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*J Immunol* 2003; 170:4380-4388; doi: 10.4049/jimmunol.170.8.4380

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T Cell Immunity to Herpes Simplex Viruses in Seronegative Subjects: Silent Infection or Acquired Immunity?1

Christine M. Posavad,2*§ Anna Wald,*†‡ Nancy Hosken,1 Meei Li Huang,* David M. Koelle,*†§ Rhoda L. Ashley,* and Lawrence Corey*†‡

During the course of investigating T cell responses to HSV among volunteers entering trials of investigational genital herpes vaccines, 6 of the 24 immunocompetent subjects with no prior history of oral/labial or genital herpes possessed HSV-specific T cell immunity but, by multiple determinants of even the most sensitive serological assays, remained seronegative to HSV-1 and -2. Of these six immune seronegative (IS; HSV-seronegative with HSV-specific T cell responses) subjects, two had transient HSV-specific T cell responses, while four had CD4+ and CD8+ T cell responses directed at HSV that persisted for up to 4 years. CD4+ T cell clones were isolated that recognized and had high binding affinities to epitopes in HSV-2 tegument proteins. All six IS subjects had potential sexual exposure to an HSV-2-infected sexual partner. Oral and genital mucosal secretions were sampled and tested for the presence of infectious HSV and HSV DNA. No evidence of HSV was detected in >1500 samples obtained from these IS subjects. The identification of persistent T cell responses to HSV in seronegative subjects is a novel finding in the herpesvirus field and suggests either undetected infection or acquired immunity in the absence of infection. Understanding the basis of these acquired immune responses may be critical in developing effective vaccines for genital herpes. The Journal of Immunology, 2003, 170: 4380–4388.

Herpes simplex viruses type 1 (HSV-1) and 2 (HSV-2) cause a variety of medically significant infections, especially in immunosuppressed subjects. HSV-1 and -2 are ubiquitous pathogens, and hence, susceptibility to infection was believed to be common, if not universal, in all populations. In prospective studies of couples discordant for HSV-2 where the HSV-seronegative partner was consistently exposed to infectious secretions, the acquisition rate of HSV-1 and HSV-2 averaged <7% yearly (1–3), suggesting that resistance to HSV infection may be acquired by persons with chronic exposure to the virus. Acquired resistance to a human herpesvirus has not been documented; however, there are precedents for acquired resistance in other human viral infections. This has been best documented in HIV infection, where multiply exposed, uninfected persons have been described by many investigators. Resistance to HIV has been in many cases attributed to the presence of HSV-specific T cell responses (4–13). More recently, T cell responses to hepatitis C virus (14, 15) and EBV (16) have been measured in the absence of detectable serum Ab. Whether these seronegative persons are infected with the organism or have developed transient infection that results in viral elimination or containment of the infection is unclear.

During the process of testing the safety and immunogenicity of a candidate HSV-2 vaccine in subjects seronegative for HSV-1 and -2, we detected HSV-specific T cell responses in blood obtained before vaccine administration in six of 24 subjects tested. In this study we detail the HSV-specific T cell and Ab responses in these six subjects with HSV-specific T cell responses. In addition, we studied mucosal secretion patterns by daily sampling of these patients for an extended time period.

Materials and Methods

Subjects

We enrolled 24 healthy subjects who were seronegative for HSV-1 and HSV-2 (HSV-seronegative) into a protocol testing the safety and immunogenicity of a candidate HSV-2 vaccine. We also enrolled 8 immunocompetent subjects who were HSV-1 seropositive only, 18 subjects who were HSV-2 seropositive, and 4 additional HSV-seronegative subjects not enrolled in the vaccine study. All subjects were enrolled in a University of Washington institutional review board-approved protocol. All subjects provided written informed consent.

Cells and viruses

PBMC were isolated by Ficoll-Hypaque and cryopreserved. Lymphoblastoid cell lines (LCL)3 were generated as previously described (17). HSV-1 strain E115 and HSV-2 strain 333 were used at an multiplicity of infection 100. Cells and viruses

3 Abbreviations used in this paper: LCL, lymphoblastoid cell line; DC, dendritic cell; ICC, intracellular cytokine staining; IS, immune seronegative; pCTL, CTL precursor; pProIF, HSV-specific lymphoproliferative precursors; LP, lymphoproliferation; VZV, varicella zoster virus.
Cryopreserved PBMC were thawed, and 1 × 10^6 cells were incubated in triplicate with HSV-2 Ag (UV-inactivated HSV-2), mock Ag (1/500), or PHA (0.8 µg/ml), and HSV-2-specific LP was determined as previously described (18).

