Interferon-\(\gamma\) Enhances Resolution of Herpes Simplex Virus Type 2 Infection of the Murine Genital Tract

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Received October 24, 1996; returned to author for revision November 27, 1996; accepted January 7, 1997

The requirement for interferon-\(\gamma\) (IFN\(\gamma\)) in resolution of an HSV-2 vaginal infection and the cellular sources of this cytokine in the vaginal mucosa were assessed. IFN\(\gamma\) levels in vaginal secretions peaked on Days 2 and 5 following HSV-2 inoculation. Natural killer (NK) cell depletion greatly diminished the early production of IFN\(\gamma\) but had no significant effect on the rate of virus clearance. CD4\(^+\) T cells were primarily responsible for the second peak of IFN\(\gamma\) levels and neutralization of this IFN\(\gamma\) beginning 3 days after virus inoculation delayed, but did not prevent, virus clearance from the vagina. HSV-2 persisted in mice depleted of both CD4\(^+\) and CD8\(^+\) T cells while clearance was delayed in CD4\(^+\), but not CD8\(^+\) T cell-depleted mice, demonstrating the T cell dependence and predominant role of CD4\(^+\) T cells in resolution of the infection. Together, these data suggest that IFN\(\gamma\) is not essential for virus clearance but plays an important role in enhancing T cell-mediated clearance mechanisms. The implication of these results is that IFN\(\gamma\) produced locally in the genital tract enhances virus clearance and may ultimately be important for reducing the amount of virus available to infect sensory ganglia.

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) infects both the oral and genital mucosa of humans. In addition to initial replication and primary disease at the site of infection, the virus can ascend sensory neurons, replicate, and become latent in the sensory ganglia. Reactivation of latent virus can result in recurrent disease at the site of initial infection. In addition to the pain associated with HSV infections, the lesions associated with primary and recurrent disease at the mucosal surface may also serve as efficient portals of entry for other sexually transmitted diseases.

Clearance of virus from infected tissue is most often T cell-mediated and involves both cytokine-mediated effector mechanisms and direct cytotoxic effects on virus-infected cells. Clearance of acute HSV infections can be accomplished by either CD4\(^+\) or CD8\(^+\) T cells depending on variables such as virus dose, tissue site, or mouse strain (Nash et al., 1987; Wildy and Gell, 1985; Leung et al., 1984). A recent study using mice lacking CD4\(^+\) or CD8\(^+\) T cells as a result of targeted gene deletions demonstrated that effective clearance from the skin was dependent mainly on CD4\(^+\) T cells (Manickan and Rouse, 1995). In a cutaneous model of HSV-1 infection using genetically resistant mice, virus clearance could be effected by either T cell subset and was dependent on the presence of interferon-\(\gamma\) (IFN\(\gamma\)) (Smith et al., 1994). Similarly, HSV-1 persisted in the eyes of mice with a targeted deletion of the IFN\(\gamma\) gene as compared to wild-type littermates in a model of herpetic stromal keratitis (Bouley et al., 1995).

The immune mechanisms responsible for clearing HSV-2 from the genital mucosa have not been well studied. Protection of mucosal surfaces is generally thought to be a function of secretory IgA. However, for pathogens such as HSV which can spread intracellularly, cellular immune mechanisms are required for resolution of infection. It has been shown that adoptive transfer of T lymphocytes from the draining lymph nodes of HSV-infected mice protected naive recipients from a lethal challenge of the genital mucosa with HSV-2 (McDermott et al., 1989). More recently, it was shown that intravaginal inoculation with an attenuated strain of HSV-2 resulted in the influx of HSV-specific Th 1-type CD4\(^+\) T cells into vaginal tissue (Milligan and Bernstein, 1995a), which apparently coincided with the rapid resolution of the infection. In the current study, a murine model of genital HSV-2 infection was used to examine the role of IFN\(\gamma\) in clearance of HSV-2 from the vaginal mucosa. Intravaginal inoculation with HSV-2 resulted in sequential production of IFN\(\gamma\) by NK cells then T lymphocytes; however, only IFN\(\gamma\) produced primarily by T cells after Day 3 played a role in rapid clearance of virus from the genital tract. The results of this study suggest that IFN\(\gamma\) was not essential for the ultimate clearance of virus from the vaginal mucosa.
but was important for increasing the effectiveness of T cell-dependent mechanisms of virus clearance.

