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

*J Immunol* 2000; 165:1146-1152; doi: 10.4049/jimmunol.165.2.1146
http://www.jimmunol.org/content/165/2/1146

**References**
This article cites 34 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/165/2/1146.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Long Term Persistence of Herpes Simplex Virus-Specific CD8⁺ CTL in Persons with Frequently Recurring Genital Herpes¹

Christine M. Posavad,²*‡ 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 clones 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).³ 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 R7015 (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), 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 R7015 (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), ³ Abbreviations used in this paper: LIL, lesion-infiltrating lymphocytes; LCL, lymphoblastoid cell line; IRV, HSV-1 × HSV-2 intertypic recombinant virus; Pt., patient.
Lesion-derived HSV-specific CD8+ CTL clones

Lesion-infiltrating T cells were expanded in bulk from vesicle fluid or culture supernatant using PHA, IL-2, and irradiated autologous PBMC. CTL clones were positively selected from PHA-stimulated lesion-derived cells, and cloned with PHA, IL-2, and irradiated allogeneic PBMC. All CTL clones were tested for lytic activity against HSV-2 infected autologous LCL or HSV-2-infected allogeneic LCL that were HLA mismatched at one or more HLA class I alleles.

Table I. Characterization of lesion-infiltrating CD8+ CTL clones

<table>
<thead>
<tr>
<th>Subject</th>
<th>Biopsy Date</th>
<th>Clone</th>
<th>Mock</th>
<th>HSV-1</th>
<th>HSV-2</th>
<th>Allo</th>
<th>HLA Restriction</th>
<th>HSV-2 Map Unit</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>

* CD8+ cells were positively selected from PHA-stimulated lesion-derived cells and cloned with PHA, IL-2, and irradiated allogeneic PBMC. Cells were screened for HSV-specific CTL activity, and positive clones were expanded with PHA. CTL clones were tested in a 51Cr release assay with autologous or allogeneic (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.

** Liquid hybridization **

PCR (product (7 μl) was mixed with 10 mM Tris (pH 8.0), 280 mM NaCl, 100 μM dNTPs, formamide, and 10 μl 32P-labeled oligonucleotide in a total volume of 25 μl as previously described (16, 17). The amount of formamide used for each probe was adjusted to Tm of 34°C. Mixtures were heated to 97°C for 5 min and cooled to room temperature during 15 min; and 10 μl were electrophoresed in 6% acrylamide gels, dried, 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. 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 × 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.

### 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. 32P-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 Tm 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 not yield a positive signal in the liquid hybridization reaction. Thus, only Pt. 3 demonstrated evidence of more than one unique lesion-derived CD8+ T cell clonotype from their original biopsy specimen.

### Clonotype-specific oligonucleotide probes

Oligonucleotide probes were designed to recognize 15 nucleotides of the CD8+ T cell clonotype from their original biopsy specimen. The probes utilized were designed to recognize the clonotype-specific region of the TCRBV3 gene, and specifically, the 3′-CDR3 region.

### 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. 32P-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 Tm 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 not yield a positive signal in the liquid hybridization reaction. Thus, only Pt. 3 demonstrated evidence of more than one unique lesion-derived CD8+ T cell clonotype from their original biopsy specimen.
For these studies, we utilized RNA from 5–10 cells from an unrelated HSV-seropositive donor (Fig. 1, A) and prospective over 2 years (February 1997), but not in PBMC-derived CD8 T cells from Pt. 3 in PBMC and lesions. Therefore, lesion-derived HSV-specific CD8+ T cells from Pt. 3 could infiltrate HSV recurrences spaced 6.5 and 7.5 years after the clone was initially isolated but not during a recurrence more than 7 years later (data not shown). Pt. 3 agreed to have biopsies of HSV lesions 6.5 (December 1997) and 7.5 (November 1998) years after enrollment. Clone 2B.1 was detected in the lesion from which it was isolated (October 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 and 7.5 years after the clone was initially isolated (May 1998) (Fig. 3, B and C).

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 October 9/8 (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.

### 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 5–10 unstimulated PBMC or CD8-selected PBMC. Liquid hybridization was performed using the clonotype-specific probe. A representative experiment on Pt. 1 is shown in Fig. 1. A positive signal was detected in PBMC-derived CD8+ T cells isolated at the time of the lesion from which clone 1B.4/5 was isolated (February 1995), 1 mo later (March 1995), 6 mo later (August 1995), and 2 years later (February 1997), but not in PBMC-derived CD8+ T cells from an unrelated HSV-seropositive donor (Fig. 1, D and E). 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-2-seropositive individual (Fig. 2A). We compared the results with liquid hybridization protocol to that of Southern blotting, an alternate technique used to longitudinally track CTL clonotypes (18). cDNA was amplified with the TCRBV10 family-specific primer as in Fig. 2A, and products were eluted on an acrylamide gel, transferred to nitrocellulose, and probed with γ32P-labeled 2B.1 clonotype-specific oligonucleotide. As in Fig. 2A, 2B.1 was detected in PBMC from Pt. 2 from all time points tested over 28 mo 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).
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 9343), 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 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 postsbiopsy 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 antibody-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.