**HSV-specific CTL and proliferative LDA**

The frequency of HSV-1- and HSV-2-specific CD8^+^ CTL precursors (pCTL) was determined by limiting dilution analysis (LDA) with negatively selected CD8^+^ T cells from cryopreserved PBMC using methods and calculations previously described (18, 19). The frequency of HSV-1- and HSV-2-specific CD4^+^ lymphoproliferative precursors (pProfil) in PBMC was determined as previously described (18, 20).

**Intracellular IFN-γ staining**

Intracellular IFN-γ staining was performed as previously described (21) using cryopreserved PBMC (1 × 10^6^) incubated with 1 µg each of co-stimulatory Abs against CD28 and CD49d (BD Biosciences, San Jose, CA). In every experiment a negative control (anti-CD28-CD49d) was included to control for spontaneous production of IFN-γ as well as a positive control (staphylococcus enterotoxin B; final concentration, 1 µg/ml; Sigma-Aldrich, St. Louis, MO) to ensure that the cells were responsive. CD8 responses to HSV-2 were stimulated using GM-CSF/IL-4 dendritic cells (DC; 1 × 10^5^) that were mock-infected (negative control) or infected overnight with HSV-2 (positive control). CD4 responses to HSV-2 were stimulated using a 1/100 dilution of mock or HSV-2 Ag. After 2-h incubation at 37°C, the secretion inhibitor brefeldin A (10 µg/ml; Sigma-Aldrich) was added, followed by an additional 4-h incubation. The cells were then kept at 4°C overnight. Cells were washed with PBS containing EDTA, followed by incubation at room temperature for 10 min with FACSLyse (BD Biosciences). Cells were washed and permeabilized using FACS permeabilization solution (BD Biosciences) for 10 min at room temperature, washed, and stained for 30 min in the dark with CD4^+^ or CD8-peridinin chlorophyll A protein, CD69-PE, and IFN-γ-PE (all from BD Biosciences). CD69-PE was used to detect activated T cells. The cells were washed and resuspended in 1% paraformaldehyde in PBS. Between 5 × 10^5^ and 1 × 10^5^ events were analyzed using a FACScan flow cytometer (BD Biosciences) and side scatter gating. The percentage of CD4^+^ or CD8^+^ T cells responding to HSV-2 was defined for each donor by subtracting the percentage of CD69^-IFN-γ^- cells using mock Ag or mock-infected DC from the percentage of cells using HSV-2 Ag or from HSV-2-infected DC. Responses were considered positive if the net percentage of CD69^-IFN-γ^- cells was >0.5% of the total CD4 T cells or >10% of the total CD8 T cells. Background CD4 responses (anti-CD28-CD49d alone or with mock Ag) were always <0.04%, and background CD8 responses (anti-CD28-CD49d alone or mock-infected DC) ranged from 0 to 0.60% (median, 0.06%).

**Generation of PBMC-derived HSV-specific T cell clones**

HSV-specific CD4^+^ T cell clones were established from PBMC as previously described (20). HLA restriction of T cell clones was determined by inhibition of LP with mAbs to HLA class II DR, DQ, and DP as previously described (20). Clones were tested in an LP assay for reactivity to HSV-1, HSV-2, varicella zoster virus (VZV), EBV, CMV, and influenza virus. CMV Ag was prepared as previously described (22) and was used at a final dilution of 1/500, VZV Ag (Advanced Biotechnologies, Columbia MD) was used at a final dilution of 1/600, EBV-infected cell extract (Advanced Biotechnologies) was used at a final concentration of 3 µg/ml, and Influenza Virus Vaccine USP (zonal purified, subviren; Pasteur Merieux, Paris, France) was used as a source of influenza Ag at a final concentration of 1 µg/ml.

**HSV-2 expression library**

HSV-2 (333) genomic DNA fragments were ligated into the pET17b expression vector. Pools (20 clones/pool) of transformed Escherichia coli (JM109 strain; Life Technologies, Gaithersburg, MD) were prepared and screened as previously described (23). E. coli clones prepared from positive pools were subsequently screened. Library inserts from positive clones were sequenced. Ags were identified by comparison of insert sequences with the HSV-2 (HG52) genomic sequence (GenBank accession no. Z86099).

