Antigen-Specific T Cells Localize to the Uterine Cervix in Women with Genital Herpes Simplex Virus Type 2 Infection

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Genital reinfection with herpes simplex virus type 2 (HSV-2) is uncommon in humans. The mechanism of acquired immunity is unknown. Because the cervix is a site of HSV exposure, we measured antigen-specific T cell responses to HSV in cervical lymphocytes during both lesional and nonlesional time periods. Cells were expanded without secondary in vitro stimulation with antigen. Proliferative and cytotoxic responses to HSV were detectable in specimens from most subjects. Limiting dilution assays showed a high frequency of antigen-specific cells. Cytotoxic T cell responses included both CD4 and CD8 components. Responses were present both during and between symptomatic infection episodes and persisted during suppressive antiviral therapy. Natural infection with HSV-2 is associated with a persistent cervical mucosal cellular immune response. This local response may possibly assist in limiting the clinical consequences of secondary HSV-2 infection, whether due to endogenous reactivation or exogenous reinfection.

Protection from exogenous infection with herpes simplex virus type 2 (HSV-2) in humans has been an elusive goal, despite intensive study of several vaccine compounds and strategies [1]. Paradoxically, natural genital infection with HSV-2 is highly effective in inducing an acquired state of immunity to exogenous reinfection. Re-exposure to HSV-2 by a genital route is believed to be relatively frequent, because 22% of adults in the United States are infected with HSV-2 [2], and shedding of virus from the genital tract occurs during 15%–40% of the days of infection, even in immunocompetent persons [3]. Restriction endonuclease studies of sequentially recovered isolates have rarely documented the isolation of >1 genotype from subjects with genital herpes [4–7]. Acquired immunity appears to be type specific, because prior HSV-1 infection appears not to protect from genital HSV-2 infection in adults [8, 9]. Acquired immunity is, at least in part, site specific: autologous auto-infection with genital HSV-2 strains may occur at cutaneous sites even after the establishment of systemic immune responses [10]. It is not presently known whether protective responses in adult humans are mediated by antibodies, T cells, or a combination of these effector mechanisms. Protective immunity against vaginal challenge can be induced in animal models, and it is particularly efficient and durable after mucosal vaccination. Antigen-specific CD4 cells and interferon-γ were shown to correlate with protection in one such animal model system [11, 12]. Local immunity may also limit HSV replication resulting from reactivation of latent HSV-2 infection. HSV-2 infection is asymptomatic in many patients [2, 13]. Almost all HSV-2-infected patients (with or without symptomatic infection) shed HSV from the genital tract [14]. Among patients with symptomatic genital HSV-2 infection, only ~40% of culture-positive shedding is associated with herpetic lesions [3]. Cervical shedding of HSV-2 is detectable by viral culture from the cervix on 0.8%–2.7% of days in the absence of lesions or symptoms [15, 16]. Although local IgA and IgG specific for HSV are present in cervical secretions [17], their levels or specificities have not been correlated with the clinical manifestations of recurrent HSV-2 infection. The purpose of this investigation was to determine the presence, effector functions, and relationship to viral infection of HSV-specific T cell responses in the cervix of HSV-infected women.

Subjects and Methods

Subjects and specimens. Healthy, human immunodeficiency virus (HIV)–uninfected women with serologic [18], clinical, and virologic [19] evidence of recurrent HSV-2 infection and HSV-seronegative women were enrolled. The initial clinic visit included
Lymphocyte culture. Lymphocytes were cultured in RPMI-
1640 with 25 mM HEPES, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-
inactivated human serum (cRPMI). Amphotericin B (2.5 µg/mL; Bio-Whittaker, Walkerville, MD) and acyclovir (50 µg/mL; Glaxo-Wellcome, Research Triangle Park, NC) were added to establish HLA-restricting loci, as described elsewhere [23]. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll-hypaque (Sigma, St. Louis) density gradient centrifugation.

