Mucosal Immunity to Herpes Simplex Virus Type 2 Infection in the Mouse Vagina Is Impaired by In Vivo Depletion of T Lymphocytes

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Intravaginal (IVAG) inoculation of wild-type herpes simplex virus type 2 (HSV-2) in mice causes epithelial infection followed by lethal neurological illness, while IVAG inoculation of attenuated HSV-2 causes epithelial infection followed by development of protective immunity against subsequent IVAG challenge with wild-type virus. The role of T cells in this immunity was studied by in vivo depletion of these cells with monoclonal antibodies. Three groups of mice were used for each experiment: nonimmune/challenged mice, immune/challenged mice, and immune depleted mice [immune mice depleted of a T-cell subset(s) shortly before challenge with HSV-2]. Mice were assessed for epithelial infection 24 h after challenge, virus protein in the vaginal lumen 3 days after challenge, and neurological illness 8 to 14 days after challenge. Monoclonal antibodies to CD4, CD8, or Thy-1 markedly reduced T cells in blood, spleen, and vagina, but major histocompatibility complex class II antigens were still partially upregulated in the vaginal epithelium after virus challenge, indicating that virus-specific memory T-cell function was not entirely eliminated from the vagina. Nevertheless, immune mice depleted of CD4$^+$ and CD8$^+$ T cells, Thy-1$^+$ T cells, or CD8$^+$ T cells alone had greater viral infection in the vaginal epithelium than nondepleted immune mice, indicating that T cells contribute to immunity against vaginal HSV-2 infection. All immune depleted mice retained substantial immunity to epithelial infection and were immune to neurological illness, suggesting that other immune mechanisms such as virus-specific antibody may also contribute to immunity.

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects the human genital tract. The prevalence of this infection is increasing worldwide, and at present over 20% of the adult U.S. population is infected with the virus (12). The virus spreads from the genital tract to the nervous system, and latent virus can persist in infected ganglia for long periods after primary infection is resolved. Activation of latent virus causes recurrent lesions in the genital tract and adjacent tissues (3). Infections are particularly severe in immunocompromised individuals and in infants who are infected during delivery through an infected birth canal. Oral treatment with acyclovir can reduce the severity of infections, but vaccination to prevent or control HSV-2 infections is highly desirable. Development of an effective vaccine to prevent genital HSV-2 infection in women is problematic at present because we do not clearly understand how to elicit strong protective immunity in the mucosa of the female genital tract. Investigations of immunity to genital HSV-2 infection in animal models are likely to play an important part in the development of a vaccine for human use. An added advantage of such investigations is that the basic information so obtained may be applicable to vaccines for other human sexually transmitted diseases.

Experimental studies of host resistance to genital herpes have been carried out by using a mouse model (7–9). In this model, intravaginal (IVAG) inoculation of wild-type, thymidine kinase-expressing HSV-2 (TK$^+$HSV-2) into young BALB/c mice caused epithelial infection followed by lethal neurological illness. The investigators also constructed an attenuated strain of the virus, ΔTK$^-$HSV-2, that contained a partial deletion of the thymidine kinase gene (9). Unlike its wild-type counterpart, the attenuated virus inoculated IVAG caused mild inflammation in the vagina and was incapable of lethal neurological spread. Importantly, IVAG inoculation of BALB/c mice with ΔTK$^-$HSV-2 induced a protective immunity to subsequent lethal challenge with TK$^+$HSV-2 (9).

Studies of immunity to vaginal HSV-2 infection in the young-mouse model were constrained by the relationship between vaginal infection and age (9, 21). Approximately 100% of weaned mice were susceptible to vaginal HSV-2 infection, but infection declined exponentially with increasing host age; fewer than 10% of mice were susceptible to HSV-2 at 14 to 16 weeks of age (9). However, several studies have shown that adult female mice treated with progesterone or sequentially with estradiol and Depo-Provera (E/DP-treated mice) became uniformly susceptible to vaginal HSV-2 infection (1, 13, 16). Vaginal infection of E/DP-treated mice with attenuated HSV-2 produced immunity that protected the mice against later infection by wild-type virus (16). Interestingly, 35 of 36 nonimmune mice showed immunostaining of virus proteins in the vaginal epithelium 24 h after IVAG inoculation of HSV-2, while only 1 of 9 immune mice challenged with the virus showed epithelial infection at this time (16). This indicates that virus infection or replication in the vaginal epithelium was rapidly and severely inhibited in the immune mice and suggests that local immune mechanisms in the vaginal mucosa were important in protection against challenge infection.

