T Lymphocytes Are Required for Protection of the Vaginal Mucosae and Sensory Ganglia of Immune Mice Against Reinfection with Herpes Simplex Virus Type 2

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T Lymphocytes Are Required for Protection of the Vaginal Mucosae and Sensory Ganglia of Immune Mice Against Reinfecion with Herpes Simplex Virus Type 2

Gregg N. Milligan, David I. Bernstein, and Nigel Bourne

Intravaginal inoculation of mice with an attenuated strain of herpes simplex virus type 2 (HSV-2) resulted in vigorous HSV-specific immune responses that protected against subsequent challenge with fully virulent HSV-2 strains. Even in the presence of high titers of HSV-specific Ab, T cell-dependent mechanisms were required for protection of the vaginal mucosae of HSV-immune mice and could be detected by 24 h after intravaginal reinoculation. Depletion of specific T cell subsets from HSV-immune mice before HSV-2 reinoculation demonstrated that CD4+ T cells were primarily responsible for this protection. Similarly, optimal protection of the sensory ganglia against reinfection with HSV-2 was dependent on the presence of T cells. Infectious HSV-2 was not detected in the sensory ganglia or spinal cord of HSV-immune mice depleted of only CD4+ or CD8+ T cells, suggesting that the T cell-mediated protection could be provided by either subset. Similarly, neutralization of IFN-γ during challenge of HSV-immune mice resulted in diminished protection of the vaginal mucosa, but not of the sensory ganglia. These results suggest that the ability to induce vigorous HSV-specific T cell responses is an important consideration in the design of vaccines to protect both the vaginal mucosa and sensory ganglia against HSV-2.


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Herpes simplex virus (HSV) normally invades its host at mucous membranes. In addition to replicating in the mucosal epithelia, HSV rapidly ascends nerve axons and gains access to the sensory ganglia (1, 2). Once in the neuronal cell body, HSV can replicate or establish a latent infection in which viral proteins and most viral RNA transcripts cannot be detected (3–5). Although the host immune system ultimately resolves the acute infection, HSV in its latent state efficiently avoids detection and clearance. As a result, HSV persists for the life of the host and may periodically reactivate, culminating in either asymptomatic shedding of the virus or recurrent disease in tissue near the original site of infection (1).

Immunization remains the best hope of preventing HSV disease. Ideally, vaccination would not only protect against disease but prevent infection of the mucosal surface. However, the task of complete protection of the mucosal epithelium against infection seems daunting given that immunization of animals with live attenuated virus or subunit vaccines rarely prevents reinfection with HSV-2 (6–11). A more realistic goal might be to prevent the establishment of a latent infection of the sensory ganglia and so prevent recurrent HSV disease and asymptomatic virus shedding. In this regard, it has been difficult to demonstrate superinfection of the sensory ganglia with HSV in animal models. Infection of mice (12–14), rabbits (15), or guinea pigs (8) with attenuated strains of HSV has been reported to prevent or severely reduce the ability of a second HSV strain to latently infect the sensory ganglia. However, the immune mechanisms responsible for this protection have not been identified and understood.

The current study examined the involvement of T lymphocytes in the immune protection of the vaginal mucosae and sensory ganglia of mice previously immunized by intravaginal inoculation with an attenuated HSV-2 strain. The results of these studies suggest that T lymphocytes played an important role in limiting HSV-2 replication in the vaginal mucosa by 24 h after intravaginal reinoculation of HSV-immune mice. Furthermore, T lymphocytes were required for optimal protection of the sensory ganglia of HSV-immune mice, suggesting that HSV-specific T cells, in addition to HSV-specific Ab, are an essential component in the protection of the sensory ganglia of the immune host against HSV reinfection. These results suggest that effective HSV vaccines will need to induce vigorous T cell-mediated immunity in addition to HSV-specific Ab responses to prevent latent infection of the sensory ganglia.

Materials and Methods

Mice

Six- to seven-week-old female BALB/c AnNHsd mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed in sterile microisolator cages in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) approved facility.

Virus

The bromodeoxyuridine-resistant mutant HSV-2 tk− strain 333, and fully virulent HSV-2 strain 186 were obtained originally from Dr. Lawrence Stanberry (Children’s Hospital Medical Center, Cincinnati, OH) and were grown and titrated on Vero cell monolayers, as described previously (7).

Intravaginal inoculation of mice

Mice were inoculated intravaginally with HSV-2 tk− or HSV-2 strain 186 by a modification of the method described previously (16). Briefly, mice were treated twice in a 1-wk period with 3 mg of medroxyprogesterone acetate (The Upjohn Company, Kalamazoo, MI). Mice were anesthetized...
with sodium pentobarbital, preswabbed, and inoculated using a calcium alginate swab (Spectrum Laboratories, Dallas, TX) soaked with 20 μl of virus suspension. Mice were immunized intravaginally with 5×10³ plaque-forming units (PFU) HSV-2 tk−. Mice were challenged 4 to 8 wk after the initial HSV-2 tk− inoculation with 5×10³ to 10⁴ PFU HSV-2 strain 186.

