Persistence of Herpes Simplex Virus Type 2 VP16-Specific CD4⁺ T Cells

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ABSTRACT: Patients with genital herpes have frequent viral reactivations. The repeated antigenic rechallenges can modulate the CD4⁺ memory T-cell repertoires during the course of infection. In this study, the CD4⁺ T-cell responses against the herpes simplex virus type 2 (HSV-2) tegument protein VP16 were studied in two HSV-2–infected subjects at two different time points that spanned a 5-year period. Although the VP16-specific T cells did exhibit variation of T-cell receptor Vβ usages at the two time points, T cells that used identical Vβ and CDR3 junction sequences were also observed at the two time points. These experiments demonstrate that the CD4⁺ T cells that are directed against HSV-2 VP16 protein in chronically infected individuals are oligoclonal and that T cells of specific clonotypes can be maintained throughout the course of the disease. Human Immunology 66, 777–787 (2005). © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: HLA tetramers; CD4⁺; herpes simplex; persistent infection; memory T cells

ABBREVIATIONS
FITC fluorescein isothiocyanate
HLA human leukocyte antigen
HSV-2 herpes simplex virus type 2

INTRODUCTION
Herpes simplex virus type 2 (HSV-2) is the major cause of genital herpes. It is estimated that 20% of the U.S. population over 16 years of age is infected with HSV-2 [1]. After initial acute viral infection, the viruses remain latent in the nerve ganglion. HSV reactivation is common, and shedding occurs in the anogenital region in 28% of days sampled among healthy seropositive individuals [2, 3]. Viral reactivation can be either symptomatic or asymptomatic [4, 5]. Symptoms tend to lessen over time [6]. It is not known whether this is the result of immunologic maturation or determinant spreading, or whether it is related to ganglionic events. The intermittent reactivation of HSV-2 viral infection provides a unique situation to examine how repeated antigenic challenges shaped the memory T-cell repertoires.

As a consequence of the life cycle of the virus, the immune system is exposed to the virus periodically throughout the lifetime of the infected individual. HSV-2–specific cytotoxic lymphocytes are critical for viral clearance. Both CD4⁺ and CD8⁺ T cells specific for HSV-2 are found to have cytolytic activity [7, 8]. The ICP47 gene encoded by the HSV-2 virus can downregulate class I expression by inhibition of the peptide transporter TAP [9]. The class I downregulation provides a mechanism by which the viral infected cells can evade the immune surveillance of HSV-specific CD8⁺ T cells in the early phase of viral infection. Production of interferon gamma by CD4⁺ and natural killer cells will eventually upregulate class I expression on an HSV-infected cell and will render the infected cell susceptible to CD8⁺ T-cell lysis. The potential evasion of CD8⁺ T cells during the first few days of viral infection, together with the cytolytic and helper activity of CD4⁺ T cells in priming and maintaining CD8⁺ T cells, underscores the role of HSV-2–specific CD4⁺ T cells in the control of HSV-2 infection.

The frequent recurrent activation of HSV-2 raises the question whether T cells that are directed against HSV-2
antigens are of different clonotypes during the course of the infection. It is also possible that different epitopes may be recognized during the course of the disease. A recent study indicated that HSV-2–specific CD8+ CTL clonotypes could persist for a long period of time [10]. However, the methodology used in the study did not evaluate the prevalence of these persistent CD8+ T cells. The maintenance of the dominant epitope during that period was also not addressed. The current study focuses on the maintenance of HSV-2–specific CD4+ T cells in peripheral blood. The CD4+ T-cell responses toward the HSV-2 tegument protein VP16 in two HSV-2–infected subjects at two time points 5 years apart were examined. The class II–restricted immunodominant epitopes for VP16 protein, the Vβ usages of the CD4+ T cells that are directed against the VP16 protein, and the clonotypes of these T cells were examined at these two time points. There was no significant change in the T-cell repertoires that were directed against VP16 protein over the 5-year period in which the peripheral blood mononuclear cells (PBMCs) were obtained. T cells of identical clonotypes were observed at both time points with similar frequency. The life cycle of HSV-2 virus in maintaining the CD4+ T-cell responses and the implication of the current results on the differentiation of CD4+ memory T cells are discussed.