One local immune mechanism that could prevent infection of the vaginal epithelium is neutralization of challenge virus by secreted antibody in the vaginal lumen. McDermott et al. (7) and Milligan and Bernstein (11) demonstrated immunoglobulin G (IgG) antibodies specific for HSV-2 in vaginal secretions of young immune mice; antiviral IgA either was not detected or was detected only at very low titers in vaginal fluids in these mice. More recently, Parr et al. (14) found IgG viral antibody

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TABLE 1. Experimental protocol for T-cell depletion experiments

Mice[a]	Primary inoculation[b]	T-cell depletion[c]	Collect tissue[d]	Vaginal wash[e]	Illness scores[f]
Nonimmune	No	No	5 Yes	No	No
			5 No	Yes	Yes
Immune	Yes	No	10 Yes	No	No
			10 No	Yes	Yes
Immune depleted	Yes	Yes	10 Yes	No	No
			10 No	Yes	Yes

[a] Fifty age-matched females were divided into three groups: 10 nonimmune, 20 immune, and 20 immune depleted. All were treated with 10 μg of estradiol benzoate in peanut oil subcutaneously followed 1 day later by 2.0 mg of Depo-Provera (Upjohn Co., Kalamazoo, Mich.) in PBS subcutaneously 5 days prior to primary inoculation. (The hormone treatment induces susceptibility to vaginal HSV-2 infection [16].) The hormone treatment was repeated 6 days prior to challenge. All mice were subjected to a vaginal wash for IgG anti-HSV-2 either 1 day prior to challenge or on the day of challenge and were challenged at 6 weeks after primary inoculation, using IVAG inoculation of 20 μl of TK$^+$HSV-2 at 10^7 PFU/ml into anesthetized mice.

[b] Immunization by IVAG inoculation of 20 μl of ΔTK$^-$HSV-2 at 1.7×10^6 PFU/ml into mice anesthetized with tribromoethanol.

[c] Seven days prior to challenge, using intraperitoneal injection of 0.35 ml of anti-CD8 ascites fluid, 0.9 ml of anti-Thy-1.2 ascites fluid, and 0.3 ml of anti-CD4 ascites fluid followed by another 0.3 ml at 3 days prior to challenge.

[d] Vagina and blood were collected 1 day after challenge from half of the mice in each of the three groups.

[e] Vaginal wash for shed virus protein in the vaginal lumen 3 days after challenge.

[f] Mice were examined for signs of illness 8 to 14 days after challenge.

in vaginal secretions of adult immune mice at a mean titer of 6,200, whereas the mean titer of viral secretory IgA (S-IgA) in the same secretions was only 1.9. The protective role of IgG and S-IgA in vaginal secretions of adult immune mice has also been studied (15). Unfractionated vaginal antibodies from immune and nonimmune mice and affinity-purified IgG and S-IgA from immune vaginal secretions were adjusted to their in vivo concentrations in the vagina. Neutralization of HSV-2 was studied by incubating the virus in the antibody preparations in vitro, followed by inoculation into vaginas of nonimmune test mice. Virus was neutralized by unfractionated immune antibody and by purified immune IgG but not by unfractionated nonimmune antibody or by purified immune S-IgA. To determine whether immune IgG alone could protect against vaginal HSV-2 infection in vivo, purified serum IgG from immune and nonimmune donors was passively transferred to nonimmune recipients 72 h prior to virus challenge in the vagina. Passively transferred immune IgG reduced virus infection of vaginal epithelium, shed virus protein concentrations in the vaginal lumen, and illness scores, even though the viral antibody titers in serum and vaginal secretions of recipient mice were only 29 and 8%, respectively, of those in standards prepared from actively immunized mice. Collectively, the data indicated that IgG viral antibody in vaginal secretions of immune mice provided early protection against vaginal challenge infection, probably by neutralizing virus in the vaginal lumen before it could infect the epithelium. In contrast, viral S-IgA antibody contributed relatively little to immune protection of the vagina in this model.

Another immune mechanism that might reduce infection of the vaginal epithelium after viral challenge is T-cell-mediated immunity. Adoptive transfer of lymphocytes from the genital lymph nodes of immune mice protected nonimmune mice against neurological illness after vaginal challenge with wild-type HSV-2 (8). This observation indicates that virus-specific T cells, if present in sufficient numbers, can protect against neurological illness, but it remains unknown whether the T cells
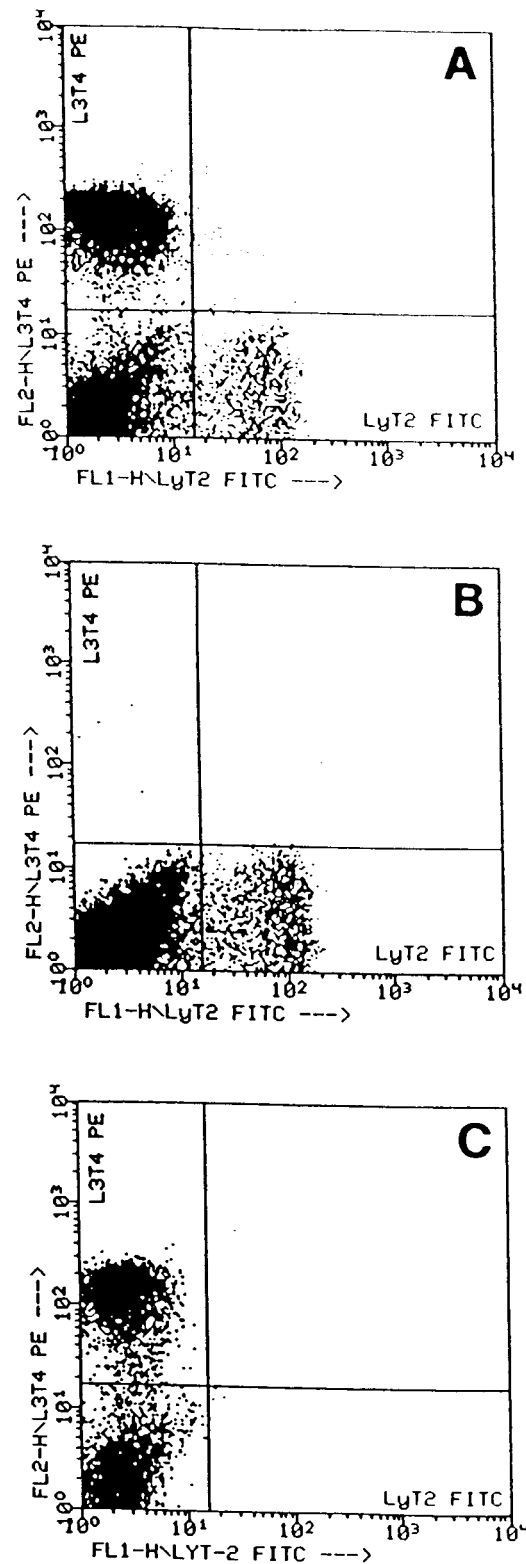