Viruses and cell lines. HSV-1 strain E115 and HSV-2 strain 333 were used throughout, as described elsewhere [21]. Epstein-Barr virus–transformed lymphoblastoid cell lines were cultured, as described elsewhere [22]. Recombinant vaccinia virus expressing the HSV-2 gD gene and wild-type vaccinia virus New York were used, as described elsewhere [23]. The HSV-1/HSV-2 intertypic recombinant virus (IRV) R7015 [24–26] was the gift of B. Roizman (University of Chicago).

Lymphocyte culture. Lymphocytes were cultured in RPMI-
1640 with 25 mM HEPES, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-
inactivated human serum (cRPMI). Amphotericin B (2.5 µg/mL; Bio-Whittaker) was included for the first 7 days in some specimen cultures; acyclovir (50 µg/mL; Glaxo-Wellcome) was used for the first 14 days of specimen culture. Cultures were evaluated for mycoplasma and, if infected, were treated with ciprofloxacin (5 µg/mL; Bayer, West Haven, CT).

Cervical mucus was reduced with 1–3 µM dithiothreitol (Sigma) and agitation for 5 min at room temperature. Volume was increased to 5 mL with PBS, and an equal volume of ficoll-hypaque (Sigma) was underlaid. Swabs of the cervix, vulva, urethra, rectal area, and throat were collected for viral culture and for polymerase chain reaction (PCR) analysis of HSV DNA, as described elsewhere [3]. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll-hypaque (Sigma, St. Louis) density gradient centrifugation.

To obtain T cell clones, CBL or T cell clones, 1×10⁶ irradiated autologous PBMC, and antigen were added to replicate wells of 96-well U-bottom plates in a 200 µL total volume. After 72 h, 1.0 µCi ³H-thymidine (³H-
Tdr) was added; wells were harvested 18 h later onto UnifilterO plates (Packard Instruments, Meriden, CT) and were read on a TopcountT (Packard) scintillation counter. Results, expressed as delta counts per min (delta cpm), were calculated as [³H-cpm - background cpm] / [mean cpm control cpm]. Proliferative responses were scored as positive if the delta cpm was >4000. A delta cpm value <20 was recorded as 0. Antigens were UV-inactivated HSV (1.0×10⁻³–1.0×10⁻³ pfu/ml before inactivation, used at 1:100 final dilution), purified HSV-2 glycoproteins gB and gD (Chiron, Emeryville, CA; used at 2.0 mg/mL), VP16 of HSV-2 (Chiron; used at 1.0 µg/mL), and tetanus toxoid (Connaught Laboratories, Toronto, used at 20 µg/mL). Negative control was UV-treated Vero cell lysate (mock). The viability of responders was established by culturing with 1×10⁴ irradiated allogeneic PBMC and 0.8 µg/mL PHA-P. Anti–HLA class II MAbS (L243, B7/21, and SPV-L3, which recognize HLA DR, DP, and DQ, respectively) were used to establish HLA-restricting loci, as described elsewhere [23].

Cytotoxic T lymphocyte (CTL) assays were performed, as described elsewhere [21], in standard 4-h ³⁵Cr release assays. Target cells were autologous or allogeneic lymphoblastoid cell lines. The effector-to–target cell (E:T) ratio was 20:1 for CBL. For T cell clones, one-eighth of 14–21-day-old clonal microcultures (which typically filled wells of 96-well U-bottom plates) were used as effectors, with 2000 targets per well, in split-well analyses. Selected clones were expanded and assayed in duplicate or triplicate at 20:1 E:T ratios. Results are reported as percentage of specific release: [(mean experimental cpm – mean spontaneous cpm)/mean cpm control cpm] × 100.

a standard medical history and physical examination. The women were asked to return to the clinic at the time of genital symptoms or lesions, so blood and cervical specimens could be obtained. Specimens were divided into “lesional” specimens, obtained ≤14 days after the onset of the most recent symptomatic recurrence, and “nonlesional” specimens, obtained >15 days after the most recent symptomatic recurrence.