MATERIALS AND METHODS

Mice

Female BALB/cAnNHsd (Harlan–Sprague–Dawley, Inc., Indianapolis, IN) were used between 6 and 15 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 grown and titrated as described previously (Milligan and Bernstein, 1995b). HSV-2 strain 186 was obtained from Dr. Lawrence Stanberry (Children’s Hospital Medical Center, Cincinnati, OH) and used as a challenge virus in these studies as described previously (Milligan and Bernstein, 1995b).

Antibodies

Rabbit anti-asialo GM1 was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Hybridomas 2.43 (anti-murine CD8), GK1.5 (anti-murine CD4), R46A2 (anti-murine IFNγ), and HO2.2 (anti-murine CD8) were obtained from the American Type Culture Collection (Rockville, MD). Hybridoma XMG1.2 (anti-murine IFNγ) was obtained with permission of Dr. Tim Mosmann (Univ. of Alberta, Alberta, Canada). Hybridomas were grown to log phase in RPMI supplemented with 20% fetal calf serum (FCS) (GIBCO Life Technologies, Gaithersburg, MD) and then seeded into 150-cm² flasks containing Hybridoma Serum Free Media (GIBCO Life Technologies). After 5–7 days, cells were removed by centrifugation and antibody was precipitated from supernatants with ammonium sulfate (Sigma, St. Louis, MO), dissolved in distilled water, and dialyzed extensively against phosphate-buffered saline (pH 7.2). Antibody purification was monitored by SDS–PAGE and antibody concentration was determined by UV spectrometry.

Genital herpes inoculation and vaginal virus titration

Mice were inoculated intravaginally with HSV-2 333 tk− or HSV-2 186 by a modification of the method described previously (Milligan and Bernstein, 1995b). Briefly, mice which had been treated twice in a 1-week period with 6α-methyl-17α-hydroxyprogesterone acetate (Sigma) at 3 mg/mouse were preswabbed with a cotton pledget, anesthetized with sodium pentobarbital, and inoculated with 20 µl of virus suspension (5 × 10⁵–10⁶ PFU HSV-2 333 tk−; 10⁶ PFU HSV-2 186) soaked onto a type 2 calgiswab. Vaginal virus titers were monitored by swabbing the vagina with a moist type 2 calgiswab which was immediately added to 1.0 ml media followed by storage at –70° until plaque titration on Vero cell monolayers as described previously (Milligan and Bernstein, 1995b).

In vivo depletion of T cell subsets and neutralization of IFNγ

Mice were injected intraperitoneally (i.p.) with 1.5 mg purified GK1.5 or 2.43 antibodies on Days 5, 4, 2, and 1 prior to HSV-2 inoculation, on the day of inoculation, and every other day thereafter to Day 6. Mice treated with a mixture of 1.5 mg each of GK1.5 and 2.43 were treated to Day 8. Splenic and gLN lymphocytes from antibody-treated and control-treated mice were analyzed on the day of inoculation by flow cytometry to determine in vivo depletion. GK1.5 antibody treatment typically resulted in 80–87% depletion of CD4⁺ gLN T cells and treatment with 2.43 antibody typically resulted in complete (100%) CD8⁺ T cell depletion. Treatment with a cocktail of GK1.5 and 2.43 antibodies resulted in an 87% depletion of splenic CD3⁺ lymphocytes. In preliminary experiments, virus clearance in mice treated with PBS or an irrelevant rat IgG monoclonal antibody was not different, therefore PBS was used as a control treatment in these studies. IFNγ was neutralized in vivo by daily i.p. injection of 1.5 mg purified XMG1.2 antibody from the day prior to inoculation through Day 7 after inoculation. In some experiments, treatment of mice was performed on Days 3 through 7 after inoculation. NK cells were depleted by intravenous injection of 50–75 µl of rabbit anti-asialo GM1 antibody (dosage based on manufacturer’s antibody titer) 2 days before and 1 day following intravaginal inoculation with HSV-2 tk⁻.

Depletion of T lymphocyte subsets in vitro

CD4⁺ or CD8⁺ T cells were depleted as described previously (Milligan and Bernstein, 1995a) with optimal dilutions of GK1.5 or HO2.2 antibody, respectively, and low tox M rabbit complement (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada). Cells were washed three times and counted prior to culture.