FIG. 1. Flow cytometric analysis of peripheral blood lymphocytes from nonimmune mice showing efficiency of T-cell depletion in vivo. Dot plots are of PE-stained CD4$^+$ cells (vertical axis) and FITC-stained CD8$^+$ cells (horizontal axis) in mice not treated with MAbs (A) and in mice treated with anti-CD4 MAb (B) or anti-CD8 MAb (C).

TABLE 2. Effects of depletion of CD4 and CD8 lymphocytes on immunity to vaginal HSV-2 infection

Mice[a]	Depleted	Mean % of cells ± SEM in blood[b]				No. of infected mice[c]	Mean % of vaginal epithelium infected ± SEM	Shed virus protein[d]	IgG anti-HSV-2[e]	Illness score
		Day 1		Day 14						
		CD4	CD8	CD4	CD8					
Nonimmune	No					9/9	2.8 ± 2.1	4,900	1.0	3.0
Immune	No	26 ± 2.5	8.1 ± 1.2	32 ± 1.5	11 ± 1.9	3/19	0.06 ± 0.4	1.2	570	0.0
	Yes	0	0.05 ± 0.05	0	1.9 ± 0.87	18/20[f]	0.87 ± 0.33[g]	62[h]	650	0.0

[a] Nine nonimmune, 19 immune, and 20 immune depleted mice were used. In each group, half of the mice were used for epithelial infection and the other half were used to measure shed virus protein titers.

[b] Measured by flow cytometry 1 or 14 days after challenge inoculation.

[c] Detected by staining of vaginal epithelium or virus protein in vaginal lumen.

[d] Geometric mean shed virus protein titer in vaginal secretions 3 days after virus challenge.

[e] Geometric mean specific viral IgG titer in vaginal secretions 1 day before virus challenge.

[f] Significantly larger than values in nondepleted immune mice by chi-square test ($P < 0.001$).

[g] Significantly larger than values in nondepleted immune mice by one-tailed Student's t test ($P < 0.0005$).

[h] Significantly larger than values in nondepleted immune mice by Wilcoxon's signed rank test ($P < 0.0001$).

that are actually present in immune mice protect against either vaginal epithelial infection or neurological illness. Few T cells were present in the vaginas of normal mice (17), but the numbers of CD4^{+}, CD8^{+}, and Thy-1.2^{+} T cells increased markedly in the vaginas of immune mice after challenge with wild-type virus (16). Similarly, we have shown that T cells with the memory phenotype continuously recirculate through the vaginal epithelium and that the number of recirculating memory cells was markedly increased when immune mice were challenged in the vagina with HSV-2 (5). The presence of specific HSV-2 memory T cells in the vaginal epithelium of immune mice is also indicated by the rapid (less than 24 h) upregulation of major histocompatibility complex (MHC) class II antigen expression in the epithelium after vaginal challenge with HSV-2. In comparison, upregulation of MHC class II antigens was not detected in the vaginal epithelium until 3 days after a primary vaginal HSV-2 infection in nonimmune mice (16). In the present study, we used the adult mouse model to examine the effects of acute in vivo depletion of T-cell subsets in immune mice on vaginal epithelial infection and neurological illness after vaginal challenge with wild-type HSV-2.