Cervical specimens were obtained, as described by Musey et al. [20]. Briefly, a cytobrush (Cytobrush PlusT; Medscan, Malmo, Sweden) was inserted into the cervical os and gently rotated. Care was taken to avoid blood contamination. Specimens with large amounts of visible blood were discarded. Samples were not taken during menstruation or pregnancy or if intercurrent cervicovaginal infection was noted. Specimens were placed in cRPMI (see below), with amphotericin B (10 µg/mL; Bio-Whittaker, Walkerville, MD) and acyclovir (50 µg/mL; Glaxo-Wellcome, Research Triangle Park, NC), for transport. Swabs of the cervix, vulva, urethra, rectal area, and throat were collected for viral culture and for polymerase chain reaction (PCR) analysis of HSV DNA, as described elsewhere [3].
the wells were pulsed with 1.0 ± antigen-presenting cells, and UV-inactivated HSV-2, for 24 repli-

CBL cultures was scored as positive if HSV-specific lysis was

Subject

Proliferative responses of cultured cervical cytobrush specimens obtained during or shortly after episodes of recurrent symptomatic

Table 2. Proliferative responses of cultured cervical cytobrush specimens obtained during or shortly after episodes of recurrent symptomatic genital herpes simplex virus (HSV) type 2 infection.

Table 2. Cell surface phenotype of expanded cervix-derived lymphocyte cultures.

HSV

serology

Specimens,

n

HSV-2 ± HSV-1

20

CD4

CD8

CD16/56

Seronegative

5

(66.9; 20–97)

(22.4; 1–61)

(7.3; 0–25)

(69.2; 47–82)

(29.4; 8–42)

(1.0; 0–2)

NOTE. HSV, herpes simplex virus.

a Significantly greater than uninfected (P = .009 by Mann-Whitney U test).

maximal cpm – mean spontaneous cpm) × 100. CTL activity in CBL cultures was scored as positive if HSV-specific lysis was >15% and the difference between autologous HSV-2 and mock-infected targets was >10%. T cell clones were scored as positive in split-well analyses if specific release for HSV-2-infected targets was >40% and specific release for mock-infected targets was <7%. MAbs W6/32 and L243, recognizing HLA class I and HLA DR, were used at a 1:4 dilution of culture supernatant in inhibition assays.

Limiting dilution analysis. Cells from cytobrush specimens were recovered, washed, and resuspended in cRPMI plus amphotericin B (Bio-Whittaker) and acyclovir (Glaxo-Wellcome). Cyto-

spin preparations of 5000–10,000 cells in 70 μL of PBS–2% human serum were made with a Cytospin 2 (Shandon, Pittsburgh) by rotating the cells at 250 g for 7 min. Cells consistent with lymphocyte size were counted and resuspended to concentrations of 20,000–50,000 cells/mL in cRPMI. The cells were plated in replicate wells of 96-well U-bottom plates in 7 serial 2-fold dilutions. To these wells were added 1 × 10^6 irradiated autologous PBMC, to serve as antigen-presenting cells, and UV-inactivated HSV-2, for 24 replic-

ate wells, or mock antigen for 12 replicates. After 5 days of culture, the wells were pulsed with 1.0 μCi of ^3H-TdR overnight and were harvested the following day. Wells were scored as positive if the cpm were >3 SDs above the mean cpm in the corresponding mock wells. The precursor frequency of HSV-specific cells was determined with the x^2 minimization method, as described elsewhere [27]. Cyto-

tosin preparations were stained with Wright-Giemsa, and a cell was classified as a lymphocyte on the basis of characteristic size, nuclear material, and morphology. This was used to adjust the input number of lymphocytes per well in the serial 2-fold dilutions. The frequency of precursor cells in PBMC with proliferative responses to HSV-2 was measured as described elsewhere [27].

Flow cytometry. Flow cytometry was done as described elsewhere [27], using mAb specific for CD3, CD4, CD8, CD16, and CD56 antigens, conjugated to phycoerythrin or fluorescein isothiocyanate (FITC; reagents from Sigma and Becton-Dickinson, respectively). Phycoerythrin- and FITC-conjugated isotype controls (Sigma) were also used.