**HSV-specific clonotypes retain cytotoxic function**

To determine whether the clones isolated over time retained cytolytic function, CD8\(^+\) T cell clones isolated from LIL from Pt. 3 in December 1997 were expanded and tested for CTL function. One of the CD8\(^+\) CTL clones that was isolated was HSV-2 type specific and restricted to HLA-B45 (Fig. 3E). With the use of the HSV-1 × HSV-2 IRV, this clone, termed 3B.51, was specific for an HSV-2 epitope contained within the region 0.0–0.18, the same region as recognized by the earlier clones 3B.4 and 3B.8. RNA from clone 3B.51 was isolated, and RT-PCR and liquid hybridization performed with the 3B.4 clonotype-specific oligonucleotide. The 3B.4 oligonucleotide hybridized with the PCR product from clone 3B.51 amplified with the TCRBV17 primer (Fig. 3A). Sequencing of this PCR product confirmed that 3B.4 and 3B.51 were identical at the nucleotide level (data not shown), proving definitively that this clonotype persisted for 7.5 years and retained cytotoxic activity.

**Lesion-derived HSV-specific CD8\(^+\) CTL clones lyse autologous HSV isolates obtained 6.5 years later**

To determine whether HSV-specific CD8\(^+\) CTL clones exerted selective pressure on HSV-2 resulting in the mutation of CTL epitopes, we evaluated the lytic activity of 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 9343), 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 an individual with frequently recurring HSV disease, no detectable mutations in viral genes to which CD8\(^+\) T cells were directed could be identified.

**FIGURE 2.** Long term persistence of 2B.1 in PBMC and presence of this clonotype in temporally spaced lesions. A, TCRBV10 transcripts were amplified by PCR using cDNA from 2B.1, Pt. 2 PBMC isolated at the time of the lesion from which these clones were originally isolated (October 1994), 16 mo (February 1996), 28 mo (February 1997), and 4.75 years (July 1999) post-lesion onset and from PBMC from an HSV-seropositive unrelated individual (ctrl PBMC). Liquid hybridization of PCR products with the 2B.1-specific oligonucleotide probe was used to detect the presence of this clone. —, no cDNA control. B, Southern blot analysis. PCR products amplified with TCRBV10 primers were eluted on a 6% acrylamide gel, transferred to a nitrocellulose membrane, and probed with the 2B.1-specific oligonucleotide. Lanes as in A, C, TCRBV10 transcripts were amplified from 2B.1, from LIL isolated and expanded from HSV buttock lesions from Pt. 2 from October 5, 1994, and October 7, 1994, on clinical lesion days 2 and 4 and from a buttock lesion occurring 1 year later (October 20, 1995). 2B.1 was originally isolated from the October 7, 1994, biopsy. Liquid hybridization of PCR products with the 2B.1-specific probe was used to detect the presence of this clonotype. —, no cDNA control.

**Lesion-derived HSV-specific CD8\(^+\) CTL clones lyse autologous HSV isolates obtained 6.5 years later**

To determine whether HSV-specific CD8\(^+\) CTL clones exerted selective pressure on HSV-2 resulting in the mutation of CTL
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

---

**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.

---

**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 cor-
responding 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 pre-
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
two 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
HSV-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-
perviruses, viruses that persist and in which antigenic variation is
not a recognized aspect of their life cycle.

Acknowledgments
We thank Dawn Mueller, Scott Whitlow, Heather Parker, Matthew John-
son, Minjun Chung, and Tony Marquardt for technical assistance; Stephen
Polyak for technical advice and many helpful discussions; and Mike Rem-
nington, Mary Shaughnessy, and Gail Barnum for specimen collection.

References
1. Fleming, D. T., G. M. McQuillan, R. E. Johnson, A. J. Nahmias, S. O. Aral,
F. K. Lee, and M. E. S. Louris. 1997. Herpes simplex virus type 2 in the United
genital herpes simplex virus 2 shedding in immunocompetent women: effect of
plex virus type 1 is mediated by CD4+ and CD8+ T cells and is restricted to the DR
4. Posavad, C. M., M. Koele, and L. Corey. 1996. High frequency of CD8+cyto-
toxic T-lymphocyte precursors specific for herpes simplex viruses in persons
with genital herpes. J. Virol. 70:8165.
recovery of herpes simplex virus (HSV)-specific T lymphocyte clones from re-
L. Corey. 1998. Clearance of HSV-2 from recurrent genital lesions correlates with
genital herpes infections in HIV-infected individuals with impaired HSV-specific
herpes simplex virus infection decreases in frequency over time. Ann. Intern.
Med. 131:14.