MATERIALS AND METHODS

Virus. Wild-type TK[+]HSV-2 and attenuated ΔTK[−]HSV-2, a strain that contains a partial deletion of the thymidine kinase gene, HSV-2-infected Vero cell
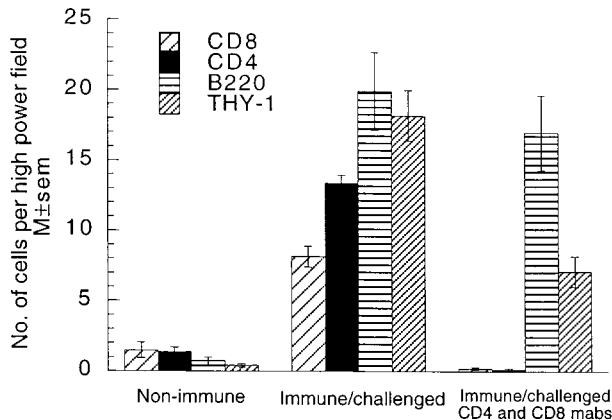

FIG. 2. Numbers of T and B lymphocytes in the vaginal mucosa of 5 nonimmune mice, 10 immune/challenged mice, and 10 immune/challenged mice that were treated with MAbs to deplete CD4^{+} and CD8^{+} T cells.

lysates, and uninfected Vero cell lysates were generously provided by Mark McDermott, McMaster University, Hamilton, Ontario, Canada (8, 9).

Animals. Virgin female BALB/c mice (Harlan/Sprague-Dawley, Indianapolis, Ind.) were used beginning at 12 to 13 weeks of age. They were housed in the SIU Vivarium and used in compliance with all institutional and federal guidelines. The protocol for treatment of the mice in the T-cell depletion experiments is summarized in Table 1.

Ascites fluids. Hybridoma cell lines 53-6.72 (anti-CD8) and 30-H12 (anti-Thy-1.2) were purchased from the American Type Culture Collection (Rockville, Md.). The GK1.5 cell line (anti-CD4) was obtained from Kevin J. Lafferty (John Curtin School of Medical Research, Australian National University, Canberra, Australia). Ascites fluids containing the monoclonal antibodies (MAbs) were produced by inoculating cultured hybridoma cells into pristane-primed outbred nude mice (Harlan/Sprague Dawley). The ascites fluids were collected, pooled, aliquoted, and stored at −70°C until used.

Vaginal washes. Vaginal secretions (mucus) were collected by pipetting 50 μl of phosphate-buffered saline (PBS) in and out of the vagina several times until a discrete clump of mucus was recovered. A second wash with 50 μl of PBS was then done to ensure more complete recovery of the vaginal secretions. The two washes were combined and centrifuged at $12,000 \times g$ to separate the mucus from the PBS wash solution. The mucus and supernatant were frozen separately at −20°C. Later, mucus samples were thawed and extracted twice for 2 h each time in 100 μl of PBS in 12-ml polystyrene tubes at 4°C with rotation at 15 rpm. The two extracts were combined with the original vaginal wash supernatant and made up to a volume of 300 μl; they were then aliquoted and frozen at −20°C for later use. Preliminary flow cytometric experiments were carried out to determine the optimal concentrations and times of administration of ascites fluid that would effectively deplete T cells in the blood and/or spleen.

Flow cytometry. Blood was collected by cardiac puncture from mice under tribromoethanol anesthesia and heparinized. Samples (100 μl) were incubated for 30 min (0°C) in a mixture of fluorescein isothiocyanate (FITC)–rat anti-mouse Lyt2 and phycoerythrin (PE)–rat anti-mouse L3T4 or FITC–rat anti-mouse Thy-1.2 at 10 μg/ml (all from Becton Dickinson Immunocytochemical Systems, Mountain View, Calif.). Control antibodies were FITC-rat IgG2a or PE-rat IgG2b (PharMingen, San Diego, Calif.). After incubation, erythrocytes were lysed in ammonium chloride and lymphocytes were collected by centrifugation. The cell pellets were washed twice with PBS and were then fixed by suspension in 1% paraformaldehyde in PBS. In preliminary experiments with normal mice, spleen lymphocytes were also analyzed. Spleens were dissociated by pressing tissue through a 70-μm-pore-size nylon cell strainer (Falcon no. 2350) into PBS. The released cells were collected, washed in PBS by centrifugation, layered over Histopaque 1083 (Sigma Chemical Co., St. Louis, Mo.), and centrifuged to equilibrium. Cells at the interface were collected by aspiration, washed in PBS, and adjusted to a concentration of 2.0×10^7 cells/ml before labeling as described above for blood cells. Labeled blood and spleen lymphocyte populations that were gated by their forward and side scatter properties were analyzed by two-color flow cytometry using a FACS Vantage (Becton Dickinson, San Jose, Calif.).

