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Analysis of Herpes Simplex Virus-Specific T Cells in the Murine Female Genital Tract Following Genital Infection with Herpes Simplex Virus Type 2

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A murine model of genital infection with a thymidine kinase-deficient (tk^-) strain of herpes simplex virus type 2 (HSV-2) was utilized to examine the development of the local T cell response in the genital mucosa and draining genital lymph nodes (gLN). HSV-specific cytokine-secreting T cells were detected in the gLN 4 days postintravaginal inoculation but not in the urogenital tract or spleen until 5 days postinoculation, suggesting the cellular immune response originates in the gLN. More $CD4^+$ than $CD8^+$ gLN T cells were detected by flow cytometric analysis following primary vaginal inoculation and the majority of HSV-specific gLN T cells detected by ELISPOT were $CD4^+$ and Th1-like based on secretion of IFNγ and not IL-4 or IL-5. A similar population of HSV-specific memory T cells persisted in the genital tract 2 months following HSV-2 tk^- genital inoculation. These data suggest that the urogenital cellular immune response elicited in mice following genital inoculation with HSV-2 tk^- is predominantly $CD4^+$ and Th1-like, resembling that observed in humans. The results of this study are important for the rational design of vaccines capable of inducing protective immunity in the genital tract.

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) infects the oral and genital mucosa of humans and is a significant cause of morbidity. HSV disease is usually of limited duration; however, primary or recurrent disease is frequently painful and herpetic lesions can be an efficient portal of entry for other sexually transmitted pathogens.

Innate and acquired immune mechanisms have been described, primarily in models utilizing HSV-1, which play important roles in the control and resolution of HSV infections (Nash *et al.*, 1985; Mester and Rouse, 1991; Rinaldo and Torpey, 1993). The ability of B-cell-suppressed mice to clear primary HSV-1 cutaneous infections (Kapoor *et al.*, 1982a) and the ability of adoptively transferred HSV-immune T cells to clear virus from the site of inoculation (Kapoor *et al.*, 1982b) suggest that viral clearance from the epithelia is primarily a function of HSV-specific T cells rather than HSV-specific antibody. The ability of both $CD4^+$ and $CD8^+$ T cell subsets to effect recovery from primary infection is well documented (Nash *et al.*, 1987; Larsen *et al.*, 1983; Sethi *et al.*, 1983) and the role each plays may depend in part on the virus dose used for challenge (Wildy and Gell, 1985).

The local T cell response to genital HSV-2 infection represents one component of a protective immune response generated by genital inoculation with an attenuated strain of HSV-2 (McDermott *et al.*, 1989; Milligan and Bernstein, 1995). McDermott *et al.* (1989) showed that passively transferred HSV-immune genital lymph node (gLN) T cells homed to the urogenital mucosa and protected mice from a lethal infection with wild-type HSV-2. However, the subsets of T cells responsible for the protective effect and the mechanisms of action were not determined.

Given the significant role of HSV-specific T cells in the resolution of HSV infections, it is likely that vaccines developed to protect against HSV-2 or other sexually transmitted pathogens should elicit the appropriate type of cell-mediated responses within the urogenital mucosa. However, little is known about the induction and function of urogenital T cells. In the present study, a previously described murine model of protective immunity to genital HSV-2 infection (McDermott *et al.*, 1984; Milligan and Bernstein, 1995) was used to examine the kinetics and type of T cell responses which occur within the urogenital mucosa and regional lymph nodes following genital HSV-2 infection. The results of this study extend previous studies of urogenital immune responses to HSV-2 and begin to define the T cell subsets involved and their mechanisms of action with regard to clearance of virus from the vaginal mucosa.

MATERIALS AND METHODS

Mice

Female BALB/cAnNHSD (Harlan Sprague–Dawley, Inc., Indianapolis, IN) were used between 6 and 15

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weeks of age. Age-matched, uninoculated mice were used as controls.

Virus

The bromodeoxyuridine-resistant mutant, HSV-2 333 tk^- (Stanberry *et al.*, 1985), was obtained from Dr. Lawrence Stanberry (Children's Hospital Medical Center, Cincinnati, OH). Virus stocks were grown in Vero cell monolayers and prepared by two cycles of freeze/thaw and removal of cellular debris by centrifugation. Virus stocks were stored at −70° and samples were titrated on Vero cell monolayers.

Virus inoculation and sampling of lymphocyte populations

Mice were inoculated intravaginally with HSV-2 333 tk^- as described previously (Milligan and Bernstein, 1995). Briefly, mice were preswabbed with a cotton pledget, anesthetized with sodium pentobarbital, and inoculated by insertion of a type 2 calgiswab soaked with 10^6 PFU virus into the vagina. At various days postinoculation, single cell suspensions of spleen and iliac and paraaortic lymph nodes were prepared by passage through stainless steel screens. Urogenital lymphocytes were obtained as described (Milligan and Bernstein, 1995) by digesting the vaginae, cervices, and uterine horns from six to eight animals with 1.5 mg/ml Dispase (Boehringer Mannheim Corp., Indianapolis, IN). Peripheral blood obtained from the orbital sinus was layered over Ficoll to obtain peripheral blood lymphocytes (PBL).