**Identification of T cell epitopes**

Overlapping 15-mer peptides (five- amino acid overlap; Mimetopes, Clayton Victoria, Australia) spanning UL21, UL29, UL46, and UL47 were synthesized and dissolved in DMSO. Peptide pools (10 peptides/pool) were prepared and screened by coculture of T cells (2 × 10^5^/well), HLA-matched DC (1 × 10^5^/well), and synthetic peptides (10 ng/ml of each peptide) in an IFN-γ ELISPOT assay (24). Individual peptides from positive pools were tested in the same fashion to identify the epitopes.

**Systemic Abs to HSV**

Standard Western blots and ECL Western blots for the detection of HSV-1 and HSV-2 were performed on sera as previously described (25, 26).

**Daily home sampling of mucosal sites and detection of HSV by culture and PCR**

Subjects were instructed on the daily sampling of genital and oral mucosal sites as described in detail previously (27, 28). Dacron swabs placed in viral transport medium were delivered to the laboratory three times per week. Viral isolation was performed as previously described (29, 30). PCR detection of HSV at mucosal sites was performed by quantitative real-time fluorescence-based PCR as previously described (31), using previously described primers and probes (32). Both the volume of sample used to extract DNA (400 µl) and the volume of purified DNA (20 µl) used in each 50-µl PCR reaction were doubled compared with those used for the detection of HSV DNA in HSV-seropositive subjects (31) to detect extremely low copy number of HSV. Samples were considered positive by PCR if they 1) contained >10 copies/reaction of HSV DNA, 2) could be repeated, and 3) could be typed.

**Results**

**Cellular immunity to HSV in HSV-seronegative subjects enrolled in a candidate HSV-2 vaccine trial: identification of immune seronegative (IS) subjects**

Subjects who were seronegative to HSV-1 and -2 by Western blot were recruited into a phase I trial testing the safety and immunogenicity of a candidate HSV-2 vaccine at University of Washington. The initial screening visit evaluated past history of oral or genital lesions, and blood was drawn for HSV Western blot analysis (25). Only subjects without a history of oral or genital lesions who were HSV seronegative were eligible for enrollment. At the enrollment visit blood was taken for determination of HSV-2-specific CTL and LP responses before immunization in 24 HSV-seronegative subjects (patients 1–24) as well as in four subjects infected with HSV-1 only (patients 30–33) and five subjects infected with HSV-2 (patients 40–44) who were used as controls (Fig. 1).

Of the 24 HSV-seronegative subjects, four (patients 1, 3, 4, and 22) had positive HSV-2-specific CTL responses (Fig. 1a) and positive HSV-2-specific LP responses (Fig. 1b). In addition, two of the 24 subjects (patients 16 and 19) had HSV-2-specific CTL responses in the absence of an HSV-specific LP response (Fig. 1). Weak positive LP responses to HSV-2 were measured in the absence of an HSV-2-specific CTL response in three of the subjects (patients 6, 8, and 14). Positive CTL and LP responses were detected in all nine HSV-infected subjects (Fig. 1, a and b).

**Persistence of HSV-specific cellular immune responses in IS subjects**

Serial blood samples were obtained from the nine HSV-seronegative subjects with positive T cell responses to HSV-2 at screening. HSV-2-specific bulk CTL and LP responses were measured in three to six separate blood samples over a period of 3–17 mo (Fig. 2). The four subjects with positive CTL and LP responses at screening (patients 1, 3, 4, and 22) had consistently positive CTL and LP responses at all time points tested (Fig. 2). In contrast, the two subjects with positive CTL responses in the absence of an LP response at screening (patients 16 and 19) had no evidence of HSV-specific LP responses, but displayed CTL responses that lasted 6–7 mo and waned over time (Fig. 2). This observation suggests that a Th response to HSV is required to maintain HSV-specific CTL responses. In addition, subjects with weak LP responses in the absence of a CTL response at prevaccine time points (patients 6, 8, and 14) had negative LP and CTL responses to HSV.
in subsequent blood samples. HSV-seronegative subjects with evidence of HSV-specific T cell responses (CTL and/or LP) in blood at more than one time point are hereafter called IS, while those HSV-seronegative subjects with no evidence of HSV-specific T cell responses or where one time point was positive for T cell responses, but negative at subsequent time points, are hereafter called HSV-seronegative.