Statistical analysis. The medians of nonparametric unpaired and paired data were evaluated for differences by using Mann-

Whitney 2-sample U tests and Wilcoxon signed-rank tests, respectively. The means of unpaired groups were compared by a 2-tailed t test.

Results

Cytobrush specimens and cultures. Median cell yield after ficoll-hypaque preparation was 180,000 cells (mean, 206,000; range, 50,000–440,000). Differential counts of Cytospin preparations after ficoll-hypaque revealed a mixture of epithelial and nonepithelial cells. Among the nonepithelial cells, means of 39% lymphocytes (range, 27%–54%), 36% monocytes, 20% PMNs, and 1% red blood cells were detected.

The cell surface phenotypes of cells expanded from cytobrush specimens varied widely. However, there were no significant differences in the percentage of expanded cells with CD4 or CD8 markers between cultures obtained from subjects with

Table 2.

<table>
<thead>
<tr>
<th>Subject</th>
<th>HSV serology (type 1/2)</th>
<th>Lesion site</th>
<th>Specimen day(s) from day of outbreak</th>
<th>Episodic acyclovir</th>
<th>Delta cpm ^3H-thymidine incorporation</th>
<th>HSV-2 culture</th>
<th>HSV PCR analysis</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>−/+</td>
<td>Buttock</td>
<td>3</td>
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<td>29,528</td>
<td>95</td>
<td>4881</td>
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<td>2</td>
<td>−/+</td>
<td>Perianal</td>
<td>9</td>
<td>Yes</td>
<td>46,133</td>
<td>311</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>10</td>
<td>Yes</td>
<td>15,824</td>
<td>0</td>
<td>401</td>
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<td></td>
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<td>14</td>
<td>Yes</td>
<td>20,122</td>
<td>655</td>
<td>507</td>
</tr>
<tr>
<td>3</td>
<td>−/+</td>
<td>Vulva</td>
<td>1</td>
<td>No</td>
<td>41,861</td>
<td>703</td>
<td>10,761</td>
</tr>
<tr>
<td>4</td>
<td>+/+</td>
<td>Leg</td>
<td>3</td>
<td>No</td>
<td>27,363</td>
<td>418</td>
<td>1238</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>No</td>
<td>14,054</td>
<td>3948</td>
<td>247</td>
</tr>
<tr>
<td>5</td>
<td>+/+</td>
<td>Vulva</td>
<td>2</td>
<td>Yes</td>
<td>ND</td>
<td>353</td>
<td>0</td>
</tr>
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<td></td>
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<td>Yes</td>
<td>2429</td>
<td>851</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>−/+</td>
<td>Perianal</td>
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<td>Yes</td>
<td>3492</td>
<td>142</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
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<td>NA</td>
<td>Day 3</td>
<td>NA</td>
<td>31,197</td>
<td>36,298</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>−/−</td>
<td>NA</td>
<td>Day 1</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>ND</td>
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</table>
genital HSV-2 infection and those obtained from subjects with no herpes simplex infection (table 1). NK-like cells (CD16/56+)
were significantly less common in cultures from HSV non-
infected subjects than in those from HSV-2–infected subjects. Cells from subjects with genital HSV were predominantly of
the TCR αβ phenotype (mean, 86.6%; n = 10 specimens), and
TCR γδ cells were rare (mean, 1.5%; n = 8 specimens).