Detection of HSV-specific Ab

Vaginal washes and serum were obtained as described previously (7). For the ELISA assay, samples were plated in duplicate in wells coated with an HSV-2 glycoprotein preparation or a glycoprotein preparation from uninfected cells (mock Ag) as a control for nonspecific binding. Plates were developed using biotinylated anti-mouse IgG Ab (CalTag, San Francisco, CA), peroxidase-conjugated anti-biotin Ab (Vector Laboratories, Burlingame, CA), and anti-phenylendiamine dihydrochloride/peroxide (Sigma, St. Louis, MO), as described previously (7). The endpoint titer was defined as the dilution of serum or vaginal wash in HSV-2 glycoprotein-coated wells resulting in an OD₅₆₄ greater than 0.1 and greater than twice the OD₅₆₄ obtained from the same sample dilution plated on mock Ag-coated wells.

Virus neutralization assay

Neutralizing serum Ab titers were determined by a modification of the technique described previously (17). Briefly, serum from groups of 6 to 10 HSV-immune or nonimmune mice was pooled and heat inactivated at 56°C for 15 min. A series of twofold dilutions of serum was then made in tubes containing titration media (DMEM + 2% newborn calf serum, 2% penicillin-streptomycin, and 2% amphotericin B) and Low Tox M rabbit C antisera, 1:15 or 1/15. Approximately 600 PFU HSV-2 strain 186 was added to each tube containing diluted serum and to control tubes containing titration media plus C (1/15 final dilution) to achieve a final volume of 600 μl. Following incubation at 37°C for 1 h, HSV-2 PFU in each tube was quantified by titration on Vero cell monolayers. The neutralizing titer was expressed as the final serum dilution effecting a greater than 50% reduction in HSV-2 PFU compared with media plus complement alone.

In vivo depletion of immune cells and neutralization of IFN-γ

NK cells were depleted by i.v. injection of polyclonal rabbit anti-asialo GM1 Ab (Wako Chemicals USA, Richmond, VA) using the manufacturer’s recommended dosage, as described previously (16). Purified stocks of anti-murine CD4 (GL1.5), anti-murine CD8 (2.43), anti-murine Thy-1.2 (30H12), and an isotype control mAb (SFR8.B6, specific for HLA-Bw6) were prepared from culture supernatants and used to deplete CD4⁺, CD8⁺, or total T cells by a modification of the method described previously (16). Depletion of T cells or T cell subsets was verified by flow cytometry using a Becton Dickinson (San Jose, CA) FACSCalibur analyzer and CellQuest software at Division of Hematology/Oncology, Children’s Hospital Medical Center. Briefly, mice were depleted of specific T cell subsets by daily i.p. injections of 2 mg GL1.5 or 2.43 beginning 6 days before intravaginal HSV-2 reinoculation. This treatment regimen routinely resulted in depletion of 80 to 85% CD4⁺ T and nearly 100% of CD8⁺ T cells from the spleens, as measured on the day of HSV-2 inoculation (data not shown). To deplete total T cells, mice were injected on alternating days with 1 mg 30H12 (anti-Thy-1.2) or a mixture of Abs GL1.5 and 2.43 (1 mg each). This treatment resulted in depletion of approximately 90% of CD4⁺ and nearly 100% of CD8⁺ splenic T cells (data not shown). IFN-γ was neutralized in vivo, as described previously (16), by i.p. injection of 2 mg anti-IFN-γ mAb (XMGI.2) beginning 1 day before HSV-2 reinoculation and daily thereafter to the termination of the experiment. Using this treatment regimen, IFN-γ was not detected in vaginal secretions of mice on days 1 to 9 following primary HSV-2 vaginal inoculation (16) or on days 1 to 9 in HSV-immune mice rechallenged with HSV-2 (data not shown).

Assay of virus clearance from the vagina and sensory (lumbosacral) ganglia

To quantitate HSV-2 present in the vagina, mice were swabbed daily with moist calcium alginate swabs that were stored at −70°C in 1 ml of titration media until titration on Vero cell monolayers, as described previously (7). To quantitate HSV-2 present in the lumbosacral ganglia and spinal cords, these tissues were dissected, weighed, and homogenized in 1 ml cold titration media. Cell debris were removed by centrifugation at 5°C, and serial dilutions of homogenates were plated immediately on Vero cell monolayers.

Detection of IFN-γ in vaginal secretions

Vaginal secretions were collected by washing the vagina twice with 60-μl vol of HBSS plus 5% FCS. The wash was clarified by centrifugation and stored at −70°C until assay. The concentration of IFN-γ in the vaginal wash was determined by capture ELISA assay, as described previously (16). Briefly, plates were coated with purified anti-murine IFN-γ mAb (R4-6A2) at 5 μg/ml and blocked with PBS plus 5% BSA. A series of twofold dilutions of rIFN-γ standards (Sigma) or undiluted vaginal washes were plated in duplicate and incubated overnight at 4°C. Plates were washed and incubated with rabbit anti-murine IFN-γ Ab (Biosource International, Camarillo, CA), followed by peroxidase-conjugated goat anti-rabbit IgG Ab (United States Biochemical, Cleveland, OH). Plates were then washed, and developed with 4-phenylendiamine dihydrochloride/peroxide (Sigma) in citrate buffer, followed by OD₅₆₄ determination. The limit of detection of the assay was considered to be the last concentration of rIFN-γ standard, which gave an OD₅₆₄ value greater than the mean OD₅₆₄ plus 3 SDs of wells receiving only diluent.