Specific viral IgG in vaginal washes. Microtiter plate wells (Falcon Pro-Bind no. 3915; Becton Dickinson, Lincoln Park, N.J.) were coated with 100 μl of UV-inactivated lysate of HSV-2-infected Vero cells in carbonate buffer at pH 9.5. The plates were centrifuged at 2,700 rpm for 3 h in a Beckman GS-6R centrifuge and then incubated overnight in a humid chamber. The next day, the plate wells were washed with PBS–0.05% Tween 20 and blocked for 30 min with 0.10% skim milk powder in PBS-Tween 20. Serial threefold dilutions of the mucus extracts in blocking medium were then placed in the wells and incubated overnight in a humid chamber. After a wash in PBS-Tween 20, the wells received horseradish peroxidase–rabbit anti-mouse IgG (Jackson ImmunoResearch Lab-
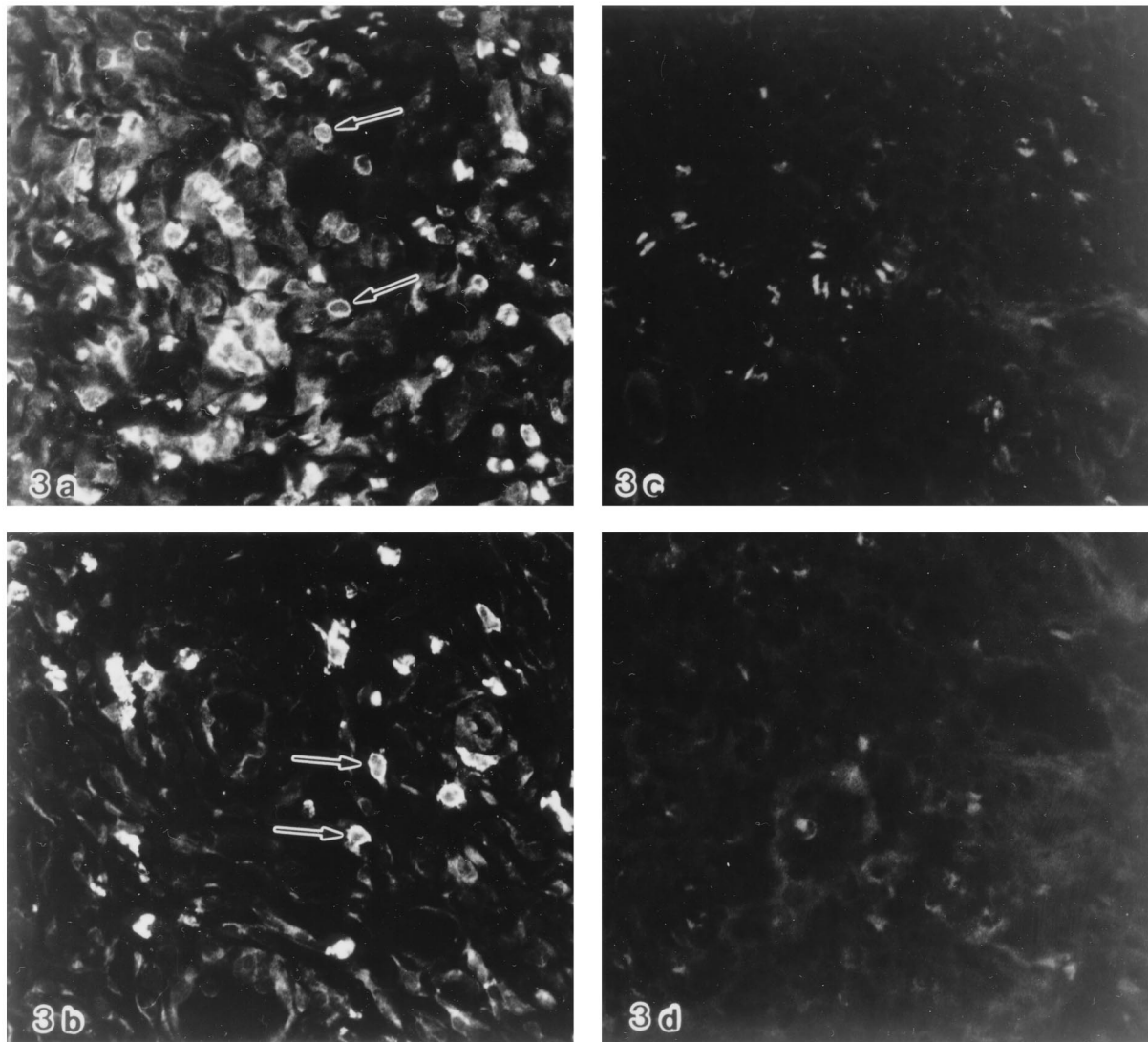
FIG. 3. Fluorescence micrographs showing immunolabeling of CD4$^+$ T cells (a, arrows) and CD8$^+$ T cells (b, arrows) in the vaginal stroma of immune mice 1 day after IVAG challenge with wild-type virus. Little or no labeling of CD4$^+$ (c) or CD8$^+$ T cells was observed in the vaginas of immune/challenged mice that were treated with MAb to CD4 or CD8 1 week before challenge. Magnifications in all panels, ×350.

oratories, West Grove, Pa.) in PBS-Tween for 2 h, followed by washing and introduction of tetramethylbenzidine substrate. The reactions were stopped with sulfuric acid, and absorbances were measured at 450 nm in a Dynatech MR700 microplate reader.

The specificity of the assay for anti-HSV-2 was indicated by lack of reaction when vaginal secretions from nonimmune mice were used and by lack of reaction when secretions from immune and nonimmune mice were incubated on lysates of uninfected Vero cells. The titer was defined as the reciprocal of the dilution at which the absorbance declined to 0.30. Log$_3$ geometric mean titers and standard errors were calculated for each group and used to determine the statistical significance of differences between groups by two-tailed Student's t test. Geometric mean titers are also presented because they indicate more clearly the relative concentrations of antiviral antibodies in the groups.