IFNγ ELISPOT assay

A modification of the procedure described previously (Milligan and Bernstein, 1995a) was used in these studies. Briefly, single cell suspensions of gLN lymphocytes from uninoculated and HSV-2 tk⁻-inoculated mice were resuspended to 10⁶ cells/ml in RPMI supplemented with 10% FCS. One hundred microliters of this cell suspension and a series of threefold dilutions was plated in duplicate on nitrocellulose filter plates (Millipore Corp., Bedford, MA) coated previously with 10 µg/ml purified R4-6A2 antibody. Plates were incubated overnight at 37°, washed thoroughly to remove cells, and incubated with rabbit anti-murine IFNγ (Biosource International, Camarillo, CA) followed by peroxidase-conjugated goat anti-rabbit IgG.
(United States Biochemical Corp., Cleveland, OH). Plates were then washed and developed with 3-aminophenyl carbazol substrate and peroxide. Spots representing specific cytokine-secreting cells were counted with the aid of a dissecting microscope.

IFNγ ELISA

IFNγ was detected in vaginal secretions by washing the vagina twice with 60 μl Hank’s balanced salt solution plus 5% FCS. The wash was clarified by microcentrifugation and stored at −70°C until quantitation by ELISA. ELISA plates were coated with purified R4-6A2 (anti-murine IFNγ) at 5 μg/ml and blocked with PBS plus 5% bovine serum albumin. A series of twofold dilutions of rIFNγ standards (Sigma) at concentrations from 125 to 0.12 Units/ml and undiluted vaginal washes were plated in duplicate and incubated overnight at 4°C. Plates were then washed and developed with the same reagents as for IFNγ ELISPOT plates except that o-phenylenediamine dihydrochloride (OPD)/peroxide (Sigma) was used as the substrate. The limit of detection of the assay was considered to be the last concentration of rIFNγ standard which gave an OD490 value greater than the mean plus 3 standard deviations of the background (media alone) wells.

Statistical analysis

Data were analyzed by unpaired, two-tailed t tests or one-way analysis of variance with the Bonferroni correction for multiple groups as appropriate.

RESULTS

IFNγ production in mice infected vaginally with HSV-2 tk−

The production of IFNγ at the vaginal mucosal surface following genital inoculation with HSV-2 tk− was biphasic (Fig. 1). The first IFNγ peak occurred on Day 2 and fell to low levels on the third day after inoculation. Mice depleted of NK cells by in vivo treatment with anti-asialo GM1 antibody produced significantly lower amounts of IFNγ in vaginal secretions on Day 2 than control mice treated with PBS or normal rabbit serum (P < 0.01) (Fig. 1). By contrast, Day 2 vaginal IFNγ levels in mice depleted of T cells were not significantly different than those of control-treated mice. IFNγ levels rose again after Day 4 in control-treated mice, peaked on Day 5, and fell to low levels on Day 8. IFNγ was detected at comparable levels in NK cell-depleted mice on these days but was undetectable in Day 5 vaginal secretions of T cell-depleted mice and was detected only at very low levels on Day 6–8 postinoculation.

In vivo neutralization of IFNγ results in delayed clearance of vaginal HSV-2 tk−

To examine the role of IFNγ in clearance of HSV-2 from the genital mucosa during a primary infection, IFNγ was neutralized in vivo by daily treatment of mice with anti-IFNγ antibody beginning the day prior to HSV-2 tk− infection. IFNγ was detected by ELISA in vaginal washes from control-treated but not in anti-IFNγ−treated mice through Day 9 postinoculation (Table 1). No significant differences in vaginal virus titers of control-treated and anti-IFNγ−treated mice were observed through the first 3 days of infection (Fig. 2A). Control-treated mice rapidly cleared virus beginning on Day 4 while virus titers decreased more slowly in anti-IFNγ−treated mice (Fig. 2A), resulting in an ultimate delay in viral clearance of approximately 2 days.

To determine if the IFNγ produced during the first 3 days of infection was sufficient to effect the rapid clearance of virus from the vagina, anti-IFNγ treatment was initiated 3 days after genital inoculation at a time coincident with the end of the NK cell IFNγ production (Days 2–3) but before T cell-mediated IFNγ production began (Days 4–7). No significant differences in the kinetics of virus clearance from the vagina were observed between control-treated and anti-IFNγ−treated mice through the first 4 days of infection (Fig. 2B). However, viral titers were significantly elevated in treated mice on Days 5–7 and, similar to the previous experiment (Fig. 2A), clearance was delayed by about 2 days.