Statistical analysis

Data were analyzed by one-way analysis of variance with the Bonferroni correction for multiple groups.

Results

T cells play an essential, early role in protection of the vaginal mucosa of HSV-immune mice

Nonimmune mice are exquisitely vulnerable to infection of the central nervous system following intravaginal inoculation with HSV-2 strain 186. Mice become symptomatic (hunched posture, ruffled fur, hind limb paralysis) usually beginning on day 5 after inoculation and die of encephalitis on days 6 to 8 after intravaginal inoculation with HSV-2 strain 186. However, mice survive infection with thymidine kinase-deficient HSV-2 strains, and the immunity elicited following primary infection with these HSV-2 strains has been shown to confer protection against subsequent challenge with normally lethal doses of wild-type HSV-2 (7, 8, 18). While HSV-specific Ab present in the vagina may be predicted to protect genital mucosal surfaces by impeding the initial infection or perhaps enhancing virus clearance, the role of HSV-specific T cells and innate immune cells such as NK cells in protection of the vaginal mucosa very early after reexposure to HSV-2 is unclear. To examine the requirement for these cell types in protection of the vaginal mucosa in HSV-immune mice, NK or T cells were depleted from mice previously immunized by intravaginal inoculation with HSV-2 tk− before challenge with the fully virulent HSV-2 strain 186. As we have shown previously (7), intravaginal inoculation of HSV-2 tk− resulted in vigorous HSV-specific Ab responses (Table I). HSV-specific Ab titers in serum and vaginal secretions and neutralizing serum Ab titers were equivalent in T cell-depleted, NK cell-depleted, and control-treated HSV-immune mice before HSV-2 reinoculation (Table I, Expt. 1). High titers of virus were detected in nonimmune mice within the first 24 h (Fig. 1), and these animals failed to clear the virus and usually died by days 6 to 7 after HSV-2 challenge. Despite the presence of HSV-specific Ab, control-treated HSV-immune mice could be reinfected with HSV-2; however, virus titers in the vaginae of these animals were reduced >90% at 24 h after reinoculation compared with nonimmune mice (p < 0.001), and virus was generally cleared from the vaginal mucosa by day 4 or 5. Vaginal HSV-2 titers in T cell-depleted HSV-immune mice were not significantly different from those of nonimmune animals on days 1 through 6 after HSV-2 challenge. Identical results were obtained if HSV-immune mice were challenged with a 20-fold lower dose of HSV-2 (G. Milligan, unpublished results).

NK cells have been identified as a source of IFN-γ in vaginal secretions during primary intravaginal infection with HSV-2 tk−, but apparently do not contribute to viral clearance (16). In contrast,
depletion of NK cells from HSV-immune mice before intravaginal reinoculation with HSV-2 resulted in significantly higher virus titers on days 1 to 6 compared with control-treated HSV-immune mice \( (p < 0.01, p < 0.001, p < 0.001, p < 0.01, p < 0.001, \text{and} \ p < 0.05 \text{ for days 1–6, respectively}) \), and a delay of greater than 2 days in virus clearance from the vaginal mucosa (Fig. 1).

Role of CD4\(^+\) and CD8\(^+\) T cells in protection of the vaginal mucosae of HSV-immune mice

Our previous studies demonstrated that Th1-type CD4\(^+\) T cells predominated the cell-mediated immune response following primary intravaginal inoculation with HSV-2 tk\(^-\) and were important for resolution of the primary vaginal infection, whereas depletion of CD8\(^+\) T cells had no effect on the ability of mice to clear virus from the vaginal mucosa (16, 19). To determine the importance of these T cell subsets in protection of the vaginal mucosae of HSV-immune mice against reinfection with HSV-2, CD4\(^+\) and CD8\(^+\) T cells were depleted from HSV-immune mice before virus challenge. As shown in the previous experiment, HSV-specific vaginal and systemic Ab levels and neutralizing Ab titers were equivalent among groups before HSV-2 challenge (Table I, Expt. 2). Depletion of CD8\(^+\) T cells from HSV-immune mice resulted in slightly higher mean vaginal HSV-2 titers on each day of infection than were detected in control-treated HSV-immune mice (Fig. 2). As a consequence, a slight delay in virus clearance was observed in CD8\(^-\)-depleted mice. However, depletion of CD4\(^+\) T cells from HSV-immune mice resulted in significantly higher virus titers compared with control-treated HSV-immune mice as early as 24 h after challenge \( (p < 0.01) \). Virus titers remained high in these mice and were not significantly different from titers of nonimmune mice on days 1, 3, and 4 after reinoculation. Although CD4\(^+\) T cell levels remained reduced through day 8 after inoculation (approximately 91\% reduction in CD4\(^+\) splenic T cells on day 8 after HSV-2 challenge), HSV-2 titers in CD4\(^+\) T cell-depleted mice fell rapidly after day 4, and virus was generally cleared from the vagina by days 6 to 7 after HSV-2 challenge.