Flow cytometry

The surface phenotype of gLN lymphocytes from normal or HSV-2 tk^--infected mice was determined by flow cytometry. Pooled lymphocytes from five mice per time point were incubated with 20% normal rat serum for 30 min at 4° to block nonspecific binding of labeled antibody, washed, then incubated with the following antibodies for 30 min at 4°: fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse B220, R-phycoerythrin (PE)-conjugated rat anti-mouse CD4, FITC-rat anti-mouse CD8a (PharMingen, San Diego, CA), and PE-rat anti-mouse CD3 (Sigma, St. Louis, MO). FITC-rat IgG2a and PE-rat IgG2b (PharMingen) were used as isotypic controls for each group of cells in each experiment. Cells were washed three times and fixed with 1.0% formaldehyde in PBS prior to analysis on an EPICS Profile II flow cytometer.

In vitro lymphocyte depletion

Lymphocyte populations were suspended at 10^7 cells/ml in Hanks' balanced salt solution alone or containing purified GK1.5 antibody (anti-CD4 at 50 μg/ml), HO2.2 ascites fluid (anti-CD8; 1:200 final dilution), a mixture of both antibodies at the indicated concentrations, or HO13.4 ascites fluid (anti-Thy1.2; 1:50 final dilution) and incubated on ice for 30 min. Cells were pelleted and resuspended in a 1:12 dilution of Low Tox M rabbit complement (Cedarlane Laboratories, Ltd, Hornby, Ontario, Canada) at one-half the original volume and incubated at 37° for 45 min. Cells were again pelleted and the antibody and complement treatments were repeated. Cells were washed three times and counted prior to culture.

Detection of cytokine-secreting cells

IL-4, IL-5, and IFNγ-secreting cells were detected by ELISPOT assay in lymphocyte populations from the urogenital tract, gLN, PBL, and spleens of mice undergoing a primary genital infection with HSV-2 333 tk^-. Lymphocyte populations were cultured at 5×10^6 cells/ml in a 1-ml volume (RPMI 1640, 10% FCS, 5 μ*M* 2-mercaptoethanol) in 24-well culture plates and stimulated with nonviable HSV-2 antigen or nonviable vaccinia virus (VV) antigen as a control (Bernstein *et al.*, 1991) for 20 hr. In other experiments, 5×10^5 uninfected or HSV-2 tk^--infected, mitomycin C-treated syngeneic spleen cells were added to cultures as control or stimulatory antigens, respectively. Following antigen stimulation, various concentrations of lymphocytes were plated in duplicate or triplicate on 96-well nitrocellulose filter plates (Millipore Corp., Bedford, MA) coated previously with purified anti-murine IFNγ (R4-6A2 hybridoma obtained from ATCC, 10 μg/ml), anti-murine IL-5 (TRFK-5, PharMingen, 4 μg/ml), or anti-murine IL-4 (11B11, hybridoma obtained from ATCC, 2 μg/ml) and incubated for 20 hr at 37°. Cells were removed by extensive washing with PBS and biotinylated anti-IL-4, anti-IL-5 (PharMingen), or rabbit anti-murine IFNγ (Biosource International, Camarillo, CA) was added to individual wells. The plates were again washed and peroxidase-conjugated goat anti-biotin antibody (Vector Laboratories, Inc., Burlingame, CA) was added to IL-4 and IL-5 wells, and peroxidase-conjugated goat anti-rabbit IgG (United States Biochemical Corp., Cleveland, OH) was added to IFNγ wells. Wells were washed thoroughly and developed with 3-amino-9-ethylcarbazole substrate. Spots representing specific cytokine-secreting cells were counted with the aid of a dissecting microscope.

CTL assay

A modification of the method of Pfizenmaier *et al.* (1977) was used to demonstrate CTL activity. gLN lymphocytes obtained 6 days after HSV-2 tk^- inoculation were cultured for 3 days without antigen at 10^7 cells/well in 12-well culture plates. A201.11 ($H\text{-}2^d$ MHC class I^+, class II^+) and EL-4 ($H\text{-}2^b$) target cells were infected with HSV-2 tk^- or VV at a multiplicity of infection of 5 and simultaneously labeled with 200 μCi ^{51}Cr for 4 hr at 37°. Targets were washed three times and 100-μl volumes