Sera from all HSV-seronegative subjects participating in the vaccine trial were tested at multiple time points by Western blot for the detection of HSV-specific Abs, and none of the 24 subjects displayed Abs to HSV-1 or HSV-2. Because of the consistent detection of HSV-specific T cell responses in the six IS subjects, we repeated the Western blots on sera obtained from all time points in these subjects. No evidence of HSV-1 or -2 Abs was detected at any time point tested (data not shown). We also examined sera from the IS subjects using an ECL Western blot, an assay that is 500-fold more sensitive than the standard Western blot and can detect HSV Abs with serum dilutions in excess of 10^6 (26). Both IgG- and IgA-specific HSV Abs were evaluated. The six IS subjects exhibited no evidence of HSV-specific bands even by this ultrasensitive assay; a representative ECL Western blot from IS subject 3 is shown in Fig. 1c. All six IS subjects had normal levels of serum IgG, IgA, IgM, IgE, and anti-tetanus Abs (data not shown), demonstrating that the IS subjects were not Ab deficient. Thus, by even the most sensitive assay available for detection of Abs to HSV, no anti-HSV Abs were measured in serum from IS subjects, including those time points when HSV-specific T cell responses were detected in PBMC.

**CD8^+ T cell responses to HSV in IS subjects**

We quantitated the frequencies of CD8^+ T cells specific for HSV-1 and -2 in the IS subjects by LDA (Fig. 3a) and intracellular cytokine staining (ICC; see below). A high frequency of HSV-2-specific CD8^+ pCTL was measured in patient 3 (250/10^6). Low frequencies of HSV-1- and HSV-2-specific CD8^+ pCTL were measured in the other three IS subjects: patients 22 (18 and 22/10^6), 4 (17 and 16/10^6), and 22 (20 and 39/10^6). LDA in HSV-1-seropositive subjects with culture-proven HSV-1 ranged from 41–218/10^6 for HSV-1 and from 9–247/10^6 for HSV-2. Responses in HSV-2-seropositive subjects with culture-proven HSV-2 ranged...
from 16–182/10^6 for HSV-1 and from 47–318/10^6 for HSV-2 (Fig. 3a). Among HSV-seronegative vaccine trial participants with no previous demonstration of HSV-specific T cell responses, the median frequency of HSV-specific pCTL was 1/10^6 in response to HSV-1 (range, <1–12/10^6 CD8s from PBMC) and 7/10^6 in response to HSV-2 (range, <1–12/10^6 CD8s from PBMC). Thus, all four IS subjects had higher frequencies of HSV-specific CD8^+ pCTL than those measured in HSV-seronegative subjects with no detectable CTL responses in bulk stimulated cultures.

We determined the frequency of IFN-γ-producing CD8^+ T cells in response to HSV-2 by ICC in 5 of the IS subjects, 12 subjects with recurrent HSV-2 infection, and 6 HSV-seronegative subjects. Fig. 3b displays representative FACS plots of CD8 responses to HSV-2 in an HSV-seronegative subject with no detectable in vitro T cell responses to HSV (patient 26), an HSV-2 infected subject (patient 49), and an IS subject (patient 3). HSV-2-specific CD8 responses were detected in all 12 HSV-2-infected subjects, ranging from 0.10–1.93% of the total CD8^+ T cells (median, 0.64%), whereas the six HSV-seronegative subjects had CD8 responses to HSV-2 ranging from 0.00–0.05% (median, 0.02%; Fig. 3d). These data are consistent with the CTL responses in these subjects; all 12 HSV-2^+ subjects and none of the six HSV-seronegative subjects had HSV-2-specific CTL responses to HSV (Fig. 1a). All five IS subjects exhibited CD8 IFN-γ responses to HSV-2 ranging from 0.30–0.79% (median, 0.41%; Fig. 3d), consistent with the detection of HSV-specific CTL responses in these subjects (Fig. 1a). These data confirm the existence of HSV-specific CD8^+ T cells in IS subjects, including T cells with CTL activity and those that produce IFN-γ.
We next quantitated HSV-specific CD4\(^+\) T cell responses by LDA (pProlif) and ICC. As with the CD8 LDAs, the IS subjects exhibited responses similar to those of HSV-seropositive subjects and higher than those measured in HSV-seronegative subjects (Fig. 3a). The four IS subjects had pProlif frequencies ranging from 19–286 cells/10\(^6\) PBMC, which, with the exception of patient 22, were within the ranges of those measured in HSV-1 (43–389/10\(^6\)).