MATERIALS AND METHODS

Donor Samples

Subjects 5101 and 5607 were seronegative for human immunodeficiency virus type 1, seropositive for HSV-2, and were neither experiencing symptomatic HSV infection nor receiving suppressive antiviral medication at the time of blood collection. Blood samples that were collected at two time points that span a period of 5 years were used in this study. For subject 5101, the average symptomatic recurrent rate was five times per year, whereas 5607 had no symptomatic recurrence during the study period. The possibility that the subjects were reinoculated with their original infecting strain or reinfected with a second strain of HSV-2 during the study period cannot be completely ruled out. Subject 5101 was engaged in a mutually monogamous relationship with a partner who was asymptomatic during the study period. Thus, it is unlikely that exogenous reinfection of HSV-2 occurred for subject 5101. Human leukocyte antigen (HLA) typing was performed by genotypic or serologic methods. Subject 5101 had the DQB1*0602 (DQ0602) allele and subject 5607 had the DRB1*0404 (DR0404) allele. Protocols were approved by the institutional review board, and informed consent was obtained from the subjects. PBMCs were cryopreserved.

Tetramer Guided Epitope Mapping

A detailed description of the generation of tetrarmers and the tetramer guided epitope mapping (TGEM) approach has been previously described [11]. Briefly, PBMCs from HSV-2–positive subject were stimulated with VP16 protein at 2 μg/ml (a gift from the Chiron Corporation). Cells were fed every other day and were stained with pooled tetrarmers on day 12. For the generation of pooled tetrarmers, a panel of 60 overlapping VP16 peptides, termed p3 to p62, were used. These peptides were 20 amino acids in length and corresponded to the entire predicted VP16 protein, with a 12 amino acid overlap between adjacent peptides. Peptides were synthesized by Mimotopes (Clayton, Australia) and were dissolved in DMSO. The peptides were divided into 12 pools, each containing five different overlapping peptides. Pooled peptides were loaded onto biotinylated class II molecules to generate phcoerythrin conjugated tetramer pools. Tetramer pools that gave positive staining were identified, and single peptides from the positive pool were used to generate single peptide tetrarmers. Analyses with single tetrarmers were carried out on day 15.

T-Cell Receptor Vβ Usages of VP16 T Cells

A panel of 20 fluorescein isothiocyanate (FITC)-conjugated T-cell receptor (TCR) Vβ Abs were used to examine Vβ usages of the tetramer-positive T cells. Vβ 5.3 and Vβ 6.7 antibodies were obtained from Endogen (Woburn, MA). The other Vβ antibodies were obtained from Beckman Coulter (Miami, FL). PBMCs were stimulated with the VP16 peptides of interest. Fourteen to 17 days later, cells were first stained with the corresponding tetramers for 40 minutes at 37°C, peridinin chlorophyll protein–conjugated CD4 Ab (Beckman Coulter, Miami, FL); FITC-conjugated Vβ antibodies were added, and the incubation was carried out at 4°C for an additional 15 minutes. Cells were washed and analyzed by BD FACSCalibur (Becton Dickinson, San Jose, CA). For flow cytometry, cells were gated on forward and side scatterings, and at least 20,000 gated events were analyzed.