Tissues. The vaginas were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4°C, 2 h), and washed with PBS containing 10% sucrose (4°C, 2 h). Tissues were embedded in OCT (Tissue-Tek; Miles Scientific, Naperville, Ill.), frozen in isopentane cooled with liquid nitrogen, and stored at −70°C until needed. Cryostat sections (5 μm) were mounted on polylysine-coated slides, air dried, and stored with desiccant at −20°C until needed.

Quantitation of epithelial infection. Cryostat sections of vagina were postfixed in methanol, blocked in 2% normal goat serum (30 min), and labeled for 60 min with FITC–rabbit IgG anti-HSV-2 (Dako Corp., Carpinteria, Calif.). The specificity of labeling was confirmed by labeling normal and infected cell cultures (Ortho Diagnostic Systems, Inc., Raritan, N.J.), by labeling vaginal sections from

infected and noninfected mice, and by using FITC-normal rabbit IgG. The percentage of vaginal epithelium infected by HSV-2 was determined by using an image analysis system. A high-resolution color camera with integration (AIC-O-VI 470, Hyper HAD CCD; Optronics Engineering, Goleta, Calif.) was attached to the fluorescence microscope. Captured images were analyzed by using a Macintosh computer (Quadra 840AV) equipped with an LG-3 frame grabber (Scion Corp., Frederick, Md.) and using NIH Image 1.55 (Wayne Rasband, National Institutes of Health). The lengths of HSV-2-labeled segments of vagina were measured in four histological sections, each derived from a different region of the vagina. The total length of vaginal epithelium was measured after staining with hematoxylin and eosin. The mean percentage of HSV-2-infected epithelium in each group was calculated, and the statistical significance of increased epithelial infection in T-cell-depleted groups was evaluated by Student's one-tailed t test.

Immunohistochemistry. Cryostat sections of vagina were postfixed in acetone (10 min), blocked in 2% normal goat serum (30 min), incubated with rat MAbs against mouse leukocyte markers (60 min at 22°C or overnight at 4°C), washed in PBS (10 min), and labeled with FITC–mouse Fab anti-rat IgG (Jackson ImmunoResearch Laboratories) (60 min). The primary antibodies used were rat anti-mouse CD4 (clones GK1.5 and YTS191.1.2; Accurate Chemical and Scientific Corp., Westbury, N.Y.), rat anti-mouse CD8 (Becton Dickinson), rat anti-mouse Thy-1.2 (Becton Dickinson), rat anti-mouse B220 (PharMingen), and rat anti-mouse Ia (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Specificity of labeling in each case was indicated by the absence of staining when
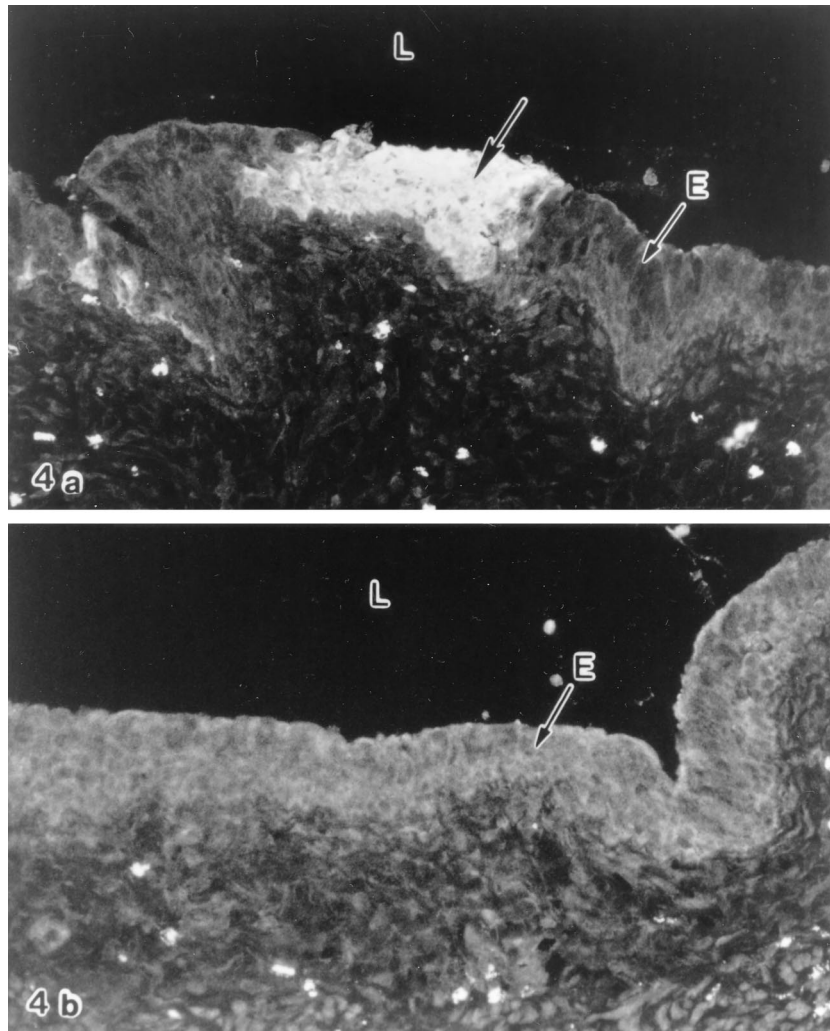
FIG. 4. Fluorescence micrograph showing immunolabeling of HSV-2 (arrow) in a portion of the vaginal epithelium of a nonimmune mouse 1 day after challenge with wild-type virus (a). Adjacent regions of the epithelium (E) were negative. No labeling of HSV-2 was detected in the vaginal epithelium when FITC-conjugated normal rabbit IgG was used in place of FITC–rabbit anti-HSV-2 (b). The fluorescence in the stroma is due to the endogenous fluorescence of granulocytes. L, lumen. Magnification in both panels, ×250.