Table 1. Characterization of HSV-specific CD4\(^+\) T cell clones from patient 1

<table>
<thead>
<tr>
<th>Clone</th>
<th>HSV-2-2 Specific Proliferation (cpm)(^a)</th>
<th>Cytokines (pg/ml)(^b)</th>
<th>HSV Ag Specificity(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-2</td>
<td>Anti-DR</td>
<td>Anti-DQ</td>
</tr>
<tr>
<td>1.6</td>
<td>26,215</td>
<td>25,840</td>
<td>–4</td>
</tr>
<tr>
<td>1.20</td>
<td>24,010</td>
<td>27,837</td>
<td>35,040</td>
</tr>
<tr>
<td>1.22</td>
<td>22,438</td>
<td>9,488</td>
<td>22,552</td>
</tr>
<tr>
<td>1.24</td>
<td>12,829</td>
<td>467</td>
<td>12,938</td>
</tr>
</tbody>
</table>

\(^a\) HSV-specific CD4\(^+\) T cell clones were isolated from PBMC from patient 1.

\(^b\) HLA restriction of each clone was determined by inhibition of HSV-specific lymphoproliferation with mAb to HLA class II DR (anti-DR), DQ (anti-DQ), and DP (anti-DP).

\(^c\) Clones were stimulated with UV-HSV-2, and supernatants were tested for cytokines by ELISA; cytokines in supernatants from mock-stimulated clones were below the level of detection of cytokine ELISAs.

\(^d\) Expression cloning was used to determine the Ag specificity of CD4\(^+\) T cell clones. The specific epitope is contained with the peptide sequences that are subscripted.
PBMC) and HSV-2 (28–484/10⁶ PBMC) seropositive subjects (Fig. 3a).

We determined the frequency of IFN-γ-producing CD4⁺ T cells in response to HSV-2 by ICC in five of the IS subjects, 13 HSV-2-seropositive subjects, and six HSV-seronegative subjects. Fig. 3c displays representative FACS plots of CD4 responses to HSV-2 in an HSV-seronegative subject (patient 26), an HSV-2-infected subject (patient 49), and an IS subject (patient 3). HSV-2-specific CD4 responses were detected in all 13 HSV-2-infected subjects, ranging from 0.12 to 0.54% of the total CD4⁺ T cells (median, 0.35%), whereas none of the six HSV-seronegative subjects had CD4 responses to HSV-2 above background (0.05%; Fig. 3e). These data are consistent with the LP responses in these subjects; all 13 HSV-2⁺ subjects and none of the six HSV-seronegative subjects had HSV-2-specific LP responses to HSV (Fig. 1b). In contrast, two of the five IS subjects exhibited IFN-γ responses to HSV-2; patients 1 and 3 had 0.30 and 0.14% of CD4⁺ T cells specific for HSV-2, respectively (Fig. 3e), consistent with the detection of HSV-specific LP responses in these subjects (Fig. 1b). Although patient 22 had persistent HSV-specific LP responses (Fig. 2), IFN-γ-producing CD4⁺ cells were below the level of detection in this IS subject (Fig. 3e). Patients 16 and 19, who each had transient CTL responses in the absence of HSV-specific LP responses, also had no detectable HSV-specific IFN-γ-producing CD4⁺ cells (Fig. 3e). When we measured IFN-γ-producing CD4⁺ longitudinally, CD4 responses to HSV-2 were detected in three of four blood draws taken over 13 mo from patient 3, although the responses were low (0.07–0.14%) (data not shown). CD4 responses to HSV-2 in patient 1 persisted for 4 years, although they were below the level of detection in one of the three blood samples (data not shown).

**Characterization of HSV-specific CD4⁺ T cell clones from an IS subject**

HSV-specific CD4⁺ clones were isolated and expanded from PBMC from patient 1, an IS subject in whom we had sufficient PBMC for cloning and subsequent characterization. Of the 48 clones from patient 1 that were screened for HSV-2-specific LP responses, 44 had net HSV-specific proliferation of >5000 cpm. Ten randomly selected clones were restimulated and expanded with PHA and IL-2, and data from four representative clones are displayed in Table I and Fig. 4. Using mAbs to inhibit HLA class II molecules, we determined that clones 1.22 and 1.24 were HLA-DR restricted, clone 1.6 was DQ restricted, and clone 1.20 was DP restricted (Table I). All four clones produced detectable levels of IFN-γ and IL-4 and/or IL-5 in response to HSV-2, indicative of a Th0 pattern of cytokine secretion. Expression cloning was used to determine the antigenic specificity of the CD4⁺ T cell clones; all four clones recognized viral tegument proteins, including UL21, UL29, UL46, and UL47. The region of the protein containing the antigenic epitope recognized by the CD4⁺ T cell clones has been narrowed to 18–20 aa (Table I). The clones were also tested for HSV-specific CTL activity, and three of the four clones (1.6, 1.22, and 1.24) specifically lysed HSV-2-infected autologous LCL, while clone 1.20 was not cytotoxic (Fig. 4a).

