were used (Table II). The concentration of formamide used in each hybridization reaction reduced the probe \(T_m\) to 34°C. Therefore, the stringency and specificity of each hybridization reaction were very high. Any single-base pair mismatch in the probe region would reduce the \(T_m\) to \(~67°C\). To test the specificity of this technique, PCR products from four lesion-derived HSV-specific TCRBV12-expressing T cells clones from Pt.7 (an unrelated HSV-2-seropositive individual) were mixed with the 1B.5-specific oligonucleotide. Clones from Pt.7 were lesion-derived CD4\(^+\) HSV-specific T cell clones that differed by 7 or 8 nucleotides at complementarity-determining region 3 (Fig. 1A). The 1B.5 oligonucleotide bound specifically to the PCR product from clone 1B.5 and did not hybridize to the PCR products from the TCRBV12-expressing clones from Pt.7 (Fig. 1B). PCR product from all clones could be visualized by ethidium bromide staining (data not shown), demonstrating that a negative signal in the liquid hybridization reaction was not due to lack of PCR product. Thus, even with \(>50\%\) homology over a 15-nucleotide region, no cross-hybridization occurred.

To determine the sensitivity of the liquid hybridization protocol for detecting HSV-specific CD8\(^+\) CTL clones, RNA was isolated from 1000 1B.5 cells, and serial 10-fold dilutions were mixed with RNA from \(3 \times 10^6\) CD8\(^+\) cells from an unrelated HSV-seropositive individual. cDNA was amplified with the TCRBV12 primer, and liquid hybridization was performed with the 1B.5-specific oligonucleotide. The clonotype-specific oligonucleotide specifically bound to the PCR product from clone 1B.5 but not to the PCR product from the control CD8\(^+\) cells amplified with the TCRBV12.
primer (Fig. 1C). A positive signal could be detected with 1 cell RNA equivalent in a background of RNA from cells not expressing the particular clonotype. Thus, this technique detected the RNA from 1 cell expressing a particular TCRBV clonotype.

**HSV-specific CD8⁺ CTL clones in PBMC**

To determine whether lesion-derived HSV-specific CD8⁺ CTL clones were present and persisted in PBMC from the same individual, we purified RNA from CD8⁺ T cells from PBMC obtained at the time of initial biopsy (if available) and prospectively over time. For these studies, we utilized RNA from 5–10⁵ uninfected PBMC from Pt. 3 and from all subsequent time points (up to 4.75 years later). Pt. 3 agreed to have biopsies of HSV lesion biopsies from recurrences spaced 6.5 (December 1997) and 7.5 (November 1998) years after the clone was initially isolated (May 1998) (Fig. 3, A and C). Thus, this clonotype was present simultaneously in a genital HSV lesion and in PBMC. Moreover, we could detect the persistence of this clone in PBMC for at least 2 years. Similar results were obtained with Pt. 2; clones 2B.1 and 2B.11 were detected in PBMC at the time the clone was isolated from the lesion (October 1994) and from all subsequent time points (n = 3) (up to 4.75 years later) but not in PBMC from an unrelated HSV-seropositive individual (Fig. 1B). Thus, both techniques generated identical results.

Each of the three unique clonotypes isolated from Pt. 3 were also found in PBMC. No PBMC were available from the time the clone was isolated from a lesion in June 1991. Clones 3B.4 and 3B.8 were detected in PBMC 2 years after they were originally isolated but not detected in PBMC 7 years later (Fig. 3A). In contrast, clones 3B.11 and 3B.22 were detected in PBMC obtained 7 years after the clone was initially isolated (May 1998) (Fig. 3, B and C).