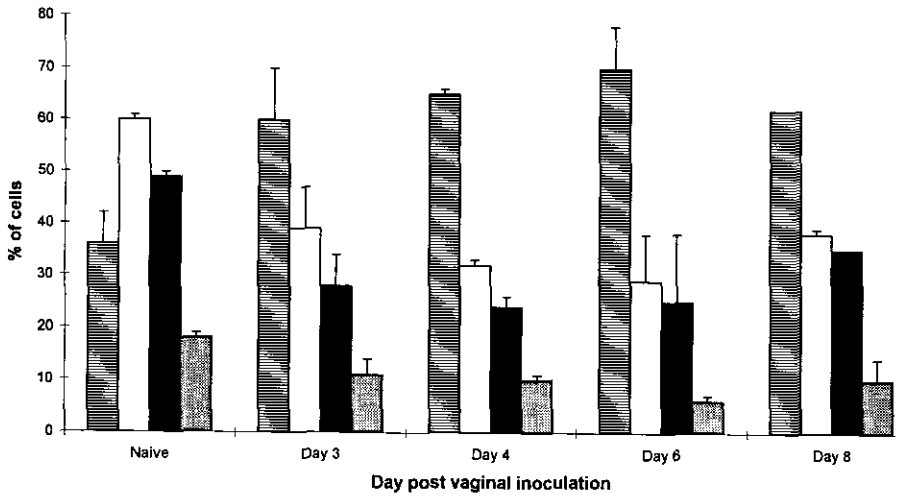

FIG. 1. Surface phenotype of gLN lymphocytes from Day 0–8 post HSV-2 tk$^-$ vaginal inoculation. Results are expressed as the mean percentage of total cells +/− SEM from two experiments with pooled lymphocytes from four to five animals per group. B220$^+$ cells, striped bars; CD3$^+$ cells, open bars; CD4$^+$ cells, solid bars; CD8$^+$ cells, stippled bars.

containing 5×10^3–10^4 cells were added to each well. Effector lymphocytes were washed, added to wells at various concentrations in 100-μl volumes, and cultured for 4 hr at 37°. One hundred microliters of supernatant was collected and counted and the percentage specific release was calculated (Milligan *et al.,* 1991).

For antibody blocking of HSV-specific lysis, effector lymphocytes in a 50-μl volume were incubated at 37° for at least 30 min with an equal volume of media or a previously determined optimally inhibitory concentration of GK1.5 (anti-CD4), 53.6.72 (anti-CD8), or TRFK-4 (anti-murine IL-5) antibody prior to addition of target cells. Supernatants were harvested and counted as described above.

T cell proliferation assay

Lymphocytes from the urogenital tracts, spleens, and gLN of normal control mice or mice inoculated intravaginally 4 weeks previously with HSV-2 tk$^-$ were cultured in triplicate at 3×10^5 cells/well in 96-well flat-bottom assay plates in 100-μl volumes. Medium (RPMI 1640, 7% FCS, and 5 μM 2-mercaptoethanol), a nonviable HSV-2 antigen preparation, or a nonviable VV antigen (Bernstein *et al.,* 1991) was added to each well for a total volume of 200 μl per well. Assays were pulsed for 20 hr with 1 μCi of [^{3}H]thymidine in a 50-μl volume on Day 3 of culture and harvested onto glass fiber filters and counted in Ecoscint scintillation fluid.

RESULTS

Surface phenotype of gLN cells following HSV-2 tk$^-$ genital inoculation

The results of flow cytometric analysis of murine gLN lymphocytes obtained on Days 3–8 following genital HSV-2 infection are shown in Fig. 1. CD3$^+$ lymphocytes constituted the majority cell population in gLN from uninfected control mice, whereas B220$^+$ (B) cells predominated the gLN population following infection. The levels of both CD4$^+$ and CD8$^+$ T cells as a percentage of the total cell number decreased and remained low through Day 6 postinfection. The CD4$^+$:CD8$^+$ T cell ratio ranged from 2.7:1 on Day 0 and 2.4:1 on Day 4 postinfection to 4.2:1 on Day 6 and 3.5:1 on Day 8 postinfection.

Detection of HSV-specific T cells in the female urogenital tract

The response kinetics and cytokine secretion profile of HSV-specific lymphocytes from Day 3 to 9 post vaginal inoculation with HSV-2 tk$^-$ were determined by ELISPOT analysis. Lymphocytes secreting IFNγ as a representative Th1-like cytokine and IL-4 and IL-5 as representative Th2-like cytokines were quantitated in populations from the spleen, peripheral blood, gLN, and urogenital tract after *in vitro* stimulation with HSV-2 or control antigen. Low levels of cytokine-secreting cells were detected from these tissues in uninfected animals (Fig. 2A) or on Day 3 postinoculation (data not shown). As shown in Fig. 2B, IFNγ-secreting lymphocytes were detected in the gLN and peripheral blood on Day 4 post vaginal inoculation but were not detected in great numbers in the spleen or genital tracts until Day 5 postinoculation (Fig. 2C). More IFNγ-secreting than IL-4- or IL-5-secreting cells were detected in all lymphocyte populations from Days 4 through 9 post vaginal inoculation (Figs. 2B–2D, and data not shown).