Clones from patient 1 were tested for reactivity to HSV-2-related human herpesviruses (HSV-1, VZV, CMV, and EBV) as well as influenza virus, a virus unrelated to HSV. Clones 1.6, 1.22, and

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**Table II. PCR studies for mucosal HSV shedding among IS subjects**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CTL/LP</th>
<th>Oral Cultures</th>
<th>Vul</th>
<th>Pen</th>
<th>PA</th>
<th>Total Cultures/ Patient</th>
<th>Oral</th>
<th>Gen</th>
<th>1st sampling</th>
<th>2nd sampling</th>
<th>Total PCRs/ Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+/+</td>
<td>0/25</td>
<td>0/25</td>
<td>0/25</td>
<td>75</td>
<td>0/25</td>
<td>0/25</td>
<td>0/25</td>
<td>0/25</td>
<td>0/25</td>
<td>158</td>
</tr>
<tr>
<td>3</td>
<td>+/+</td>
<td>0/100</td>
<td>0/100</td>
<td>300</td>
<td>200</td>
<td>0/101</td>
<td>0/101</td>
<td>0/100</td>
<td>0/26</td>
<td>0/26</td>
<td>178</td>
</tr>
<tr>
<td>16</td>
<td>+/+</td>
<td>0/112</td>
<td>0/111</td>
<td>334</td>
<td>334</td>
<td>0/114</td>
<td>0/114</td>
<td>0/114</td>
<td>0/25</td>
<td>0/25</td>
<td>342</td>
</tr>
<tr>
<td>19</td>
<td>+/+</td>
<td>236</td>
<td>236</td>
<td>338</td>
<td>912</td>
<td>242</td>
<td>101</td>
<td>239</td>
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<td>1,583</td>
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<tr>
<td>Total/site</td>
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<td>236</td>
<td>236</td>
<td>338</td>
<td>912</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*During the first sampling study, samples were collected for PCR from the oral and genital (Gen) sites. For the genital site, one swab was used to sample the penis (Pen) and perianal area (PA) from male subjects (patients 1, 3, 16, and 22), and one swab was used to sample the cervicovaginal, vulvar (Vul), and perianal area from the female subject (patient 19). In the second sampling study, samples were obtained for both viral cultures and PCR.*
1.24 were HSV type common because they proliferated in response to HSV-1 and HSV-2, while clone 20 was HSV-2 type specific (Fig. 4b). The clones were not cross-reactive with VZV, CMV, EBV, or influenza virus (Fig. 4b), supporting the hypothesis that these clones were generated by exposure to or infection with HSV. We next tested the TCR affinity of each clone by diluting each peptide and measuring lymphoproliferation. The proliferative response of each clone was achieved at low peptide concentrations (EC$_{50}$ = $<1$ ng/ml), providing further evidence that clones from this IS subject were not generated by cross-reactive epitopes. A representative peptide dose-response experiment using clone 22 and the UL46$_{621-639}$ peptide is displayed (Fig. 4c).

No evidence of HSV infection in IS subjects

We next evaluated whether the IS subjects were infected with HSV in the absence of seroconversion. It was of course not possible to evaluate HSV infection in sensory neural root ganglia in IS subjects. We could, however, evaluate mucosal reactivation of HSV by daily sampling of genital and oral secretions. We asked the six IS subjects to enroll in a daily mucosal shedding trial in which oral and genital secretions were sampled for HSV by both culture and PCR, the most sensitive method of detecting mucosal HSV shedding. Four IS subjects (patients 3, 16, 19, and 22) collected samples for viral culture and PCR detection, and one IS subject (patient 1) collected samples for PCR only. Three subjects (patients 3, 16 and 19) performed two sampling studies for PCR (Table II). Patient 4 declined participation in this study. Patient 16 agreed to the collection of oral samples for one PCR sampling study only. Subjects collected samples for PCR for 26–114 days and for viral culture for 25–112 days (Table II). For individual subjects, 75–334 viral cultures and 158–505 PCRs were collected for a total of 912 viral cultures (236 oral and 676 genital) and 1583 PCRs (573 oral and 1010 genital) for all IS subjects (Table II). We anticipated that if virus was present, it would be at low levels, and thus we increased the sensitivity of the real-time PCR by doubling the sample volume of purified DNA used in the assay. All 912 viral cultures and all 1583 PCRs were negative for HSV.