Role of IFN-\(\gamma\) in protection of the vaginal mucosae of HSV-immune mice against HSV-2 reinfection

The elevated vaginal HSV-2 titers in HSV-immune mice depleted of total T cells (Fig. 1) or CD4\(^+\) T cells (Fig. 2) suggested an important role for T cells in protection of the vaginal mucosa as early as 24 h after reinoculation. IFN-\(\gamma\) has been shown to be essential for resolution of primary cutaneous and ocular HSV-1 infections (20, 21) and is an important component in conjunction with CD4\(^+\) T cells in the rapid clearance of HSV-2 tk\(^-\) from the

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Table I.  
**HSV-specific IgG Ab in serum and vaginal secretions following HSV-2 tk\(^-\) intravaginal immunization**

<table>
<thead>
<tr>
<th>Experiment 1(^a)</th>
<th>HSV-Specific Serum IgG (log 10 ± SEM)</th>
<th>Neutralizing Serum Ab Titer</th>
<th>HSV-Specific Vaginal IgG (log 3 ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-NK cell treated, HSV-immune</td>
<td>5.2 ± 0</td>
<td>1.640</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>Anti-T cell treated, HSV-immune</td>
<td>5.2 ± 0</td>
<td>1.640</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>Control treated, HSV-immune</td>
<td>5.2 ± 0</td>
<td>1.640</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>&lt; 1.7</td>
<td>&lt; 1:10</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Experiment 2(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CDS treated, HSV-immune</td>
<td>5.6 ± 0.1</td>
<td>1.640</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>Anti-CD8 treated, HSV-immune</td>
<td>5.8 ± 0.1</td>
<td>1.640</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Control treated, HSV-immune</td>
<td>5.8 ± 0.1</td>
<td>1.640</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>&lt; 1.7</td>
<td>&lt; 1:10</td>
<td>&lt; 1.0</td>
</tr>
</tbody>
</table>

---

\(a\) Serum and vaginal washes were taken from the HSV-immune mice used in the experiment described in Figure 1 prior to intravaginal HSV-2 challenge. HSV-specific IgG and neutralizing Ab titers were determined as described in Materials and Methods. The limit of antibody detection was 1.7 (log 10) for serum IgG, 1:10 for neutralizing antibody, and 1.0 (log 3) for vaginal IgG.

\(b\) HSV-specific and neutralizing Ab titers from the mice used in the experiment described in Figure 2 prior to intravaginal HSV-2 challenge.

---

FIGURE 1.  
Effect of depletion of T cells or NK cells on clearance of HSV-2 from the vaginal mucosae of HSV-immune mice. Mice inoculated intravaginally 2 mo previously with HSV-2 tk\(^-\) were treated as described in Materials and Methods with anti-Asialo GM1, or a mixture of Abs to murine Thy-1.2 (30-H12), CD4 (GK1.5), and CD8 (2.43) to deplete NK cells \( (n = 7) \) or T cells \( (n = 8) \), respectively. These mice, control-treated HSV-immune mice \( (n = 7) \), and age-matched nonimmune mice \( (n = 8) \) were then challenged intravaginally with 10\(^6\) PFU HSV-2 strain 186. Daily vaginal swabs were taken and titered on Vero cell monolayers. All nonimmune mice died by day 7. Results are expressed as the mean vaginal virus titer (log 10) ± SEM. Data from one representative experiment of two performed are shown.
vaginal mucosa of nonimmune mice (16). To determine when IFN-γ was present at the site of infection, vaginal secretions were sampled from nonimmune, T cell-depleted, NK cell-depleted, and control-treated HSV-immune mice and tested for the presence of IFN-γ. As reported previously (16), vaginal IFN-γ in nonimmune mice rose 2 days after vaginal HSV-2 inoculation, declined on day 3, and began increasing again on day 4 (Table II). Vaginal IFN-γ levels in control-treated HSV-immune mice were significantly higher than those of nonimmune mice at 24 h after HSV-2 challenge (p < 0.001) and continued rising on day 2 before declining on days 3 and 4. Depletion of T cells from HSV-immune mice before HSV-2 challenge resulted in significantly lower IFN-γ production at 24 and 48 h after virus challenge compared with HSV-immune controls (p < 0.001). NK cell-depleted HSV-immune mice also had significantly higher IFN-γ levels compared with nonimmune mice at 24 h (p < 0.001) and maintained high levels relative to control-treated HSV-immune mice on days 3 and 4 after HSV-2 reinoculation.

To test the importance of IFN-γ in protection of the vaginal mucosa from HSV-2 reinfection, HSV-immune mice were treated daily with anti-IFN-γ Ab beginning the day before HSV-2 challenge. The results of one such experiment of four performed are shown in Figure 3. HSV-immune mice could be reinfected with HSV-2, although vaginal virus titers were significantly less than those of nonimmune mice at 24 h after reinoculation (p < 0.001). Vaginal HSV-2 titers of anti-IFN-γ-treated HSV-immune mice varied somewhat among experiments at 24 h from slightly lower to slightly higher than the titers of control-treated HSV-immune mice. However, in all experiments, HSV-2 titers in anti-IFN-γ-treated HSV-immune mice rose after the 24-h time point and remained high through at least day 5 after reinoculation.