Phenotype of cytokine-secreting cells

The lymphocyte subset responsible for the IFNγ production was determined by depletion of CD4$^+$, CD8$^+$,

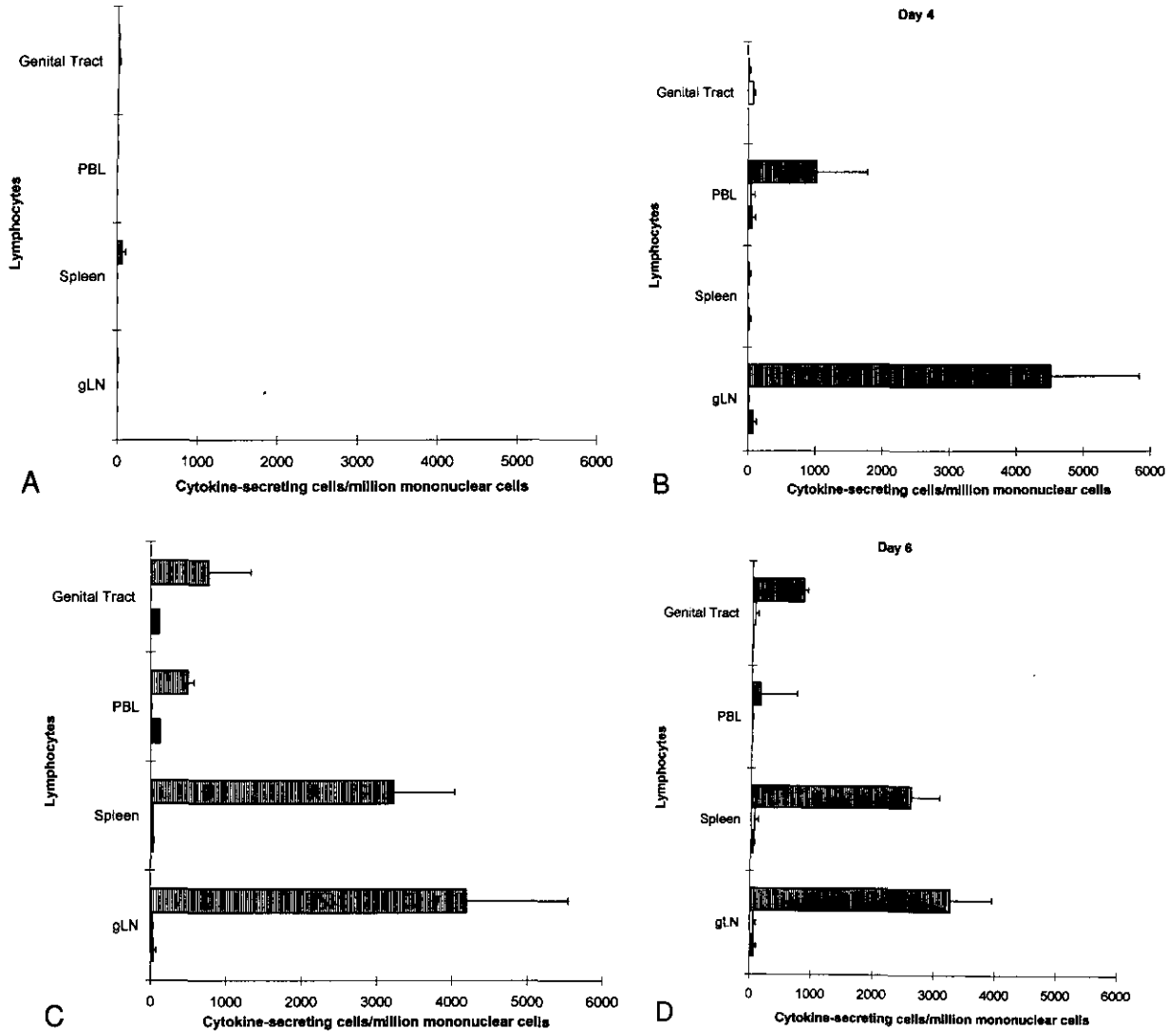

FIG. 2. Kinetics of the HSV-specific cytokine-secreting cell response following primary vaginal inoculation with HSV-2 tk^-. Lymphocytes from the various tissues were stimulated with nonviable HSV or VV antigen prior to culture on ELISPOT plates for detection of IL-4 (solid bars)-, IL-5 (open bars)-, or IFNγ (striped bars)-secreting cells. The number of cytokine-secreting cells (CSC) from VV-stimulated cultures was subtracted from the number obtained from HSV-stimulated cultures in each experiment to obtain the number of HSV-specific cytokine-secreting cells. The results are expressed as the mean number of CSC/10^6 mononuclear cells +/− SEM from three to five separate experiments. The ranges of the mean numbers of cytokine-secreting cells from vaccinia virus-stimulated cultures from all tissues were 0−55 for IL-4-secreting cells; 0−178 for IL-5-secreting cells; and 0−433 for IFNγ-secreting cells. See experiments in Tables 1 and 5 for typical experimental values for vaccinia virus stimulation. (A) Normal mice, Day 0; (B) Day 4 postinoculation; (C) Day 5 postinoculation; (D) Day 6 postinoculation.