Sequencing of the TCR Vβ Chain

PBMCs from HSV-2–positive individuals were obtained from two different time points. PBMCs from subject 5101 were stimulated with 10 μg/ml of the VP16 369–379 peptide, and PBMCs from subject 5607 were stimulated with the VP16 443–455 (p58) peptide. On day 14, cells from subject 5101 were stained with DQ0602/VP16 369–379 tetramer and anti-TCR Vβ (either Vβ 2 or Vβ 6.7) Abs; cells from subject 5607 were stained with DR0404/P58 tetramer and anti-TCR Vβ (either Vβ 2 or Vβ 5.3) Abs. Cells found to be double positive for the anti-TCR Vβ Ab and the tetramer were single cell sorted.
RESULTS

Immunodominant Epitopes of VP16 Protein Restricted by DR0404

The TGEM approach was used to compare the DR0404-restricted CD4+ T-cell responses directed against the VP16 protein from peripheral blood samples of a DR0404 HSV-2–infected subject 5607 collected 5 years apart. In the TGEM approach, overlapping peptides corresponding to the entire VP16 protein were synthesized. These peptides were subsequently divided into 12 different pools. Tetramers were loaded with the different peptide pools and assembled. PBMCs from the DR0404 subject were stimulated with the VP16 protein and stained with the pooled tetramers 12 days later. The dominant antigenic epitope was identified to be located in the pool of 12-peptide mixtures for both samples (Figure 1). Tetramers were generated from individual peptides of pool 12, and the staining was repeated with the single-peptide-loaded tetramers. Tetramers generated by individual peptides from pool 12 indicated that the major epitope was located at VP16 443–455 (p58) of both samples (Figure 2). The result indicated that the major DR0404 restricted epitopes of VP16 was confined to the VP16 443–455 region, and no further determinant spreading was observed during the 5-year time span.

Vβ Usages of DR0404 VP16 Specific T Cells

The Vβ usages of VP16-specific T cells were assayed by staining for DR0404/p58 tetramers in conjunction with a panel of TCR Vβ antibodies. The results are illustrated in Figures 3 and 4. Tetramer-positive T cells with Vβ 2 and Vβ 5.3 usage were observed at both time points. For the year 0 time point, approximately 4% of the T cells were tetramer positive after 14 days of expansion, and 28% and 18% of the tetramer-positive T cells used Vβ 2 and Vβ 5.3, respectively. For the year 5 time point, approximately 6% of the T cells were tetramer positive after expansion by in vitro peptide stimulation, and 28% and 14% of the tetramer-positive T cells used Vβ 2 and Vβ 5.3, respectively. Before expansion, approximately 7% and 1% of the PBMCs used Vβ 2 and Vβ 5.3, respectively, at both time points (data not shown). Thus, there is a selective usage of Vβ 2 and Vβ 5.3 for the tetramer-positive T cells. The uses of additional TCR Vβs that were unique to a specific time point were also observed. For example, Vβ 14 was being used at the year 5 sample, but not at the year 0 sample (Figures 3 and 4).

In order to evaluate whether T cells that were being expanded in the earlier time point could persist, both DR0404/p58-specific Vβ 2- and Vβ 5.3-positive T cells at year 0 and year 5 were isolated by FACS sorting. The antigenic specificity of these T-cell clones was confirmed by tetramer and Vβ staining. The CDR3 regions of the Vβ chain of the clones were sequenced (Table 1).

The experiments demonstrated that T cells with identical Vβ CDR3 sequences were present at the two time points for the VP16 443–455–specific Vβ 5.3 T cells. Limited data from the Vβ 2-specific T cells precluded us from drawing any firm conclusions regarding whether the Vβ 2 VP16 443–455 T cells persist during the 5-year period. Although we have not examined the Vα sequences of these clones, the data strongly suggest that T cells with identical TCR are present over the 5-year period for the VP16-specific Vβ 5.3 T cells. As the percentages of VP16-specific Vβ 5.3 T cells are fairly similar (18% vs. 14%) after in vitro expansion at the two time points, the frequency of these T cells has not changed significantly over a 5-year period.