normal rat IgG (Jackson ImmunoResearch Laboratories) was substituted for the primary antibody. The numbers of stained lymphocytes per high-power field were counted in captured images with the image analysis system described above. Cells in five high-power fields were counted in each of 5 nonimmune, 10 immune/challenged, and 10 immune/challenged depleted mice. Labeling of MHC class II

antigens in the vaginal epithelium was evaluated qualitatively in coded sections as bright, moderate, or absent.

Shed virus proteins in the vaginal lumen. Shed virus proteins were measured by enzyme-linked immunosorbent assay in extracts of vaginal mucus that was collected 72 h after vaginal challenge with wild-type virus. The vaginal mucus was

TABLE 3. Effects of depletion of Thy-1.2 lymphocytes on immunity to vaginal HSV-2 infection

Mice[a]	Depleted	Mean % Thy-1.2 ± SEM in blood[b]	No. of infected mice[c]	Mean % of vaginal epithelium infected ± SEM	Shed virus protein[d]	IgG anti-HSV-2[e]	Illness score
Nonimmune	No		9/10	2.6 ± 1.4	2,200	1.0	3.0
Immune	No	28 ± 5.8	3/19	0.2 ± 0.1	1.0	270	0.0
	Yes	0.8 ± 1.8	14/20[f]	0.7 ± 0.3[g]	23[h]	380	0.0

[a] Ten nonimmune, 19 immune, and 20 immune depleted mice were used. In each group, half of the mice were used for epithelial infection and the other half were used to measure shed virus protein titers.

[b] Measured by flow cytometry 1 day after challenge inoculation.

[c] Detected by staining of vaginal epithelium or virus protein in vaginal lumen.

[d] Geometric mean shed virus protein titers in vaginal secretions 3 days after virus challenge.

[e] Geometric mean specific viral IgG titers in vaginal secretions 1 day before virus challenge.

[f] Significantly larger than values in nondepleted immune mice by chi-square test ($P < 0.001$).

[g] Significantly larger than values in nondepleted immune mice by one-tailed Student's t test ($P < 0.003$).

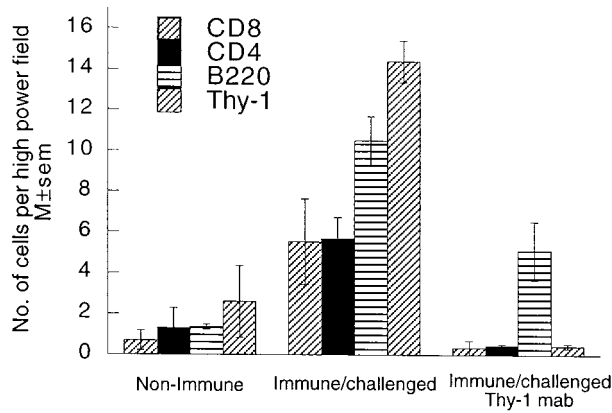[h] Significantly larger than values in nondepleted immune mice by Wilcoxon's signed rank test ($P < 0.002$).

FIG. 5. Numbers of T and B lymphocytes in the vaginal mucosa of 5 nonimmune mice, 10 immune/challenged mice, and 10 immune/challenged mice that were treated with MAb to deplete Thy-1.2^+ T cells.

collected and extracted as described above, aliquoted, and frozen. For measurement, 135 μl of each extract was combined with 15 μl of 1.0 M carbonate buffer (pH 9.5) in the top wells of microtiter plates (Falcon Pro-Bind no. 3915; Becton Dickinson) and serially diluted threefold in 0.10 M carbonate buffer. The plates were covered with sealing film, centrifuged at 2,700 rpm for 3 h in a Beckman GS-6R centrifuge, and then incubated overnight at 4°C. The next day, plate wells were washed with PBS–0.05% Tween 20, blocked with 0.10% skim milk powder in PBS-Tween 20, and incubated in rabbit IgG anti-HSV-2 (Dako) at 1/2,000 in blocking medium for 2 h. After a wash in PBS-Tween 20, wells were incubated 2 h in horseradish peroxidase–donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at 1/2,000, washed, and incubated in tetramethylbenzidine substrate. The specificity of the assay for HSV-2 was confirmed by substituting normal rabbit IgG for rabbit anti-HSV-2 and by using rabbit anti-HSV-2 on vaginal secretions from noninfected mice. The titer was defined as the reciprocal of the dilution at which the absorbance declined to 0.30. Log_3 geometric mean titers and standard errors were calculated for each group and were used to determine the statistical significance of increased virus protein in depleted mice by Wilcoxon's signed rank test. Geometric mean titers are also presented because they indicate more clearly the relative concentrations of shed viral proteins in the groups.