or $Thy1^+$ populations prior to antigen stimulation with either a nonviable HSV antigen preparation (Table 1) or mitomycin C-treated, HSV-2-infected spleen cells (Table 2). As shown in Table 1, stimulation of gLN lymphocytes with nonviable HSV-2 antigen resulted in an increased number of IL-4-, IL-5-, and IFNγ-secreting cells compared to gLN lymphocytes stimulated with a nonviable VV antigen or HSV-stimulated gLN lymphocytes from uninoculated animals. Treatment of cells with anti-Th1.2 or anti-CD4 antibody and complement reduced the number of IL-4-, IL-5-, and IFNγ-secreting cells to below background. Treatment with anti-CD8 antibody and complement had no negative effect on numbers of cytokine secreting cells (CSC) compared to cells treated with complement only.

Following stimulation with HSV-2-infected syngeneic spleen cells, both $CD8^+$ and $CD4^+$ CSC were detected (Table 2). Although total abrogation of IFNγ secretion was observed only in lymphocyte cultures treated with a mixture of anti-CD4 and anti-CD8 antibody and complement, the majority of IFNγ-secreting cells were removed by treatment with anti-CD4 antibody and complement compared to treatment with anti-CD8 antibody and complement. IL-4- and IL-5-secreting cells were completely depleted by treatment of cells with anti-CD4 antibody and complement while depletion of

TABLE 1

Phenotype of HSV-Specific Cytokine-Secreting Cells Detected after Stimulation with Nonviable HSV-2 Antigen

Source	Treatment[b]	Stimulation	Mean CSC/10^6 cells ± SD[a] IL-4	IL-5	IFNγ
Naive gLN	none	HSV	0 ± 0	0 ± 0	0 ± 0
Immune gLN	none	VV	7 ± 2	13 ± 8	386 ± 70
Immune gLN	C	HSV	138 ± 23	128 ± 16	2240 ± 684
Immune gLN	α CD4 + C	HSV	0 ± 0 (100)[c]	0 ± 0 (100)	43 ± 11 (98)
Immune gLN	α CD8 + C	HSV	193 ± 10 (0)	283 ± 20 (0)	3252 ± 252 (0)
Immune gLN	α CD4 + α CD8 + C	HSV	0 ± 0 (100)	4 ± 4 (97)	225 ± 19 (90)
Immune gLN	α Thy1.2 + C	HSV	0 ± 0 (100)	0 ± 0 (100)	84 ± 14 (96)

[a] Results are expressed as the mean numbers of cytokine-secreting cells (CSC) ± SD per 10^6 mononuclear cells. Results are shown from a typical experiment of three performed.

[b] Lymphocytes were harvested 6 days after genital HSV-2 tk^- inoculation and depleted of $CD4^+$, $CD8^+$, or $Thy1^+$ T cells by treatment with the appropriate antibody and complement (C) prior to HSV-2 antigen stimulation and assay by ELISPOT.

[c] Percentage of inhibition is shown in parentheses and was calculated by the formula 100 × [1 − (the number of CSC obtained from an antibody + C-treated group/the number of CSC from C-treated immune cells)].

$CD8^+$ T cells did not reduce the number of cells secreting these cytokines.

HSV-specific CTL

HSV-specific CTL were detected in the genital lymph nodes as early as Day 4 and as late as Day 8 following primary intravaginal inoculation with HSV-2 tk^- (data not shown). Antigen-specific cytolysis of HSV-infected A201.11 target cells (MHC class I^+, class II^+) could be inhibited by addition of anti-CD4 but not control anti-IL-5 antibody to gLN effector cells at the time of assay (Table 3). Addition of anti-CD4 antibody consistently resulted in greater inhibition of lysis than did addition of anti-CD8 antibody. In a similar set of experiments, specific lysis of HSV-infected A201.11 target cells was markedly decreased by depletion of $CD4^+$ T cells (Fig. 3). By contrast, the depletion of $CD8^+$ T cells had less effect.

Persistence of HSV-specific T lymphocytes in the urogenital mucosa

To determine if HSV-specific T lymphocytes persisted in the urogenital tract following genital HSV-2 infection, urogenital, splenic, and gLN lymphocytes were obtained from control mice or mice inoculated intravaginally 3 weeks previously. As shown in Table 4, lymphocytes from control mice did not proliferate in response to either the control VV antigen or the HSV antigen. By contrast, HSV-immune lymphocytes from the urogenital tract, spleen, or gLN proliferated to HSV, but not VV antigen.