Proliferative responses to HSV during the lesional period of
genital HSV-2. We obtained cervix specimens from 6 subjects
with recurrent genital HSV-2 infections during or within 14 days
of symptomatic external lesions. Five of the 6 subjects were
sampled more than once during the recurrence (table 2). HSV-
specific T cell responses were detected in cervical cells from 5
of the 6 recurrent HSV-2 episodes. HSV-2–specific proliferative
responses were detected in 9 of 12 specimens, HSV-1–specific
responses in 8 of 11, and tetanus-specific responses in 3 of 10.
HSV-specific proliferative responses were not detected in cul-
tures from HSV-seronegative subjects, whereas control re-
sponses to PHA were similar between the HSV-infected and
the HSV-uninfected subjects. Proliferative responses to HSV-2
antigen tended to be higher than those to HSV-1 antigen (mean
delta cpm, 25,893 and 21,194, respectively). T cell responses to
whole inactivated virus were more prevalent, and of greater
magnitude, than responses to purified HSV-2 proteins. Prolif-
erative responses to gB2 were detected in 2 of 12 samples, gD2
in 3 of 12, and VP16 in 3 of 12. As reported elsewhere in regard
to specimens from genital lesions [21], no significant differences
were seen between proliferative responses taken from early
Proliferative responses to HSV during intercurrent, virologically quiescent periods. To determine whether HSV-specific T cell responses were present during nonlesional periods of time, we sampled the HSV-2-infected subjects after lesion resolution. In addition to collecting mucosal lymphocyte samples, we also sampled the HSV-2-infected subjects after lesion resolution.

Table 3. Comparison of herpes simplex virus (HSV)-specific proliferative responses of bulk expanded cervix-derived cells obtained during or <14 days after recurrent symptomatic genital HSV-2 infection and cells obtained between episodes of symptomatic infection.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Lesional (n = 7)</th>
<th>Intercurrent (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Delta cpm</td>
<td>Delta cpm</td>
</tr>
<tr>
<td>HSV-1</td>
<td>11 21,193 (15,511)</td>
<td>11 13,653 (13,006)</td>
</tr>
<tr>
<td>HSV-2</td>
<td>12 25,893 (23,583)</td>
<td>12 25,281 (27,025)</td>
</tr>
<tr>
<td>PHA-P</td>
<td>12 77,460 (41,299)</td>
<td>11 60,632 (38,487)</td>
</tr>
<tr>
<td>Tetanus</td>
<td>10 2440 (3546)</td>
<td>8 2417 (3894)</td>
</tr>
</tbody>
</table>

NOTE. Data are mean (SD) delta counts per minute (cpm) 3H-thymidine incorporation. PHA-P, phytohemagglutinin purified. PHA-P, phytohemagglutinin purified.

* By Mann-Whitney U test.

HSV-specific T cell responses were detected in all 6 subjects and in 7 of the 11 samples taken during nonlesional time intervals. The mean proliferative responses to HSV-1, HSV-2, PHA, and tetanus were similar in the specimens from nonlesional versus lesional time periods (table 3). Comparison of the T cell proliferative responses during nonlesional and lesional time periods (figure 1) in 4 subjects sampled during both time periods shows similar responses to antigens and mitogen.

Cytobrush specimens from 2 subjects were analyzed by limiting dilution assay to estimate the precursor frequencies of HSV-2–specific T cells with proliferative responses in cervical mucosal cells, in comparison with PBMC. One subject was sampled during a nonlesional time period; the precursor frequency of cells with HSV-specific proliferative responses was 1 in 60, compared with a frequency of 1 in 4530 in the PBMC for specimens obtained during a nonlesional time period. A second subject was sampled twice, 4 days apart, during a symptomatic external lesion episode. The precursor frequency increased ~7-fold, from 1 in 3810 to 1 in 573, during a recurrence of external genital HSV-2, compared with a frequency of 1 in 554 in the PBMC obtained at the second time point.

Table 4. Cytotoxic responses of cultured cervical herpes simplex virus (HSV) cytobrush specimens, expressed as percentage of specific release at an effector-to-target cell ratio of 20:1.