Clearance of HSV-2 from the vaginal mucosa requires T cells but not NK cells

To determine if NK cells played a role in clearance of HSV-2 from the vaginal mucosa, BALB/c mice were depleted of NK cells by treatment with anti-asialo GM1 antibody prior to genital HSV-2 tk− inoculation. For comparison, separate groups of mice were control treated or made deficient in T lymphocytes by treatment with antibodies to CD4 and CD8 (Fig. 3). No significant differences in mean vaginal virus titers were observed among NK cell-depleted, T cell deficient, and control-treated mice for the first 3 days of infection (Day 1, P = 0.47; Day 2, P = 0.13; Day 3, P = 0.8). However, the mean vaginal virus titer in anti-CD4/CD8 antibody-treated mice was significantly greater than that of the other groups on Day 4 (P < 0.05). Further, NK cell-depleted mice and control-treated mice cleared the virus rapidly on Days 5 and 6, whereas virus persisted in vaginal secretions of T cell-deficient mice through at least Day 10.

Role of CD4+ and CD8+ T cells in IFNγ production and clearance of virus from genital mucosa

BALB/c mice were depleted of CD4+ or CD8+ T cells prior to genital inoculation with HSV-2 tk− to examine the importance of these T cell subsets for vaginal IFNγ production and virus clearance from the vaginal mucosa. Treatment of mice with anti-CD4 antibody resulted in altered kinetics of vaginal virus clearance (Fig. 4A). There
FIG. 1. Detection of IFN-γ in vaginal secretions following intravaginal HSV-2 tk- inoculation. Mice were treated with antibodies to CD4 and CD8 (triangles), anti-asialo GM1 (diamonds), or normal rabbit serum (squares) as described under Materials and Methods. Vaginal washes were taken from BALB/c ANnhsd at 24-hr intervals after genital HSV-2 tk- inoculation. The concentration of IFN-γ in vaginal washes was determined by ELISA (limit of detection 0.5 U/ml). Results are expressed as the mean Units of IFN-γ/ml ± SEM for 9 anti-asialo GM1-treated, 5 anti-CD4/CD8-treated, and 5 normal rabbit sera-treated mice. Less than 0.5 U/ml IFN-γ were detected in vaginal washes from 8 uninoculated mice (not shown). Comparable results were obtained in a separate experiment.

was no significant difference in viral titers between anti-CD4-treated and control-treated mice on Days 1–3 but virus clearance was delayed and virus persisted in vaginal secretions through Day 8 in anti-CD4-treated mice.

A reduction (87%) in the number of IFN-γ-secreting cells in the genital lymph nodes (gLN) of anti-CD4-treated mice as compared to control-treated mice was detected on Day 6 following HSV-2 tk- infection (Table 2). The remaining IFN-γ-secreting cells from these mice were predominantly CD8+ as treatment of gLN lymphocytes from these mice with anti-CD8 antibody and complement resulted in nearly complete (88%) depletion of IFN-γ-secreting cells (Table 3). Treatment of the same population of cells with anti-CD4 antibody and complement did not further reduce the number of IFN-γ-secreting cells.

Mice depleted of CD8+ T cells by in vivo treatment with anti-CD8 antibody cleared virus from the vaginal mucosa and resolved the infection with similar kinetics as control-treated mice (Fig. 4B). Further, IFN-γ-secreting cells were detected in the gLN of these mice in numbers comparable to PBS-treated controls (Table 2) and the majority of these cells were eliminated by in vitro treatment with anti-CD4 and complement (Table 3).

DISCUSSION

The immune mechanisms directly responsible for virus clearance are not well understood and may depend on variables such as the virus type, dose, and route of inoculation. Historically, virus-specific CD8+ T cells have been

| TABLE 1 |

Neutralization of IFN-γ in Vaginal Secretions by in Vivo Treatment with Anti-IFN-γ Antibody

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>Days post vaginal inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control treated</td>
<td>5.6 ± 3.3</td>
</tr>
<tr>
<td>Anti-IFN-γ treated</td>
<td>&lt;0.5b</td>
</tr>
</tbody>
</table>

a Results are expressed as the mean IFN-γ Units/ml present in vaginal washes from 6 PBS or anti-IFN-γ antibody treated, HSV-2 tk- inoculated BALB/c mice.