**HSV-specific CD8⁺ CTL clones infiltrate subsequent lesions**

Once we developed the techniques to detect unique clonotypes in lesions, we sought to determine whether the same clone infiltrated subsequent herpetic lesions. For these studies, RT-PCR was performed on RNA from LIL isolated from different episodes of HSV-2 reactivation. Pt. 2 agreed to undergo two additional genital lesion biopsies from recurrences 1 and 4.75 years after initial enrollment. Clone 2B.1 was detected in the lesion from which it was isolated (October 5, 1994, and October 7, 1994), from a lesion 1 year later on October 20, 1995 (Fig. 2C) and from a lesion 4.75 years later (data not shown). Pt. 3 agreed to have biopsies of HSV recurrences 6.5 and 7.5 years postenrollment. Clone 3B.4, isolated from a genital lesion in June 1991, was detected in two subsequent lesions 6.5 (December 1997) and 7.5 (November 1998) years after the clone was initially isolated but not during a recurrence on 10/98 (Fig. 3A). Clone 3B.11, also isolated from a lesion in June 1991, was detected in biopsies from October 1998 and November 1998 (Fig. 2B), and 3B.22 was detected in the November 1998 biopsy (Fig. 3C). Fig. 3D summarizes the detection of clones from Pt. 3 in PBMC and lesions. Therefore, lesion-derived HSV-specific CD8⁺ CTL clones could infiltrate HSV recurrences spaced >7 years apart. Pt. 1 did not agree to undergo further biopsies, precluding study of local long term memory.
To determine whether HSV-specific CD8+ CTL clones to the viral isolate obtained from a buttock recurrence of Pt. 3, 6.5 years after the CTL clones were initially obtained. Two separate clinical isolates of HSV-2 obtained from lesion swabs on December 15, 1997 (strain 9349), and December 18, 1997 (strain 9344), were harvested and used to infect autologous LCL. CTL clones 3B.8 and 3B.22, isolated in June 1991, were tested for lytic activity against autologous LCL infected with these 1997 viruses. Both clones lysed autologous LCL and LCL matched at the appropriate HLA-restricting allele (B45 for 3B.8 and A2 for 3B.22) infected with either clinical isolate (Fig. 4). This demonstrates that in an individual with frequently recurring HSV disease, no detectable mutations in viral genes to which CD8+ T cells were directed could be identified.

**Discussion**

HSV as a recurrent mucocutaneous infection offers an opportunity to study in vivo survival and persistence of memory T cell responses to Ags that appear not to undergo antigenic variation. We developed several sensitive techniques to define and follow HSV-specific CD8+ CTL clonotypes. We demonstrated that individual clonotypes were consistently detected in PBMC for as long as 7 years. Most fascinating was the finding that identical clonotypes infiltrated herpetic lesions spaced as long as 7.5 years apart and that these clonotypes retained cytotoxic function and recognized and killed autologous clinical HSV-2 isolates. HSV-specific CD8+ CTL clonotypes were not compartmentalized to the skin but were found simultaneously in the skin and in peripheral blood.

One of the intriguing aspects of our study was the limited number of unique CD8+ memory T cell clonotypes we were able to derive from LIL and the fact that all the clones were specific for the infecting strain of virus, HSV-2, and were not cross-reactive with HSV-1. We may have skewed our results by the timing of biopsies in that HSV-specific CD8+ T cells infiltrating at times postbiopsy would not be expanded. Although this is a possibility, we did perform multiple biopsies at different stages and time points during lesion evolution. In each of these cases, consistency in the TCRBV usage in LIL was noted. Unfortunately, the total number of T cells that can be isolated from a single biopsy is extremely low, precluding direct analysis of T cells from lesions without expansion. Thus, it is possible that the clones we identified possess a unique stability compared with other clones. Highly restricted TCRBV usage has been observed in lymph nodes draining HSV infection in the murine model, providing further support of a restricted CD8+ T cell response to HSV; the vast majority of T cells recognize a single epitope of glycoprotein B (19, 20). Our data suggest that HSV-specific CD8+ T cell responses appear highly restricted in nature in terms of both TCRBV usage and immunogenic viral epitopes. We studied only persons with frequently recurring HSV, and it is possible that persons with infrequent disease have a more diverse response.

Immune evasion strategies used by HSV are myriad and include latency in immunologically privileged neurons, down-regulation of HLA class I (21–23), infection and inactivation of immune effector cells (13, 24–26), and inhibition of complement- and Ab-mediated viral neutralization (reviewed in Ref. 27). Selection of viral mutants that can escape immune surveillance has not been thought to be a mechanism of viral immune evasion utilized by herpesviruses due to the high fidelity of DNA polymerase. However, an example of viral mutation of a CTL epitope in a herpesvirus, namely EBV, has been described (28) although this mechanism does not appear to be universal (29). Few studies have performed detailed sequence analyses of sequential HSV isolates.
from individuals over time; however, “hot spots” for genetic change in the thymidine kinase gene have been described under drug selection (30). Our study is the first to use in vivo selection of T cell sequences as a mechanism to probe genetic changes in the virus. Our novel albeit limited look at this issue suggests no evidence of viral escape variants. The clinical isolates used to infect autologous target cells were recognized and killed by the CD8\(^+\) CTL clones, which is consistent with no mutation(s) of HSV-2 in

![Image 66x415 to 520x734]