<table>
<thead>
<tr>
<th>Subject</th>
<th>HSV serology (type 1/2)</th>
<th>Time since beginning of last outbreaka</th>
<th>Clinical statusb</th>
<th>Auto mock</th>
<th>Auto HSV-2</th>
<th>Allo mock</th>
<th>Allo HSV-2</th>
<th>HSV-2–specific proliferationc</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>–/+</td>
<td>3 d</td>
<td>L</td>
<td>16.0</td>
<td>67.4</td>
<td>5.9</td>
<td>39.4</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43 d</td>
<td>I</td>
<td>3.6</td>
<td>12.4</td>
<td>1.1</td>
<td>11.7</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>–/+</td>
<td>9 d</td>
<td>L</td>
<td>2.0</td>
<td>51.0</td>
<td>22.0</td>
<td>19.0</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 mo</td>
<td>I, SA</td>
<td>8.0</td>
<td>38.0</td>
<td>7.0</td>
<td>22.0</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>–/+</td>
<td>3 d</td>
<td>L</td>
<td>11.2</td>
<td>19.5</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 d</td>
<td>I</td>
<td>0.0</td>
<td>3.4</td>
<td>0.0</td>
<td>5.0</td>
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<tr>
<td></td>
<td></td>
<td>1 d</td>
<td>L</td>
<td>1.7</td>
<td>9.9</td>
<td>7.3</td>
<td>18.5</td>
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</tr>
<tr>
<td>4</td>
<td>–/+</td>
<td>7 d</td>
<td>L</td>
<td>20.0</td>
<td>61.0</td>
<td>34.0</td>
<td>59.0</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 d</td>
<td>I</td>
<td>2.2</td>
<td>1.8</td>
<td>9.0</td>
<td>8.2</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>+/+</td>
<td>2 d</td>
<td>I</td>
<td>19.9</td>
<td>20.5</td>
<td>3.1</td>
<td>6.8</td>
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</tr>
<tr>
<td>9</td>
<td>–/+</td>
<td>10 d</td>
<td>L</td>
<td>7.3</td>
<td>5.8</td>
<td>3.5</td>
<td>6.2</td>
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</tr>
<tr>
<td>10</td>
<td>–/+</td>
<td>8 mo</td>
<td>I, SA</td>
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<td>37.7</td>
<td>0.8</td>
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<tr>
<td>7</td>
<td>–/+</td>
<td>27 d</td>
<td>I, SA</td>
<td>2.7</td>
<td>2.1</td>
<td>13.8</td>
<td>16.2</td>
<td>Yes</td>
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<tr>
<td>8</td>
<td>–/+</td>
<td>6 mo</td>
<td>I, SA</td>
<td>12.1</td>
<td>30.8</td>
<td>2.1</td>
<td>60.3</td>
<td>No</td>
</tr>
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</table>

NOTE. Auto, autoinfection; Allo, allogeneic infection; ND, not done; NA, not applicable.

a For control subjects 7 and 8, repeated specimens were obtained 2 and 17 days after an initial specimen, respectively.
b L, during or <14 days after onset of last symptomatic recurrent genital HSV-2 episode; I, >14 days after last symptomatic genital HSV-2 infection; SA, subject receiving suppressive acyclovir therapy at the time of specimen collection.

c Yes, delta counts per minute (cpm) of proliferative responses of cultured cervical lymphocytes in response to HSV-2 antigen was >4000 cpm; no, response was <4000 cpm.
Figure 2. Herpes simplex virus (HSV)-specific cytotoxic T lymphocyte activity in lymphocyte populations cultured from the cervix of women with recurrent genital herpes. Specimen from subject 1 was obtained while a culture-positive buttock lesion was present. Auto, autologous; allo, allogeneic.

Table 5. Characterization of CD4 T cell clones derived from cervical cytobrush specimens in women with recurrent genital herpes simplex virus (HSV) type 2 infection.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Clone group</th>
<th>Clones, n</th>
<th>HSV type specificity</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>6</td>
<td>TS</td>
<td>0.8–1.0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>10</td>
<td>TC</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>10</td>
<td>TC</td>
<td>ND</td>
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<tr>
<td>4</td>
<td>2</td>
<td>10</td>
<td>TC</td>
<td>gD</td>
</tr>
</tbody>
</table>

NOTE. All clones displayed cytolytic activity against HSV-2-infected autologous target cells in standard ^51Cr release assays, as detailed in Subjects and Methods. ND, not done.

a TS, proliferates to HSV-2 antigen only; TC, proliferates to both HSV-1 and HSV-2 antigens.

b Reacted with an HSV-2 type-specific epitope mapping to 0.8–1.0 map units (see table 2).
type-specific clones further evaluated from subject 1 recognized an HSV-2 antigen mapping to 0.8–1.0 map units of the HSV-2 genome, on the basis of reactivity with HSV-2 and IR V R7015. The gene for gD2 is in the 0.8–1.0 map region, these T cell clones were checked for proliferative responses to gD2, which were absent (delta CPM <1000). Therefore, at least 1 additional HSV-2 type-specific T cell epitope is encoded by an HSV-2 gene in this region of the genome.