b Limit of detection for this assay was 0.5 Units/ml. IFN-γ content of vaginal washes from unoinoculated BALB/c mice was less than 0.5 Units/ml.
FIG. 2. Effect of in vivo neutralization of IFN-γ on clearance of virus during an acute vaginal HSV-2 tk− infection. Six BALB/c AnNHsd mice were treated with anti-IFN-γ (squares) or control (diamonds) beginning the day prior to inoculation (A) or 3 days following (B) intravaginal HSV-2 tk− inoculation. The data are presented as the mean vaginal virus titer (log 10) ± SEM and are representative of two repetitions of each experiment. Data points indicated with an asterisk are significantly different than control values (P < 0.004 for A and P < 0.035 for B).

credited with virus clearance via direct cytolysis of infected cells (Zinkernagel and Doherty, 1979; Lukacher et al., 1984). However, evidence has also been reported for secreted factors such as IFN-γ mediating clearance of viruses such as vaccinia virus (Ruby and Ramshaw, 1991; Huang et al., 1993), ectromelia virus (Karupiah et al.,
FIG. 3. Kinetics of HSV-2 tk- clearance from the genital tracts of NK cell-depleted or T cell deficient mice. Mice were treated with PBS (X) or normal rabbit sera (squares) as controls or treated with antibody to deplete either T cells (triangles) or NK cells (diamonds) and then inoculated vaginally with HSV-2 tk-. The data are presented as the mean vaginal virus titer (log 10) +/- SEM from the mice used in Fig. 1. Data points in the anti-CD4/CD8-treated group indicated with an asterisk are significantly different (P < 0.05) from values in normal rabbit sera-treated group. A representative experiment of two performed is shown.

1993), murine cytomegalovirus (Lucin et al., 1992), and HSV-1 (Smith et al., 1994). The results of the present study demonstrate that IFN-γ augments T cell-mediated virus clearance but is not essential for the ultimate clearance of HSV-2 from the vaginal epithelia.

During the first 3 days of infection, IFN-γ was apparently produced predominantly by NK cells while IFN-γ detected on Days 4–7 was produced primarily by T cells (Fig. 1). Because greater numbers of IFN-γ secreting cells were detected in genital lymph nodes from CD8+, compared to CD4+ T cell-depleted mice (Tables 2 and 3), and CD4+ T cell depletion, but not CD8+ T cell depletion, resulted in low levels of IFN-γ in vaginal secretions on Days 4–5 (G. Milligan, unpublished results), it seems most likely that Th1-type CD4+ T cells were primarily responsible for the second period of IFN-γ-secretion. These data are in agreement with our previous studies, demonstrating the appearance of HSV-specific, Th1-type CD4+ T cells in vaginal tissue on Day 5 after infection (Milligan and Bernstein, 1995a) but differ from those of Smith et al. (1994) in which either CD4+ or CD8+ T cells apparently could provide the IFN-γ required for clearance of HSV-1 in a cutaneous infection model. The failure of CD8+ T cell depletion to affect virus clearance kinetics (Fig. 4B) as well as the meager CD8+ T cell response to HSV-2 in this model (Milligan and Bernstein, 1995a) may reflect low numbers of HSV-specific CD8+ T cell precursors which develop following HSV-2 infection (Jennings et al., 1985), presumably due to the ability of HSV-2 to decrease the surface expression of MHC class I molecules (Jennings et al., 1985; York et al., 1994).

Depletion of NK cells with the consequent loss of early IFN-γ production (Fig. 1) did not significantly affect the rate of HSV-2 clearance from the vagina (Fig. 3). In contrast, neutralization of IFN-γ beginning Day 3 after inoculation effectively delayed virus clearance (Fig. 2B), suggesting that CD4+ T cell-derived IFN-γ and not NK cell-produced IFN-γ played a role in rapid virus clearance. NK cell-produced IFN-γ has been shown to play an important role in skewing the developing immune response to a Th1-type response in Leishmania major infections (Scharton and Scott, 1993). However, in preliminary experiments, depletion of NK cells resulted in severe dampening of early IFN-γ release but did not change the ratio of IFN-γ secreting T cells to IL-4 secreting T cells following HSV-2 tk- vaginal inoculation (G. Milligan, unpublished results). Therefore, in these studies of a primary HSV-2 infection we could not detect a role for NK cells in virus clearance from the vagina or in influencing the nature of the developing HSV-specific CD4+ T cell response. It remains possible that NK cells may have influenced the local vaginal immune response in other ways. For example, based on the ability of IFN-γ to enhance expression of antigens such as mouse endothelial cell antigen 325 (Duijvestijn et al., 1986), intercellular adhe-
FIG. 4. Clearance of virus from the genital mucosa of mice deficient in CD4+ or CD8+ T cells. Groups of 5 mice were control-treated (diamonds) or treated with anti-CD4 antibody (squares) (A) or anti-CD8 antibody (squares) (B) and inoculated intravaginally with HSV-2 tk+. In A, data points indicated by an asterisk are significantly different than control-treated values (P < 0.002). The data from representative experiments of two performed for each antibody treatment are presented as the mean vaginal virus titer (log 10) +/- SEM.