Vβ Usages of VP16 Specific DQ0602 Restricted T Cells

HSV-2 VP-16–specific DQ0602 restricted T cells from a DQ0602 subject 5101 was also studied. The VP16\(_{369–379}\) epitope has previously been identified as a dominant DQ0602 restricted epitope [12]. PBMCs from 5101 at 2 time points that were 5 years apart were used as starting materials. Cells were stimulated with the VP16\(_{369–379}\) peptide, and were examined by DQ0602/VP16\(_{369–379}\) tetramers and anti-TCR Vβ antibodies staining 14 days later. Dominant Vβ usages for the DQ0602 restricted T cells as assayed by Vβ antibody screening was not observed at the year 0 time point. Approximately 14% of the tetramer-positive T cells used
FIGURE 1  Tetramer-guided epitope mapping of DR0404-restricted HSV-2 VP16 epitopes with pooled peptide tetramers. PBMCs obtained at two time points 5 years apart from subject 5607 were stimulated with 2 μg/ml of VP16 protein and analyzed by flow cytometry on day 12. Cells were stained with phycoerythrin-conjugated DR0404 tetramer loaded with peptides from 12 peptide pools that cover the VP16 protein sequence. Adapted from figure 1 of Novak et al. [11] with permission (Copyright 2001, The American Association of Immunologists, Inc.).
Vβ 3, other tetramer-positive T cells used Vβ 2, 5.1, 5.3, 6.7, 12, 13.1, 21.3, and 22. For the year 5 samples, approximately 22% of the tetramer-positive T cells use Vβ 2, other tetramer-positive T cells used Vβ1, 5.1, 6.7, 13.1, 13.6, 17, and 21.3 (Figure 5). Tetramer-positive T cells from the Vβ 2 and Vβ 6.7 samples at the 2 different time points were again sorted and their antigen specificity was confirmed. The CDR3 region of the TCR of these T-cell clones were examined by DNA sequencing. Although identical TCR CDR3 sequences were not observed with the Vβ 2 T cells at the 2 different time points, identical CDR3 regions were observed for the Vβ6.7 T cells at the two time points (Table 2). It should be pointed out that frequencies of this particular clonotype were very similar in the two time points examined.

DISCUSSION

The generation and maintenance of CD8 memory T cells have been studied in both murine models and humans. Experiments in the murine models tend to support the hypothesis that selection of memory T cells is stochastic, and the memory T-cell repertoires is a reflection of the T-cell repertoires during the primary responses [13–15]. Data in humans do not completely agree with a simple stochastic theory in selecting memory T cells from naive T cells, and data differ from those that were observed in the murine model. CD8+ T cells that were massively expanded in the primary response could still be detected 1 year later, but they were no longer the dominant clonotype [16]. It was speculated that massive expansion of the T cells led to clonal deletion of the T cells, thus reshaping both the epitope immunodominance and clonotype dominance of the memory repertoires [17, 18].

In contrast to the studies in CD8+ T cells, studies for the generation and maintenance of memory CD4+ T cells were very limited. Recent data indicated that CD4+ and CD8+ T-cell responses during viral and bacterial infection are distinct, with different kinetics in expansion and contraction [19–22]. CD8+ T-cell responses can be massive, in which up to 50% of peripheral T cells can be directed toward a class I–restricted epitope [23]. In contrast, CD4+ T-cell responses are less robust [24]. Recent data indicated that CD4+ T cells could only undergo a limited number of cell divisions compared with the massive proliferative response for CD8+ T cells [25]. The rate of cell division for CD4+ T cells is slower compared with CD8+ T cells, whereas the threshold for activation is higher [26–28]. CD4+ T cells was also postulated to have a shorter half-life compared with CD8+ T cells [20]. The different dynamic of CD4+ T-cell and CD8+ T-cell responses implies different ho-
Figure 3  Vβ expression of DR0404/p58-specific T cells. PBMCs from subject 5607 were stimulated with the VP16 443–455 peptide. Fourteen days after the in vitro stimulation, cells were stained with CD4^+, DR0404/p58 tetramers, and a panel of 20 different anti-TCR Vβ antibodies. (A) Year 0. (B) Year 5. Major TCR Vβs used by DR0404/p58 T cells are marked by a rectangle.
FIGURE 4  Vβ expression of DR0404/p58-specific T cells. Percentage of DR0404/p58 tetramer-positive T cells with a particular Vβ is illustrated. Data derived from Figure 3.
TABLE 1  Sequence of CDR3 regions of Vβ chain of clones for subject 5607