The presence and function of memory HSV-specific T lymphocytes residing in the urogenital tract was next tested by ELISPOT assay. Two months following intravaginal inoculation of HSV-2 tk^-, lymphocytes were isolated from spleen, gLN, and urogenital tracts and cultured with nonviable VV or HSV antigen. As shown in Table 5, urogenital, splenic, and gLN lymphocytes ob-

TABLE 2

Phenotype of HSV-Specific Cytokine-Secreting Cells Detected after Stimulation with Live HSV-2 Antigen

Source[b]	Treatment	Stimulation	Mean CSC ± SD/10^6 cells[a] IL-4	IL-5	IFNγ
Naive gLN	None	HSV spc	0 ± 0	0 ± 0	2 ± 2
Immune gLN	C	Uninf. spc	0 ± 0	0 ± 0	178 ± 2
Immune gLN	C	HSV spc	463 ± 46	228 ± 10	5166 ± 376
Immune gLN	α CD4 + C	HSV spc	0 ± 0 (100)[a]	0 ± 0 (100)	871 ± 31 (83)
Immune gLN	α CD8 + C	HSV spc	659 ± 83 (0)	258 ± 4 (0)	4020 ± 339 (22)
Immune gLN	α CD4 + α CD8 + C	HSV spc	0 ± 0 (100)	0 ± 0 (100)	26 ± 0 (99)

[a] As in *Table 1*. Results are shown from a typical experiment of two performed.

[b] Cells from the indicated sources were untreated or treated with complement alone or the indicated antibody and complement prior to stimulation with mitomycin C-treated HSV-2-infected or uninfected syngeneic splenocytes (spc).

TABLE 3

Inhibition of HSV-Specific Cytolysis with Anti-CD4 and Anti-CD8 Antibody

Antibody[a]	% Specific lysis	% Inhibition
Experiment 1		
None (media)	51	—
Anti-IL-5	47	8
Anti-CD4	27	47
Anti-CD8	45	12
Anti-CD4 + CD8	7	86
Experiment 2		
None (media)	37	—
anti-IL-5	41	0
Anti-CD4	24	35
Anti-CD8	35	5
Anti-CD4 + CD8	14	62

[a] Lymphocytes from genital lymph nodes taken 6 days after genital HSV-2 tk$^-$ inoculation were cultured as described under Materials and Methods. Fifty microliters of the indicated antibodies were mixed with 50 μl effector cells for at least 30 min prior to addition of target cells. Data from E:T ratios of 75:1 are shown. Standard deviations were less than 10%. Specific lysis of HSV-infected EL-4 targets was <5%.

tained from HSV-2 tk$^-$-immune animals secreted predominantly IFNγ in response to HSV antigen. IL-4-secreting cells were detected at lower levels in the genital tract and gLN of HSV-2 immune animals.

DISCUSSION

Organized lymphoid follicles such as the mucosal inductive sites found in the digestive and respiratory tracts are not found in the murine female genital tract (Thapar *et al.,* 1990). Consequently, the vaginal mucosa is a poor inductive site for immune responses. However, both HSV-specific B cells (Milligan and Bernstein, 1995) and T cells (this study) apparently home to this mucosal site following vaginal HSV-2 tk$^-$ inoculation.

HSV-specific CD8$^+$ T cells and the majority of CD4$^+$ T cells secreted IFNγ following intravaginal HSV-2 infection (Tables 1 and 2). Interestingly, a similar population of urogenital IFNγ-secreting cells has been recently described by Cain and Rank (1995) following intravaginal infection of mice with *Chlamydia trachomatis.* This predominance of IFNγ-secreting cells in the regional lymph nodes and genital tract suggests an important role for this cytokine in controlling genital tract infections by sexually transmitted pathogens. Recently, Smith *et al.* (1994) found that anti-IFNγ antibody treatment of mice receiving HSV-immune T cells, including CD8$^+$ cytotoxic lymphocytes, resulted in a diminished capacity to clear HSV-1 from cutaneous tissue. These results suggest that locally produced IFNγ may be more important than T-cell-mediated cytolysis of infected cells in clearance of HSV from cutaneous sites.

IFNγ may restrict primary HSV genital infection by direct antiviral effects including activation of 2′,5′-oligoadenylate synthetase and P1/eIF-2 protein kinase pathways (Samuel, 1991) or by promotion of antigen presentation resulting from increased expression of class I and class II MHC proteins (Pestka *et al.,* 1987; Kappes and Strominger, 1988), proteasome subunits (Yang *et al.,* 1992), and TAP peptide transporters (Bahram *et al.,* 1991). Besides regulating humoral and T cell responses (Snapper and Paul, 1987; Mosmann and Coffman, 1991; Gajewski *et al.,* 1989), IFNγ also increases secretory component levels in reproductive tract epithelial cells resulting in increased levels of IgA in uterine secretions (Prabhala and Wira, 1991). IFNγ-activation of mononuclear phagocytes results in increased production of high affinity Fc receptors (Erbe *et al.,* 1990) and reactive oxygen and nitrogen metabolites (Ding *et al.,* 1988), therefore augmenting antibody-dependent cell-mediated cytotoxicity and macrophage-mediated resistance to HSV (Karupiah *et al.,* 1993; Croen, 1993).