sion molecule 1 (ICAM 1), and platelet endothelial cell adhesion molecule 1 (PECAM1) on high endothelial venules (Tang and Hendricks, 1996), it is possible that NK-produced IFNγ may play a role such as enhancing recruitment of lymphocytes and inflammatory cells into the infected mucosa.

Neutralization of IFNγ in vivo delayed, but did not prevent, virus clearance (Figs. 2A and 2B). Similar results were obtained using BALB/c mice with a targeted deletion of the IFNγ gene (IFNγ−/−) where the delay of clearance was approximately 1 day as compared to control animals (G. Milligan, unpublished results). These results are different than those obtained by Smith et al. (1994) and Bouley et al. (1995) in which in
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viral clearance kinetics. That is, virus might have ultimate viral clearance among these studies may seem to argue against the hypothesis that the disparate results reflect only a difference in viral clearance kinetics. That is, virus might have ultimately been cleared in the studies of Smith et al. (1994) and Bouley et al. (1995) but at times later than were assayed. It is also possible that other cytokines capable of replacing the function of IFNγ or different clearance mechanisms with different dependencies on IFNγ may predominate at certain anatomical sites.

Although it can be envisioned that IFNγ in vaginal secretions may act on epithelial cells at the surface of the vaginal lumen as well as on immune cells in the vaginal exudate to enhance antigen processing and presentation and immune effector cell function, the importance and role of IFNγ at this site relative to that present throughout the cell layers of the mucosa remains to be ascertained. IFNγ may act alone on vaginal epithelial cells (Kimura et al., 1994; Klotzbucher et al., 1990) or in synergy with other cytokines such as TNFα, IFNα, or IFNβ (Feduchi and Carrasco, 1991; Balish et al., 1992; Czarniecki et al., 1984; Zerial et al., 1982) to exert direct antiviral effects. The presence of vaginal IFNγ in the absence of a detectable effect on virus clearance during the first 3 days of primary infection (Table 1, Fig. 2) would seem to argue against IFNγ acting directly as an antiviral agent. Alternatively, cytokines capable of acting in synergy with IFNγ such as TNFα may have been present in sufficient concentration at the mucosal surface only later in the infection after the arrival of T cells and macrophages.

A significant portion of the inflammatory exudate cells found in the vagina following intravaginal inoculation of HSV-2 tk− was composed of macrophages (G. Milligan, unpublished results). Macrophages activated by IFNγ release a number of antimicrobial compounds including nitrogen metabolites and TNFα (Nathan, 1987; Ding et al., 1988). The ability of nitric oxide to inhibit HSV infection in vitro (Croen, 1993) and TNFα administration to increase survival of HSV-1-infected mice (Rossol-Voth et al., 1991) suggest that these molecules may have contributed to resolution of the vaginal infection in this model. Studies to determine the role of vaginal macrophages and their products in the protection of the vaginal mucosa are in progress.

Depletion of both CD4+ and CD8+ T cells resulted in persistent virus shedding through Day 10 (Fig. 3), suggesting an essential role for T cells in virus clearance from the vagina. Depletion of only CD8+ T cells had no affect on virus clearance, whereas clearance was delayed, but not prevented by depletion of CD4+ T cells (Figs. 4A and 4B). It seems very likely that CD8+ T cells were responsible for virus clearance in CD4+ T cell-depleted mice because IFNγ-secreting CD8+ T cells were detected in CD4+ T cell-depleted mice (Tables 2 and 3) and mice depleted of both T cell subsets shed virus through Day 10 (Fig. 3). Taken together, these results are consistent with the hypothesis that both T cell subsets can effect virus clearance during an acute HSV-2 vaginal infection but that CD4+ T cells