**FIGURE 3.** Long term persistence of CD8\(^+\) CTL clones from Pt. 3 in PBMC and in temporally spaced lesions. A, TCRBV17 transcripts were amplified by PCR using cDNA from the following sources: clone 3B.4 (isolated in June 1991); PBMC from Pt. 3 isolated in August 1993 and May 1998 (2 and 7 years after the clone was initially isolated); and from LIL isolated in June 1991 (lesion from which 3B.4 was isolated), December 1997, October 1998, and November 1998. Liquid hybridization of PCR products with the 3B.4/8-specific oligonucleotide probe was used to detect the presence of this clone. —, no cDNA control. B, TCRBV5S2 transcripts were amplified by PCR using cDNA from clone 3B.11; PBMC from Pt. 3 isolated in August 1993, March 1998, and May 1998; and from LIL from June 1991, October 1998, and November 1998. Bottom panel, longer exposure to show the detection of weaker bands. Liquid hybridization was performed with the 3B.11-specific probe. C, TCRBV21 transcripts were amplified by PCR using cDNA from 3B.22, PBMC from Pt. 3 isolated on August 1993, July 1998, and May 1998 and from LIL from June 1991, December 1997, October 1998, and November 1998. Liquid hybridization was performed with the 3B.22-specific probe. D, Summary of clones detected in PBMC and lesions. E, Clonotype 3B.4/8 retains cytolytic function after 6.5 years. Clone 3B.4 isolated from a lesion in June 1991 and clone 3B.51 isolated from a lesion in December 1997 were tested for killing against autologous LCL that were mock infected or infected with HSV-1, HSV-2, or RE6 or with HSV-2-infected LCL that were completely mismatched at the HLA class I locus (allo) or matched at HLA-B45. Data represent the mean of triplicate wells at E:T 10:1.

![Image 98x77 to 488x209]

**FIGURE 4.** HSV-specific CD8\(^+\) CTL clones lyse targets infected with autologous clinical HSV-2 isolates: no evidence of CTL escape variants. Clones 3B.8 (HLA B45-restricted) and 3B.22 (HLA A2-restricted), isolated from a lesion in June 1991, were tested for lytic activity against autologous LCL (auto), LCL matched at HLA-A2 or B45, or LCL mismatched at all HLA class I loci (allo). LCL were mock infected or infected with HSV-1, HSV-2, or HSV-2 strains 9349 and 9434. HSV-2 strains 9349 and 9434 were isolated from Pt. 3 on December 15, 1997, and December 18, 1997, respectively. E:T ratio was 10:1.
the genes providing the CTL epitopes. Confirmation of this awaits the identification of the epitope and sequencing of HSV-2 corre- sponding to this region. However, this does not exclude the pos- sibility that other epitopes had succumbed to immune pressure but that changes were not detected based on our experimental design. Clonal exhaustion also does not appear to promote viral persistence to this human pathogen in immunocompetent adults. Even in the pres- ence of frequent Ag exposure, shown in certain infections to cause clonal exhaustion or deletion of Ag-specific T cells (reviewed in Ref. 31), all the HSV-specific CD8+ CTL clones that we isolated from our three patients with frequently recurring HSV disease persisted for long periods of time and retained cytolytic function.

Our findings raise several questions related to the complex in- teraction between the virus and host. Is it the person with fre- quently recurring disease who has a restricted response whereas those with less frequent disease display a broader response? T cell diversity is likely to be an important component in the establish- ment and maintenance of a balanced host-virus coexistence. In HIV infection, another persistent human infection, polyclonal HIV-specific CD8+ T cell responses have been associated with better clinical status (32, 33). Do quantitative differences in mem- ory T cell responses make a difference in disease severity in im- munocompetent persons? In immunosuppressed persons, severe HSV disease was more closely correlated with low to undetectable numbers of HSV-specific CD8+ memory CTL (7). In the present report, we studied only people with longstanding infection; do the clones isolated at later stages of disease represent clones with more or less avidity/efficacy in vivo than clones isolated earlier in the disease process? Perhaps the major implication of our work is the development of a reproducible tracking assay to define the persis- tence and localization of a clonotype over time. With the elucida- tion of specific epitopes recognized by HSV-specific CD8+ CTL, use of the tetramer binding technology (34) will likely be helpful in defining more of the quantitative aspects of viral-host interac- tions for HSV. However, the novel techniques we outline here provide a useful model for studying the immunobiology of her- pesviruses, viruses that persist and in which antigenic variation is a major mechanism for evading the immune system. In all cases, it is likely that other epitopes had succumbed to immune pressure but that changes were not detected.

References