**Requirement for T cells in protection of the sensory ganglia and central nervous system**

Using a guinea pig model of genital HSV-2 infection, Stanberry et al. (8) showed that a previous genital infection with HSV-2 tk− prevented reinfection of the lumbosacral ganglia with a fully virulent strain of HSV-2. To extend these findings and investigate the immune basis for this protection, mice made immune by intravaginal inoculation with HSV-2 tk− were depleted of T cells before reinoculation with HSV-2, and the effect on protection of the lumbosacral ganglia and spinal cord was determined. As shown in Table III, infectious HSV was detected at high levels in the lumbosacral ganglia and spinal cord of nonimmune mice on days 4 to 8 after challenge. In contrast, no infectious virus was detected during the same time period at either site in control-treated HSV-immune mice. Depletion of T cells from HSV-immune mice resulted in the presence of infectious HSV-2 in the lumbosacral ganglia in two of five mice on day 4, three of five mice on day 6, and four of four mice on day 8 after reinoculation. Virus titers in infected ganglia of T cell-depleted HSV-immune mice were slightly lower than in nonimmune mice on day 4, but were similar on days 6 and 8 after reinoculation. Similar results were obtained following examination of spinal cords for infectious HSV-2 (Table III).

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**FIGURE 2.** Clearance of HSV-2 from the vaginal mucosa of HSV-immune mice depleted of CD4+ or CD8+ T cells. Mice were immunized against HSV-2 by intravaginal inoculation with HSV-2 tk−. Two months later, groups of 10 HSV-immune mice were treated as described in Materials and Methods with anti-murine CD4 (OK1.5), anti-murine CD8 (2.43), or the isotype-matched control Ab SFR8.B6. These mice and age-matched nonimmune mice were then challenged intravaginally with 5 × 10⁴ PFU HSV-2 strain 186. HSV-2 titers were determined as described in Materials and Methods. The results are expressed as the mean vaginal HSV-2 titer (log 10) ± SEM. The results of one representative experiment of two performed are shown.

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**Table II.** Detection of IFN-γ in vaginal secretions of HSV-immune mice reinoculated intravaginally with HSV-2

<table>
<thead>
<tr>
<th>Mice</th>
<th>IFN-γ (U/ml ± SEM)</th>
<th>Day −1</th>
<th>Day +1</th>
<th>Day +2</th>
<th>Day +3</th>
<th>Day +4</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell depleted, HSV-immune</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.7 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>NK cell depleted, HSV-immune</td>
<td>0.0 ± 0.0</td>
<td>4.1 ± 0.9</td>
<td>34.8 ± 3.7</td>
<td>55.3 ± 6.9</td>
<td>41.2 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>Control treated, HSV-immune</td>
<td>0.0 ± 0.0</td>
<td>18.2 ± 0.1</td>
<td>31.1 ± 2.6</td>
<td>9.8 ± 0.2</td>
<td>2.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Nonimmune</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>27.8 ± 2.6</td>
<td>1.5 ± 0.2</td>
<td>3.2 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Vaginal washes were taken on the indicated day and IFN-γ in vaginal secretions was quantified by ELISA as described in Materials and Methods. Similar results were obtained in a separate experiment.

* Groups of 7 to 8 HSV-immune mice were treated as described in Materials and Methods to deplete the indicated cell populations. These mice and nonimmune mice (8) were then inoculated intravaginally with 5 × 10⁴ PFU HSV-2 strain 186.

* Indicates day vaginal secretions were sampled relative to the day of HSV-2 challenge.
To further explore the T cell requirement for protection of the sensory ganglia of HSV-immune mice, the requirements for specific T cell subsets or IFN-γ were determined. Based on the kinetics of HSV-2 detection in the sensory ganglia of T cell-depleted HSV-immune mice (Table III), the presence of infectious HSV-2 in the sensory ganglia and spinal cords of HSV-immune mice depleted of CD4+ or CD8+ T cells or treated with anti-IFN-γ Ab was determined on days 6 and 8 after HSV-2 challenge. Depletion of CD8+ T cells from HSV-immune mice before reinoculation did not abrogate protection of the sensory neurons (Table IV). Similarly, although depletion of CD4+ T cells or neutralization of IFN-γ in HSV-immune mice resulted in high titers of HSV-2 in the vaginal mucosa (Figs. 2 and 3), no infectious virus was detected in either the lumbosacral ganglia or spinal cord of anti-IFN-γ-treated or CD4-depleted HSV-immune mice (Table IV).

Discussion

Mice inoculated intravaginally with an attenuated strain of HSV-2 develop vigorous Ab and cell-mediated immune responses (7, 18, 19) that have been shown to protect the animals from death following reinoculation with normally lethal doses of fully virulent HSV-2 (7, 18, 22, 23). We used this system as a paradigm to examine the immune basis for the protection of the vaginal mucosa and sensory ganglia. Although prior intravaginal inoculation with HSV-2 tk- did not prevent reinfection of the vaginal mucosa (Figs. 1–3), it did result in a significant reduction in the magnitude of HSV-2 replication in the vagina. Importantly, intravaginal HSV-2 tk- immunization resulted in protection of the sensory ganglia and central nervous system from HSV-2 reinfection, as measured by the absence of detectable infectious HSV-2 in the lumbosacral ganglia and spinal cord after intravaginal reinoculation. The results also demonstrate that HSV-specific T cells played an essential role in protection within the first 24 h after challenge of HSV-immune mice. Furthermore, while it is generally accepted that HSV-specific Ab plays an important role in preventing or limiting the spread of HSV within the sensory ganglia (24–26), HSV-specific T cells were also necessary for optimal protection of the sensory ganglia of HSV-immune mice.