The results of cell depletion experiments (Tables 1 and 2) and flow cytometric analysis (Fig. 1) in the current study suggest that a large component of the gLN cellular immune response to HSV-2 genital infection was CD4$^+$ and Th1-type. Similarly, the greater inhibition of specific lysis of HSV-2-infected target cells detected following depletion of CD4$^+$ T cells compared to CD8$^+$ T cell depletion (Fig. 3) and the greater capacity of anti-CD4 antibody to block HSV-specific target cell lysis (Table 3) are also consistent with a predominant HSV-specific CD4$^+$ T cell response.

HSV-1 has been shown to block transport of human class I MHC proteins *in vitro* as a result of the HSV protein ICP47 binding to human TAP proteins (York *et al.,* 1994; Fruh *et al.,* 1995; Hill *et al.,* 1995). Similarly, HSV-2 infection greatly decreases the surface expression of MHC class I antigens on murine cells resulting in resistance to lysis by CD8$^+$ CTL (Carter *et al.,* 1984, Jennings

TABLE 4

HSV-Specific Memory T Cells in Genital Tracts of HSV-Immune Mice

		[^{3}H]Thymidine uptake (mean cpm ± SD)[a]	
Source[b]	Media	VV antigen	HSV antigen
Naive spleen	2581 ± 1594	4664 ± 263	6274 ± 1808
Naive genital tract	ND	4845 ± 2229	3831 ± 2371
Naive gLN	1007 ± 329	1437 ± 737	257 ± 323
Immune spleen	4889 ± 496	6090 ± 1915	24,647 ± 6511
Immune genital tract	621 ± 302	822 ± 373	16,516 ± 9165
Immune gLN	2857 ± 1699	2989 ± 1405	11,497 ± 6437

[a] Results are expressed as the mean cpm ± SD for triplicate cultures. Results are shown from a typical experiment of two performed.

[b] Pooled lymphocytes (8 animals/group) were cultured in triplicate with nonviable VV or HSV antigens.