<table>
<thead>
<tr>
<th>Year 0</th>
<th>Vβ</th>
<th>CDR3</th>
<th>JB</th>
<th>Frequency</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>ICS VRRGLPDVQLSPL</td>
<td>HFG</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ICS KKVGGGVDTQ</td>
<td>YFG</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>5.3</td>
<td>CAS SYYQENNSPL</td>
<td>HFG</td>
<td>1.6</td>
</tr>
<tr>
<td>Year 5</td>
<td>2</td>
<td>ICS PRGPGNTI</td>
<td>YFG</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>5.3</td>
<td>CAS SYYQENNSPL</td>
<td>HFG</td>
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T-helper mechanisms may be involved in dictating the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell pool sizes and affects the generation and maintenance of the CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells. Longitudinal study of antigen-specific CD4<sup>+</sup> T cells is difficult because of their low frequency. Recent data did indicate that antigen-specific CD4<sup>+</sup> T cells persist for decades after vaccination [29]. However, the clonotype of these long-persistent T cells was not addressed in the study. A study in cytomegalovirus (CMV)-infected subjects did demonstrate the persistence of a dominant clonotypic CD4<sup>+</sup> T cells in a 12-month period [30]. As CMV CD4<sup>+</sup> T-cell responses are robust, it is unclear whether the persistence clonotypic CD4<sup>+</sup> responses observed in CMV can also be observed in other viral infections.

Samples from HSV-2–infected individuals provide an unique scenario for studying maintenance of memory T cells in chronic infection. It has been documented that almost everyone with HSV-2 seropositivity does shed virus, regardless of whether or not they have symptomatic recurrences. As a consequence of the periodic reactivation of HSV-2 virus, the immune system is being rechallenged with viral antigens intermittently [3]. Posavad et al. have used PBMC samples from HSV-2–infected individuals to demonstrate the persistence of CD8<sup>+</sup> CTL up to 7 years [10].

In this particular study, we examined the effect of repeated antigen rechallenges on the CD4<sup>+</sup> memory pools. Class II tetramers were used to monitor the CD4<sup>+</sup> T cells longitudinally from two HSV-2–infected subjects who have frequent symptomatic reactivations during a 5-year period of time. Because primary HSV-2 infection can be asymptomatic [4], it is unclear whether the year 0 samples from both subjects were obtained immediately after seroconversion. However, the study result did provide a glimpse of the evolution of the VP16 responses during the span of 5 years.

The HLA haplotype of the first subject is DR0404, and the p58 epitope (VP16 443–455) of the VP16 protein was identified as the dominant epitope at both time points.

Examine of Vβ usages of the DR0404/p58 T cells demonstrate that these T cells mainly use Vβ 2 and Vβ5.3 of the TCR Vβ gene at the year 0 time point, and Vβ 2, Vβ5.3, and Vβ14 at the year 5 time point. Thus, major change in the Vβ usages of the immunodominant epitope was not observed over this period. The bias of TCR Vβ usages used may represent a bias of the peptide in selecting certain Vβ alleles. In addition, the bias can also represent the oligoclonal expansion of DR0404/p58-specific T cells, which are being preserved throughout that period. In order to examine the clonality of these T cells, the Vβ2 and Vβ5.3 T cells were sorted and expanded. These T cells were again stained with the DR0404/p58 tetramer to confirm the specificity. The CDR3 region of these T-cell clones was sequenced. Although only a limited numbers of T-cell clones were being sequenced, identical CDR3 sequences were obtained from the Vβ5.3 T cells at both time points. The VP16-specific T-cell responses of a DQ0602 subject was also examined. No dominant Vβ usages for the DQ0602 restricted VP16-specific T cells was observed as assayed by TCR Vβ antibody staining of tetramer-positive T cells. It is possible that the dominant Vβ could not be detected by the panel of Vβ antibodies used in the experiment. Nevertheless Vβ2 and Vβ6.7 positive DQ0602/VP16<sub>369–379</sub> tetramer-positive T cells were observed at both time points. These T cells were again sorted and the TCR CDR3 region of these T-cell clones were sequenced. Identical CDR3 sequences were observed for the Vβ6.7 TCR from both the year 0 and year 5 time point. Although we have only sequenced the Vβ chain of the VP16-specific TCR from both subjects, and really cannot exclude the possibility that T cells of identical Vβ chain paired with different Vα chain; however, the chance of the later is remote. With the assumption that these are T cells of the same origin, it is remarkable that T cells at time points that are 5 years apart can be of the same clonotype amidst repeated viral activations. T cells of a particular clonotype were present at similar frequency at both time points.