Demographics and sexual history of IS subjects: assessment of exposure to HSV

None of the IS subjects reported a history of oral or genital herpes. All six of the IS subjects were Caucasian. Patients 1, 3, 16, and 22 were men, and patients 4 and 19 were women. The ages of the IS subjects ranged from 23–38 years. The six IS subjects reported number of lifetime sexual partners ranging from 9–24 (median, 10 partners). Three of the six IS subjects reported that they had partners infected with HSV, two stated that previous partners were infected with HSV-1 (patients 1 and 19), and one reported a current partner with genital HSV-2 (patient 22). The human subjects review committee did not allow access to these partners.

Discussion

We detected CD4$^+$ and CD8$^+$ T cells directed at HSV in 6 of 24, or 25%, of HSV-seronegative subjects enrolled in a candidate HSV-2 vaccine study. T cell responses were persistent in four of the IS subjects and lasted up to 4 years in one subject. Both cytotoxic and IFN-γ-producing CD4$^+$ and CD8$^+$ T cells specific for HSV were detected in the IS subjects, although in general the frequencies of HSV-specific T cells tended to be lower in IS subjects compared with HSV-seropositive subjects. CD4$^+$ T cell clones were isolated and were shown to be specific for multiple HSV tegument proteins. No cross-reactivity to other herpesviruses, including the other human α-herpesvirus, VZV, was shown. Even using the most sensitive assays for HSV-specific Abs, we obtained no evidence of HSV seroconversion in the IS subjects. In addition, despite analysis of >1500 samples, we did not detect any infectious HSV or HSV DNA at mucosal sites in the IS subjects we studied. Taken together, these novel findings indicate the existence of HSV-seronegative subjects with T cell immunity to HSV in the absence of clinical or subclinical HSV infection and suggest that the characterization of T cell responses in these subjects may lend insight into the mechanisms involved in resistance to HSV infection or inhibition of HSV disease expression.

One possible explanation for the detection of HSV-specific T cell responses in HSV-1 seropositive IS subjects is that these responses were primed by cross-reacting Ags from related or unrelated organisms. CD8$^+$ T cell cross-reactivity in humans has been recently documented between nonhomologous viruses, including influenza A virus with hepatitis C virus (33). While no study documents T cell cross-reactivity between human α-herpesviruses (VZV and HSV), human CD4$^+$ T cell cross-reactivity has been detected between human β-herpesviruses, namely CMV, HHV-6, and HHV-7, although this is rare (34). In at least one IS subject (patient 1), T cell cross-reactivity seems unlikely, since four CD4$^+$ T cell clones isolated from this subject recognized four different epitopes from four different HSV-2 proteins. Further, these CD4$^+$ T cell clones did not proliferate in response to VZV (the most closely related virus to HSV) or to CMV or EBV (other related human herpesviruses). Moreover, the clones responded to low concentrations of peptide (EC$_{50}$ = $<1$ ng/ml), providing additional evidence that the clones were generated by exposure to or infection with HSV; the concentration of peptide required to stimulate cross-reacting responses tends to be $>1$ μg/ml (35). Thus, our data strongly argue that the HSV-specific T cell responses identified in this IS subject are not due to cross-reacting T cells, but are the result of exposure to or infection with HSV.

The sexual histories of the IS subjects related to potential HSV exposure suggest that at least three of six IS subjects have knowingly been exposed to HSV-1 or -2. However, considering 1) the high percentage of subjects who are seropositive for HSV-1 or HSV-2 (36), 2) the number of previous sexual partners (range, 9–24), 3) the high percentage of HSV-2-infected subjects who do not recognize the signs and symptoms of genital herpes (37), and 4) the potential of HSV-infected subjects not to inform partners of their HSV status (38), it is highly likely that the other three IS subjects were exposed to at least one partner infected with genital herpes. Analysis of T cell immunity to HSV in subjects with known and documented exposure to HSV will provide stronger evidence of a link between exposure to HSV mucosally and acquired T cell immunity to HSV in the absence of seroconversion. Currently, we are investigating couples discordant for HSV-2 where the susceptible partner is seronegative for HSV-1 and -2. At present, we have identified four of 10 HSV-seronegative partners who possess HSV-specific T cell responses in the absence of infection or seroconversion (C. M. Posavad and L. Corey, unpublished observations), corroborating the hypothesis that exposure to HSV can induce HSV-specific cellular immunity in the absence of seroconversion.