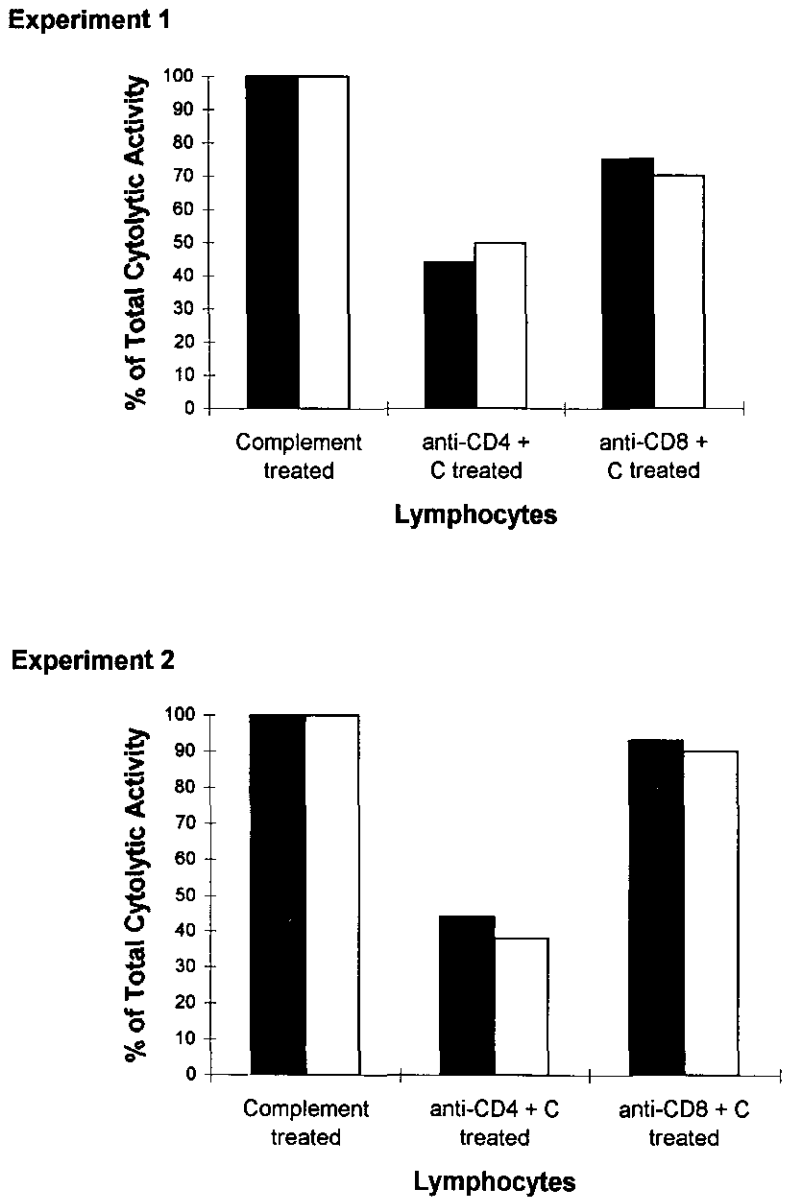

FIG. 3. HSV-specific $CD4^+$ CTL in the gLN following primary vaginal inoculation with HSV-2 tk^-. gLN lymphocytes were cultured without antigen prior to treatment with anti-CD4 +C, anti-CD8 +C, or C alone. Data from two separate experiments with HSV-2-infected A201.11 cells and effector:target ratios of 75:1 (filled bars) and 38:1 (open bars) are shown. Depletion of $CD8^+$ cells was complete and depletion of $CD4^+$ cells was approximately 80% as determined by flow cytometry.

et al., 1985). This property may explain the low level of lysis of HSV-2-infected A201.11 target cells by HSV-specific gLN $CD8^+$ T cells detected in the present study. Currently, it is unknown what effect this property may have on class I MHC expression or the induction of $CD8^+$ CTL *in vivo*, but it is intriguing to speculate that such a mechanism may explain the predominance of $CD4^+$ HSV-specific T cells observed following human HSV infections (Yasukawa and Zarling, 1984, Schmid, 1988), the lower $CD8^+$ CTL precursor frequencies observed following infection of mice with HSV-2 compared to HSV-1 (Jennings *et al.*, 1985), and the predominance of HSV-specific $CD4^+$ T cells in the present study. The induction and quantitation of the HSV-2 specific urogenital $CD4^+$ and $CD8^+$ T cell responses are currently under investigation.

MHC class II^+ cells are obviously required to present antigen to HSV-specific $CD4^+$ T cell precursors and to trigger the release of cytokines by mature effector cells in the vaginal mucosa. In this regard, Parr *et al.* (1994) demonstrated class II MHC^+ Langerhans cells under vaginal mucosal cells 1 day after vaginal infection and enhanced class II MHC expression on vaginal epithelial cells 3 days postinoculation with a thymidine kinase-deficient strain of HSV-2. Langerhans cells may acquire antigen in the genital tract, migrate to the regional lymph node, and present antigen to HSV-specific T cell precur-

TABLE 5

HSV-Specific Memory T Cells in the Urogenital Tracts Secrete Predominantly IFNγ

Lymphocytes[a]	*In vitro* antigen stimulation	Mean CSC/10^6 mononuclear cells ± (SEM)	
		IL-4	IFNγ
Naive genital tract	VV	5 ± (5)	97 ± (61)
	HSV	4 ± (4)	150 ± (88)
Naive gLN	VV	2 ± (2)	15 ± (11)
	HSV	0 ± (0)	19 ± (10)
Naive spleen	VV	15 ± (9)	101 ± (28)
	HSV	56 ± (28)	236 ± (54)
Immune genital tract	VV	8 ± (8)	30 ± (19)
	HSV	67 ± (39)	816 ± (126)
Immune gLN	VV	1 ± (1)	71 ± (38)
	HSV	121 ± (30)	2051 ± (606)
Immune spleen	VV	36 ± (18)	319 ± (54)
	HSV	132 ± (44)	2412 ± (299)

[a] Lymphocytes from the indicated sources were stimulated with nonviable HSV or VV antigen prior to plating on ELISPOT plates. Results are shown as the mean number of CSC-secreting cells/10^6 mononuclear cells from four individual experiments ± SEM.

sors. Following maturation and migration of the T cells to the infected vaginal epithelia, both MHC class II^+ Langerhans cells and vaginal epithelial cells might serve as the focus of either the cytolytic activity or the local cytokine release by effector $CD4^+$ T cells in the vaginal mucosa.

Vaccines designed to induce a cellular immune response at the vaginal mucosal surface would obviously be beneficial to the host not only in terms of limiting the primary epithelial infection, but perhaps by limiting the number of virions that gain access to neurons thus preventing or limiting the extent of latent infection. In the current study, HSV-specific T cells persisted in the urogenital tract at least 2 months after primary genital infection. Studies of genital *Chlamydia* infections in guinea pigs have correlated resistance to reinfection with the presence of *Chlamydia*-specific T cells in the urogenital tract. Resistance waned with time and was lost when antigen-specific T cells could no longer be detected in the vaginal tract (Igietseme and Rank, 1991). Antigen-specific T cells may be lost over time from the vaginal mucosa due to the constant shedding of mucosal cells during the normal female reproductive cycle. Studies are currently underway to assess the duration of HSV-specific memory T cell residence in the genital tract and develop ways to enhance recruitment of immune T cells to this mucosal surface.

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