TABLE 2
Effect of Anti-CD4 or Anti-CD8 Antibody Treatment in Vivo on the Number of IFNγ Secreting Cells in Genital Lymph Nodes (gLN) of HSV-2 tk− Inoculated Mice

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>IFNγ secreting cells/gLN±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>PBS treated</td>
<td>7415 ± 2728</td>
</tr>
<tr>
<td>Anti-CD4 treated</td>
<td>962 ± 313</td>
</tr>
<tr>
<td>Uninoculated, untreated</td>
<td>29</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>PBS treated</td>
<td>8528 ± 1279</td>
</tr>
<tr>
<td>Anti-CD8 treated</td>
<td>7474 ± 1502</td>
</tr>
<tr>
<td>Uninoculated, untreated</td>
<td>7</td>
</tr>
</tbody>
</table>

* Genital lymph nodes were removed on Day 6 following HSV-2 tk− inoculation. Results are expressed as the mean number of IFNγ secreting cells per gLN ± SEM (Experiment 1, n = 3 control, 4 anti-CD4-treated mice; Experiment 2, n = 4 control and 4 anti-CD8-treated mice).

* Results are from a single representative experiment of three performed for Experiment 1 and two for Experiment 2. Results of uninoculated, untreated gLN analysis are from a single animal.

* The number of IFNγ secreting cells from anti-CD4-treated mice is significantly less than from control treated mice (P < 0.02).

* The number of IFNγ secreting cells from anti-CD8-treated mice is not significantly different from control treated mice (P = 0.61).

TABLE 3
Surface Phenotype of IFNγ Secreting Lymphocytes from the gLN of Anti-CD4 or Anti-CD8 Antibody Treated, HSV-2 tk− Inoculated Mice

<table>
<thead>
<tr>
<th>Treatment in vitro</th>
<th>C</th>
<th>Anti-CD4 + C</th>
<th>Anti-CD8 + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ secreting cells/10^6 mononuclear cells±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>309 ± 8</td>
<td>80 ± 7</td>
<td>279 ± 72</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>75 ± 31</td>
<td>82 ± 32</td>
<td>9 ± 7</td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>164 ± 5</td>
<td>5 ± 3</td>
<td>191 ± 16</td>
</tr>
</tbody>
</table>

* Groups of 4 BALB/c mice were treated in vivo with PBS (Control), anti-CD8 (2.43), or anti-CD4 (5K1.5) as described under Materials and Methods. Genital lymph node cells were harvested on Day 6 after inoculation, pooled, and treated in vitro as indicated.

* Results are expressed as the mean number of IFNγ secreting cells/10^6 mononuclear cells ± SD. The percentage of inhibition of the number of IFNγ secreting cells by a given in vitro treatment is given in parentheses.
appear to play the dominant role in a normal animal. CD4⁺ cytotoxic lymphocytes (CTL) and smaller numbers of CD8⁺ CTL have been demonstrated in the draining lymph nodes of mice infected intravaginally with HSV-2 tk⁻ (Milligan and Bernstein, 1995a). IFNγ may have augmented T cell recognition of HSV-2-infected cells and enhanced HSV-specific CTL function by increasing the expression of both class I and class II MHC proteins as well as the enzymes and peptide transport proteins necessary for antigen processing and presentation in the vaginal epithelium.

The results of experiments involving T cell depletion and IFNγ neutralization in mice inoculated intravaginally with HSV-2 tk⁻ suggest that once HSV-2 infects the vaginal epithelia, IFNγ production is important for the rapid, T cell-dependent clearance of virus from the epithelia. The importance of IFNγ in limiting the amount of HSV-2 which reaches the sensory ganglia and establishes a latent infection is currently under investigation. The results of this study suggest that priming for appropriate T cell responses at the site of viral infection in addition to the induction of high titers of mucosal antibody should be an important consideration in the development of vaccines designed to protect the genital mucosa from HSV-2 and other sexually transmitted pathogens.

ACKNOWLEDGMENTS

We thank Jennifer Hannah-Hardy for technical assistance, Dr. Helen Mullen for helpful discussions on cell depletion, and Dr. Lawrence Stanberry and Dr. Nigel Bourne for useful review of this manuscript. This work was supported by the National Institute of Allergy and Infectious Disease Grant AI-23482 and the Gamble Center for Clinical Studies.

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