To date we have no evidence of HSV infection in the IS subjects we have identified. In a recent study of 53 HSV-2-seropositive subjects who reported no history of genital herpes, 52 of 53 subjects demonstrated HSV reactivation by PCR on the mucosal shedding sampling protocol we used in this study (37). If IS subjects are infected, we would have expected to detect HSV considering the total number of days sampled (1583 days), especially with the doubling in sample volume we used. It is possible that 1) virus was shed at too low a copy number (<10 copies/reaction) to be detected by real-time PCR, 2) IS subjects reactivated HSV, but at even lower frequencies than we sampled, or 3) HSV does not reactivate
from latency in these subjects; for example, subjects are infected with a defective or less virulent or replication-defective mutant of HSV (39).

Although the current study has described the immune response to HSV in IS subjects as a potential mechanism for resistance of HSV infection or lack of disease, viral and genetic factors may also contribute to the IS status. One genetic mechanism may be immunologic determinants of disease severity that are associated with HLA expression. A prospective study of 146 subjects has identified several HLA alleles that are associated with HSV-2 infection or with frequent symptomatic genital recurrences (40). Most significantly, the presence of HLA-B8 and the absence of HLA-B27 and -Cw2 were associated with symptomatic disease in HSV-infected subjects, and HLA-Cw4 was significantly associated with HSV-2 infection (40). These HLA associations suggest that immunologic factors linked to the MHC influence the risk of HSV-2 infection and disease expression. Four of the six IS subjects (patients 1, 3, 4, and 16) did not express HLA-Cw4, consistent with a decreased susceptibility to HSV-2 infection, although clearly many more IS subjects than we have identified will be required to detect an association between HLA and IS status.

A second potential genetic factor may be mutations in genes encoding HSV receptors, a situation analogous to the HIV system, where resistance to HIV infection in some multiply exposed HIV seronegative persons has been associated with the homozygous inheritance of a defective HIV-1 coreceptor, CCR5 (41, 42). We have recently sequenced three known HSV receptors, herpesvirus entry mediator, HVEM (HveA) (43), nectin-1 (HveC, PRR1) (44–46), and nectin-2 (HveB, PRR2) (47, 48), in patients 1, 3, and 4 to determine whether coding polymorphisms in these genes could cause altered susceptibility to HSV infection in IS subjects (49). Coding polymorphisms were detected in the HVEM and nectin-1 genes, but these mutations were not exclusive to IS subjects, and the receptors encoded by these variant genes were indistinguishable from the wild-type forms tested in vitro HSV entry activity assays (49). Additional subjects will be needed to determine whether an association exists between HSV receptor polymorphisms and susceptibility to HSV infection, especially in HSV-seronegative subjects in long term monogamous relationships with HSV-infected individuals.

This is the first documentation of persistent HSV-specific T cell responses, including T cell clones, in HSV-seronegative subjects with no history of oral or genital herpes infections. We found no evidence of HSV infection when mucosal samples were tested for HSV or HSV DNA, suggesting either silent infection or acquired immunity to HSV. Our preliminary data suggest that further evaluation of the unique cellular immune responses seen in these subjects may provide insights into defining protective immunity and the more rational design of vaccines for genital herpes.

Acknowledgments

We thank the subjects for their participation; Lin Zhao, Dawn Mueller, Jennifer Moses, and Suzanne McMullen for their excellent technical assistance; Mary Shughnessy for specimen collection; Hilary Olsen, Hui-Fang Wang, and Wendy Lin for their accurate performance of the HSV PCR; Julie Dalesio and Jeremy Lee for performing the ECL Western blots; Craig Day for preparation of the HSV-2 expression library; Lisa Anest and Felicia Wagener for performing T cell expression cloning and peptide-screening assays; Dr. Mark Wener and Maggie Mayes for analyzing serum IgG and anti-tetanus Ab levels; Stacy Selke for data management; and Dr. Patricia Spear for critical reading of the manuscript.

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