The involvement of HSV-specific T cells in resolution of HSV infections has been well documented (25, 27–29), and as expected, depletion of T cells from HSV-immune mice abrogated the ability of these animals to resolve an HSV-2 vaginal infection (Fig. 1). Interestingly, these results also suggest that the greater than 90% reduction in virus titer observed in control-treated HSV-immune animals compared with nonimmune mice within the first 24 h following reinoculation was not attributable solely to prevention of

Table III. Effect of depletion of T cells on protection of the sensory ganglia and spinal cords of HSV-immune mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Tissue</th>
<th>Day 4*</th>
<th>Titer (log_{10} ± SEM)</th>
<th>Day 6</th>
<th>Titer (log_{10} ± SEM)</th>
<th>Day 8</th>
<th>Titer (log_{10} ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmune</td>
<td>Ganglia</td>
<td>5/5</td>
<td>4.56 ± 0.12</td>
<td>5/5</td>
<td>4.61 ± 0.18</td>
<td>2/2</td>
<td>3.98 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>5/5</td>
<td>3.47 ± 0.16</td>
<td>5/5</td>
<td>4.38 ± 0.09</td>
<td>2/2</td>
<td>4.39 ± 0.32</td>
</tr>
<tr>
<td>T cell depleted, HSV-immune</td>
<td>Ganglia</td>
<td>2/5</td>
<td>3.35 ± 0.05</td>
<td>3/5</td>
<td>4.55 ± 0.28</td>
<td>4/4</td>
<td>4.77 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>1/5</td>
<td>3.14 ± 0.00</td>
<td>2/5</td>
<td>3.88 ± 0.23</td>
<td>4/4</td>
<td>4.43 ± 0.46</td>
</tr>
<tr>
<td>Control treated, HSV-immune</td>
<td>Ganglia</td>
<td>0/5</td>
<td>&lt;</td>
<td>0/5</td>
<td>&lt;</td>
<td>0/5</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>0/5</td>
<td>&lt;</td>
<td>0/5</td>
<td>&lt;</td>
<td>0/5</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

* The results of a single experiment are shown for the given day relative to HSV-2 challenge. Similar results were obtained for days 6 and 8 after HSV-2 challenge in a separate experiment.

b Three of five nonimmune mice died on day 7.

HSV-2 titers indicated by “<” fell below the level of detection of 50 PFU/ml.
Table IV. Effect of depletion of T cell subsets or neutralization of IFN-γ on protection of the sensory ganglia and spinal cord of HSV-immune mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Tissue</th>
<th>Day 6</th>
<th>Titer (log10 ± SEM)</th>
<th>No. Positive/total incidence</th>
<th>Titer (log10 ± SEM)</th>
<th>No. Positive/total incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonimmune</td>
<td>Ganglia</td>
<td>14/15</td>
<td>4.19 ± 0.38</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>14/15</td>
<td>3.78 ± 0.45</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-IFN-γ treated, HSV-immune</td>
<td>Ganglia</td>
<td>0/8</td>
<td>&lt;c</td>
<td>0/9</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>0/8</td>
<td>&lt;c</td>
<td>0/9</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Anti-CD4 treated, HSV-immune</td>
<td>Ganglia</td>
<td>0/10</td>
<td>&lt;c</td>
<td>0/11</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>0/10</td>
<td>&lt;c</td>
<td>0/11</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Anti-CD8 treated, HSV-immune</td>
<td>Ganglia</td>
<td>0/10</td>
<td>&lt;c</td>
<td>0/10</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>0/10</td>
<td>&lt;c</td>
<td>0/10</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Control treated, HSV-immune</td>
<td>Ganglia</td>
<td>0/12</td>
<td>&lt;c</td>
<td>0/14</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>0/12</td>
<td>&lt;c</td>
<td>0/14</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

*Results obtained from at least two separate experiments are shown for the given days relative to HSV-2 challenge.

a All nonimmune mice in each experiment died prior to day 8. Values on this day were not determined (ND).

b HSV-2 titers indicated by “<” fell below the level of detection of 50 PFU/ml.

HSV-2 infection by neutralizing Ab. Our results suggest that both T cell subsets played a role in clearance of HSV-2 from the vaginal mucosa. However, the early T cell-dependent protection was apparently provided primarily by CD4+ T cells, as selective depletion of this subset from HSV-immune mice had a more detrimental effect on clearance of virus from the vagina than did depletion of CD8+ T cells (Fig. 2). Given that mice depleted of both T cell subsets do not clear HSV-2 from the vagina (Fig. 1), we believe the late clearance of virus from CD4-depleted mice was most likely achieved by infiltrating HSV-specific CD8+ T cells. Given the low numbers of HSV-specific CD8+ T cells in this model (19), CD8+ T cells may not arrive in the vagina in numbers sufficient to affect clearance until later in the infection. It may be that CD4+ T cells were responsible for early virus clearance, while CD8+ T cells were important later to remove remaining foci of infected cells.