The results suggested HSV-2 memory T cells are capable of continual renewal amidst multiple episodes of viral activation. There are a number of possibilities that may account for the long-term persistence of these CD4<sup>+</sup> T cells. HSV-2 memory T cells may be distributed in
FIGURE 5  Vβ expression of DQ0602/VP16_{369–389}-specific T cells. PBMC T cells from subject 5101 were stimulated with the VP16_{369–389} peptide. Fourteen days after *in vitro* stimulation, cells were stained with CD4^+^, DR0404/VP16_{369–389} tetramers, and a panel of 20 different Vβ antibodies. Percentage of tetramer-positive T cells with a specific Vβ is illustrated.
various lymphoid organs and lymph nodes throughout the body. Although exposure of memory T cells close to the site of HSV-2 infection might convert these proximal cells to effectors, reactivation of the virus may not activate all the memory T cells in distal lymphoid tissues. Thus, CD4\(^{+}\) memory T cells at distal sites could maintain the capacity for self-renewal, and continue to provide a source of T cells for immune surveillance. In an alternate explanation, it is possible that memory T cells can convert or give rise to both effector and memory T cells upon antigenic stimulation, thus allowing the perpetuation of the memory T-cell pool. The generation of additional CD4\(^{+}\) memory T cells from memory T cells upon antigenic encounter can be related to the small burst size of the CD4\(^{+}\) T-cell expansion. For HSV-2 infection, the frequency of T cells in PBMCs that directed against the entire spectrum of viral proteins during chronic infection is ~0.2%–0.5% of the total T-cell population [5]. The magnitude of the initial burst response to primary HSV has not been reported in humans. A smaller burst size for HSV-2 may limit deletions of T cells after expansion, allowing T cells to evolve into memory T cells.

Cameron et al. used class II tetramer analyses to demonstrate that memory repertoires specific for influenza antigens were much broader [31]. This different observation as compared with the current results may be a reflection of the different viruses being used. Influenza viruses cause acute infection, and the viruses are completely clear after the infection. In contrast, HSV-2 is a chronic infection with frequent viral reactivation. The observation that HSV-2–specific T cells are oligoclonal indicated that viral reactivation could shape the memory repertoires specific for HSV-2 antigens.

In summary, the studies suggested that HSV-2–specific CD4\(^{+}\) T cells of specific clonotypes were maintained during the course of HSV-2 infection. Repetitive challenge of the CD4\(^{+}\) T cells with antigens during viral reactivation could also narrow the TCR V\(\beta\) repertoire of the VP16-specific T cells, leading to the oligoclonal expansion of specific clonotypes. Further longitudinal analyses of CD8\(^{+}\) T and CD4\(^{+}\) cells in the HSV-2 system and other viral infections in human are needed to elucidate the evolution of memory T cells from primary and naive T cells.

**ACKNOWLEDGMENTS**

We thank Matt Warren for preparation of the manuscript. This work was supported by the Immune Tolerance Network, NIH contract HHSN266200400028C and grants AI30731 and AI50132.

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