Discussion

This investigation provides the first evidence that HSV-2–specific T cells, including CTL, are present in the cervical mucosa of infected women. Cervical HSV-specific T cells with proliferative responses were detected at least once in 5 of 6 women; cells with CTL activity were detected at least once in 4 of 7 women. Reactivity against diverse HSV antigens, including gB2, gD2, and VP16, was present. Using intertypic recombinant viruses, we also demonstrated the presence of reactivity to at least 1 antigen mapping to ~0.8–1.0 map units. Thus, HSV-2 infection induces a strong CD4 and CD8 T cell response against diverse antigens, not only in the PBMC [29, 30] but also at a physiologically relevant mucosal site.

With regard to the phenotype of antigen-specific CTL, we detected CD8 effectors in 2 subjects at the bulk culture level (figure 2). Clonal analysis was performed for only 1 subject, but it revealed that HSV-specific CD4 cells with CTL activity were also present in the cervix, similar to our findings regarding herpetic skin lesions [21, 23]. We did note relatively high levels of non–HLA-restricted CTL activity in some specimens (table 4), regardless of HSV infection of the allogeneic target cells. We believe that it is unlikely that the effects of lectin (PHA) stimulation account for this nonrestricted CTL activity, because such activity was very rarely observed in a large number of cell lines derived from normal skin biopsy specimens [21] and was not present in the cervical specimens from the HSV-uninfected women (table 4). Additional studies are required to determine the phenotype of the cells responsible for the non–HLA-restricted cytotoxicity present in some cultures.

Cells required for inductive and effector functions of the cellular immune responses are present in the cervix. Antigen-presenting cells, such as Langerhans’ cells and dendritic cells, are present in the endocervical and ectocervical epithelia and submucosa [34–38]. Human Langerhans’ cells are functional antigen-presenting cells for recall responses to HSV [39–43], although HSV infection may have modulatory effects on dendritic cell maturation [44]. Cervical T cells are generally enriched in the transition zone between endocervix and exocervix [35]. For the effector phase, ectocervical, endocervical, and squamous metaplastic cervical epithelial cells can become HLA class II positive when inflammation is present [34]. Interferon-treated keratinocytes can present HSV antigen to HLA class I– and II–restricted memory/effector T cells [45, 46]. Recently, Musey et al. presented evidence that HIV-specific CD4 and CD8 CTL were present in cervical mucosal T cells [20], confirming that functional T cell responses against chronic viral pathogens can be generated and maintained at sites of infection.

Of potential biological importance, CTL and proliferative responses were detected among cells harvested >14 days after the last clinical recurrences of HSV-2. Among these subjects with symptomatic genital HSV-2, periodic local stimulation of HSV-specific T cells in regional lymph nodes or in the mucosa may give rise to cells that persist in the mucosa. It is possible that asymptomatic shedding provides adequate stimulation to maintain these responses. Recovery of HSV-specific T cells after prolonged treatment with suppressive doses of acyclovir could be due to local persistence of long-lived memory cells or periodic emigration of such cells from the blood. A low level of viral reactivation can be detected even during suppressive therapy [3], possibly enough to maintain local responses. Some of our subjects were seropositive for HSV-1, and it is possible that immune effector cells primed by orofacial HSV-1 infection might also localize to the cervix as part of a common mucosal immune response. Studying subjects who have documented orofacial HSV-1 infection, but no genital HSV infection, may address this possibility. Our data, albeit preliminary, indicate that locally persistent antigen-specific T cell responses may be present in the cervix and, as such, may be available to provide an early response to endogenous or exogenous infection.