Several mechanisms may account for the role of CD4+ T cells in protection of the vaginal mucosa. CD4+ CTL have been detected following infection with a number of viruses, including HSV (19, 30); however, the role of cytotoxicity mediated by these cells in virus clearance remains uncertain. It is possible that the protection in the current study may reflect the ability of CD4+ CTL residing in the vaginal mucosa as a result of the initial HSV-2 tk− inoculation to recognize and kill HSV-2-infected vaginal epithelial cells. In this regard, we have demonstrated previously CD4+ CTL precursors in the genital lymph nodes of mice following intravaginal inoculation of HSV-2 tk− (19). Additionally, Parr et al. (31) demonstrated that vaginal epithelial cells of mice previously inoculated with HSV-2 tk− expressed high levels of MHC class II Ags 1 day after intravaginal HSV-2 rechallenge. This increase in MHC class II expression was most likely the result of exposure to the IFN-γ present in vaginal secretions early after HSV-2 challenge (Table II). Although it is possible that HSV infection may alter MHC class II expression in murine vaginal epithelial cells, Mikloska et al. (32) have shown that MHC class II expression by IFN-γ-treated human keratinocytes was not altered by HSV infection. It therefore seems possible that CD4+ memory CTL present in the vaginal mucosa as a result of HSV-2 tk− immunization may have been activated following HSV-2 rechallenge and recognized processed HSV-2 peptides in association with MHC class II proteins on infected vaginal epithelial cells.

While CD4+ CTL may play some role in virus clearance, it is unlikely that sufficient numbers of effector CTL would be present over the entire mucosal surface of the vagina within the first 24 h of rechallenge to limit the virus infection. Alternatively, the protection may be attributable to CD4+ T cell production of cytokines such as IL-3, TNF-α, or IFN-γ, which have been shown to play a role in resolution of HSV infections (16, 20, 21, 33, 34). In this regard, we detected IFN-γ in vaginal secretions of HSV-immune mice 24 h after HSV-2 rechallenge (Table II). The diminished IFN-γ levels in T cell-depleted HSV-immune mice compared with NK cell-depleted mice suggest that the presence of IFN-γ in vaginal secretions at 24 h is primarily dependent on T cells (Table II). We previously showed that neutralization of IFN-γ delayed resolution of a primary intravaginal infection with HSV-2 tk− (16). In the present study, neutralization of IFN-γ in HSV-immune mice resulted in a dramatic increase in vaginal virus burden compared with control-treated HSV-immune mice. Interestingly, the absence of IFN-γ during the first 24 h after challenge of HSV-immune mice appeared to have little effect on vaginal virus titers (Fig. 3). However, as the infection progressed, neutralization of IFN-γ resulted in significantly higher virus titers and a failure to clear the HSV-2 infection by day 6 after intravaginal challenge. These results suggest that IFN-γ produced by T cells, although important for the ultimate resolution of the challenge infection, is not solely responsible for the T cell-mediated protection observed at 24 h after rechallenge. Other cytokines, such as TNF-α, or granulocyte/macrophage CSF may have acted in synergy with IFN-γ to promote protection of the vaginal mucosa by augmenting innate immune responses or interfering with HSV-2 replication (35).

Delayed-type hypersensitivity mediated by Ag-specific CD4+ T cells has been suggested to play a role in clearance of HSV-1 from the skin (27). It is possible that this mechanism, induced by HSV-specific CD4+ T cells, may also be responsible for HSV clearance from the vaginal mucosa of HSV-immune mice in the present study. The role of delayed-type reactions in clearance of virus from the vagina is supported by the infiltration of large numbers of macrophages and neutrophils into the vaginal tissue soon after intravaginal challenge of HSV-immune mice (G. Milligan, unpublished results) as well as the detection of large quantities of IFN-γ in vaginal secretions of HSV-immune mice early following intravaginal rechallenge (Table II). Activation of macrophages and granulocytes by cytokines such as IFN-γ or TNF-α may result in clearance of HSV by mechanisms such as direct phagocytosis of virus particles (36, 37), antibody-dependent cellular cytotoxicity of infected cells (38, 39), production of nitrogen metabolites (40), or direct lysis of virus-infected cells (41). It is possible that the diminution of virus clearance from the mucosa observed following neutralization of IFN-γ (Fig. 3) may have been a reflection of...
insufficient macrophage activation for efficient viral clearance. The regulation of innate immune cells such as macrophages and neutrophils by HSV-specific T cells and their role in protection of the vaginal mucosa are currently unclear and are the subject of ongoing investigation in our laboratory.

Depletion of either NK cells (Fig. 1) or CD8+ T cells (Fig. 2) from HSV-immune mice also delayed HSV-2 clearance, but less dramatically than depletion of CD4+ T cells. These results suggest that these cells, while capable of effecting virus clearance, play a lesser role relative to that of CD4+ T cells in clearance of HSV-2 from the vaginal mucosa of HSV-immune mice. The contribution of CD8+ T cells and NK cells in HSV-2 clearance from HSV-immune mice contrasts with their apparent lack of involvement in resolution of a primary HSV-2 intravaginal infection (16). This difference may reflect greater numbers of these cell populations residing in the vagina of mice previously immunized by intravaginal inoculation of HSV-2 tk- compared with nonimmune mice. Alternatively, infiltration of these cells into the vagina may have been enhanced after re inoculation of HSV-immune mice with HSV-2. NK cell activity in HSV-immune mice may also have been enhanced by cytokines such as IL-2 and IFN-γ produced by vaginal HSV-specific T cells. Because NK cells are present in the vaginal mucosa before infiltration of HSV-specific T cells following primary HSV-2 inoculation (26), the lack of involvement of NK cells in resolution of a primary HSV infection might reflect insufficient T cell-derived cytokines necessary for complete NK cell effector function.