The clinical correlates and functional activities of cervical HSV-specific T cells require further study. This initial study did not include enough specimens collected during asymptomatic time periods to compare responses between episodes of asymptomatic shedding (as defined by PCR analysis or culture) and responses during virus-free time periods. However, the methods described in this report are amenable to a high frequency of sampling. Studies of additional genital HSV recurrences, with and without clinically and virologically proven HSV cervicitis, and intensive longitudinal or cross-sectional studies correlating local responses with asymptomatic shedding will be necessary to determine whether local CTL activity is associated with mucosal viral clearance. The functional role of local HSV-specific T cell responses in protection from reinfection with HSV-2 will be difficult to define in humans. Careful local and systemic studies after vaccination, should an effective human vaccine be identified, may indicate whether local CTL or proliferative responses correlate with immunity in women.

To collect cervical mucosal lymphocytes, we used a method that routinely yields cervical epithelial cells for diagnostic purposes and has been shown to selectively collect mucosal HIV-specific T cells from HIV-infected women [20]. We did not collect specimens during the menstrual period or if visible blood
or ulcerative lesions were seen on speculum examination. Cytobrush specimens were collected gently, and visibly bloody specimens were rejected. Detection of HSV-specific CD4 and CD8 CTL activity in mitogen (PHA)-expanded lymphocytes, without the use of secondary in vitro stimulation with antigen, also supports the conclusion that mucosal lymphocytes were preferentially obtained by the specimen collection method used in this study. We used a polyclonal activator (PHA) that should be equally stimulatory for all T and NK cells [28]. However, it is possible that differential growth of subsets of cervical lymphocytes could also have led to the performance of assays that did not accurately reflect the distribution of cell types in the original specimens. Possibly, expansion in the presence of IL-2 might have led to lymphokine-activated killer activity. However, detection of high levels of cytotoxicity against uninfected, autologous target cells was infrequent. Our limiting dilution assay data, which stimulated oligoclonal or monoclonal populations of cells, also suggest that cells with HSV-specific proliferative responses were enriched in some of the cervical specimens. Because the present study used in vitro–expanded cells, study of mucosal homing or memory/effector markers was not performed.

The phenotype of expanded cervix-derived T cells was extremely variable. No differences were noted in the median or mean percentages of CD4, CD8, or NK-like cells when cultures from lesional and interlesional time periods were compared. The CD4/CD8 ratio was >1 and was similar to that normally present in PBMC. Several factors, including cyclic hormonal factors and other infections [47], may influence the phenotype of cervical lymphocytes. These data are similar to those reported by Moscicki et al. [48], who cultured predominantly CD4+ lymphocytes from cervical biopsy specimens. Of interest, NK cells were more common in specimens from women with recurrent genital HSV-2 than in specimens from women without HSV infection. These findings are similar to the finding of local enrichment of NK cells at sites of recurrent cutaneous HSV-2 infection, in comparison with normal skin, again, after expansion of cells with PHA [21].

Only limited information is available concerning the fine specificity of the HSV-specific responses detected in this study. At the bulk culture level, responses to individual HSV proteins gB2, gD2, and VP16 were each observed in only a minority of persons, similar to the findings of our studies of cutaneous lesion–infiltrating T cells [21]. This is in contrast to the findings of studies of PBMC CD4 responder cells, among which responses to membrane glycoproteins appear to be ubiquitous in HSV-infected donors [49, 50]. For the 2 donors studied at the clonal level, secondary in vitro stimulation with whole virus antigen was used to increase the recovery of HSV-specific cells. Thus, the relative predominance and diversity of the repertoire cannot be estimated from the limited panel of antigens and intertypic recombinant viruses used to define the activities of the resultant clones. However, reactivity with at least 1 other HSV-2 type-specific antigen mapping to 0.8–1.0 map units, in addition to gD2, was detected, as in our studies with skin-derived cells [45]. Application of expression cloning [51] is under way to address the specificity of these cells.

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