Based on the results of studies involving passive transfer of HSV-specific Ab to HSV-infected nonimmune mice (42–46) or HSV infection of B cell-suppressed mice (47), HSV-specific Ab is assumed to limit the spread of HSV to and within the nervous system. In the current study, although high titers of HSV-specific, neutralizing Ab were present in serum and vaginal secretions, T cells were necessary for optimal protection of the sensory ganglia from HSV infection. While it is possible that HSV-2 reached the sensory ganglia of control-treated HSV-immune mice at levels below detection by our infectious virus assay, the results of these experiments still demonstrate that effective protection of the ganglia was achieved by prior intravaginal immunization with an attenuated strain of HSV-2 and that T cells were responsible in part for this protection. Depletion of either T cell subset had no apparent detrimental effect on protection of the sensory ganglia (Table IV), suggesting that the protection could be provided by either subset. Surprisingly, vaginal HSV-2 titers in HSV-immune mice depleted of CD4+ T cells or treated with anti-IFN-γ were comparable with those of nonimmune mice, although these mice still displayed effective protection of the sensory ganglia. These results suggest that multiple immune mechanisms are responsible for protection of the sensory ganglia and are effective even in the presence of high titers of virus in the mucosa.

The mechanism by which T cells protect the sensory ganglia is not understood. Passive transfer of HSV-immune lymphocytes has been shown to restrict the acute infection of the sensory ganglia (24) and reduce the number of latently infected neurons (48). Because the protective effect was only observed if transfer of lymphocytes occurred before virus reached the sensory ganglia (24), it is tempting to speculate that T cells function in protection of the sensory ganglia in an indirect manner by effecting rapid viral clearance from the epithelia. Given the ability of HSV to reach the ganglia within 24 to 48 h after inoculation (24, 49, 50), HSV-specific memory T cells must be activated and provide their effector function very soon after virus inoculation to provide significant protection of the ganglia. The results of the T cell-depletion experiments in the current study are consistent with this assumption and suggest that vaginal T cells primed by an initial intravaginal HSV infection play a critical role in virus clearance from the genital mucosa by the first 24 h of re inoculation. HSV-2 titers in the vaginas of control-treated HSV-immune mice were reduced greater than 90% compared with nonimmune or T cell-depleted HSV-immune mice at 24 h after re inoculation. At these lower virus titers, neutralizing Ab or innate immune mechanisms may be sufficient to protect the nerve endings from HSV-2 infection.

It is also possible that HSV-specific T cells act within the sensory ganglia to clear virus that escapes neutralization by HSV-specific Ab. T lymphocytes have been shown to infiltrate sensory ganglia by 5 days after primary HSV inoculation and remain at this site for at least 6 mo (51, 52). Furthermore, CD8+ T cells that infiltrate the ganglia following cutaneous HSV-1 infections play a role in virus clearance from the peripheral nervous system by a nonlytic mechanism (53). In the present study, T cells that infiltrated the sensory ganglia as a result of HSV-2 tk- immunization may have prevented superinfection of the ganglia by the challenge virus in a similar fashion. Experiments are currently underway to further establish the mechanisms by which T cells cooperate in the protection of the sensory ganglia as well as to identify the anatomical sites at which they act.

Mucosal Ab may serve as a barrier that prevents or limits the infection of mucosal cells. The present studies did not directly address the role of neutralizing Ab in protection, but do demonstrate effective protection of the vaginal mucosa and sensory ganglia in the presence of both HSV-specific T cells and Ab. These results suggest that HSV-specific T cells in the vaginal mucosa were required to clear virus that penetrated the mucosal Ab barrier. In fact, the data are consistent with a model in which HSV-specific T cells and Ab work synergistically to limit the infection and clear virus that penetrates the Ab barrier in vaginal secretions. In this regard, passive transfer of an HSV glycoprotein D-specific mAb has been shown to prevent HSV-1-induced encephalitis in the absence of one, but not both, T cell subsets (54).

The very rapid T cell response to HSV-2 intravaginal re inoculation in these studies may reflect the presence of virus-specific memory T cells in the vaginal mucosa. In this regard, we have shown previously that HSV-specific memory T cells reside in the vaginal mucosa for at least 2 mo following intravaginal HSV-2 tk- inoculation (19). The results of the current studies indicate that very efficient protection of the vaginal mucosa and sensory ganglia against HSV-2 infection may be achieved by intravaginal immunization. Because the vagina is a poor immune inductive site for nonreplicating vaccines (55), further studies are currently underway to determine whether similar levels of humoral and cell-mediated immune protection can be provided to the vaginal mucosa and sensory ganglia by immunization at alternative sites.

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References
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