Illness scores. Illness was indicated by ruffled fur, arched backs, feeble movements, paralysis of one or both hindlimbs, and a swollen red vulva. An illness score of 3.0 was assigned to mice that died or became so ill that euthanasia was desirable by 9 days after inoculation of wild-type virus. Mice that died or required euthanasia 10 to 14 days after infection were scored 2.0; mice that developed some sign of illness but survived beyond 14 days were scored 1.0; and mice that never showed signs of illness were scored 0.0.

RESULTS

Depletion of T cells in nonimmune mice. MAbs to CD4, CD8, or Thy-1 administered in vivo to E/DP-treated naive mice depleted the respective T-cell subset in spleen and/or

blood as determined by flow cytometry (Fig. 1). These antibodies were therefore administered to immune mice shortly before vaginal challenge with wild-type virus in order to assess the role of T cells in immunity against vaginal infection.

Depletion of $CD4^+$ and $CD8^+$ T cells in immune mice. Combined administration of both CD4 and CD8 MAbs to immune mice effectively depleted these T cells in the blood (Table 2) and in the vaginal mucosa (Fig. 2 and 3), and it diminished the immunity of these mice to vaginal challenge infection by wild-type HSV-2. Diminished immunity was seen in the increased number of mice in which vaginal epithelial infection was observed, in the increased percentage of the epithelium that was infected, and in the increased titer of shed virus protein in the vaginal lumen after challenge (Fig. 4; Table 2). However, despite essentially complete depletion of detectable $CD4^+$ and $CD8^+$ T cells, the immune depleted mice retained substantial immunity to epithelial infection, were completely resistant to neurological illness, and exhibited upregulation of MHC class II antigens in the vaginal epithelium after challenge (see below). Depletion of T cells had no significant effect on IgG anti-HSV-2 titers in vaginal secretions 1 day before virus challenge. $CD4^+$ T cells remained absent from blood 2 weeks after virus challenge, while $CD8^+$ cells had recovered slightly at this time (Table 2).

Depletion of Thy-1^+ T cells in immune mice. Administration of a Thy-1.2 MAb to immune mice depleted these cells in the blood (Table 3) and in the vaginal mucosa (Fig. 5), and it diminished the immunity of these mice to vaginal challenge infection by wild-type virus (Table 3). Diminished immunity was seen in the increased number of mice in which vaginal epithelial infection was observed, in the increased percentage of the epithelium that was infected, and in the increased titer of shed virus protein in the vaginal lumen after challenge. As in the previous experiment, immune depleted mice retained substantial immunity to epithelial infection and were completely resistant to neurological illness. Depletion of Thy-1^+ cells had no significant effect on IgG anti-HSV-2 titers in vaginal secretions 1 day before virus challenge.

Depletion of $CD8^+$ T cells in immune mice. Administration of a CD8 MAb to immune mice effectively depleted these cells in blood (Table 4) and in the vagina (Fig. 6), and it reduced immunity to vaginal HSV-2 infection (Table 4). As in previous experiments, immune depleted mice retained substantial immunity to epithelial infection and were completely resistant to neurological illness.

Depletion of $CD4^+$ T cells in immune mice. In vivo administration of monoclonal antibody to CD4 depleted these cells in

TABLE 4. Effects of depletion of CD8 lymphocytes on immunity to vaginal HSV-2 infection

Mice[a]	Depleted	Mean % of cells ± SEM in blood[b]		No. of infected mice[c]	Mean % of vaginal epithelium infected ± SEM	Shed virus protein[d]	IgG anti-HSV-2[e]	Illness score
		CD4	CD8					
Nonimmune	No			10/10	6.3 ± 2.1	4,200	1.0	3.0
Immune	No	41 ± 3.4	6.5 ± 0.6	2/19	0.08 ± 0.1	1.0	560	0.0
	Yes	50 ± 2.0	0	11/19[f]	1.3 ± 0.5[g]	6.5[h]	730	0.0

[a] Ten nonimmune, 19 immune, and 19 immune depleted mice were used. In each group, half of the mice were used for epithelial infection and the other half were used to measure shed virus protein titers.
[b] Measured by flow cytometry 1 day after challenge inoculation.
[c] Detected by staining of vaginal epithelium or virus protein in vaginal lumen.
[d] Geometric mean shed virus protein titers in vaginal secretions 3 days after virus challenge.
[e] Geometric mean specific viral IgG titers in vaginal secretions 1 day before virus challenge.
[f] Significantly larger than values in nondepleted immune mice by chi-square test ($P < 0.002$).
[g] Significantly larger than values in nondepleted immune mice by one-tailed Student's t test ($P < 0.0001$).
[h] Significantly larger than values in nondepleted immune mice by Wilcoxon's signed rank test ($P < 0.03$).

FIG. 6. Numbers of T and B lymphocytes in the vaginal mucosa of 5 non-immune mice, 10 immune/challenged mice, and 10 immune/challenged mice that were treated with MAb to deplete $CD8^+$ T cells.
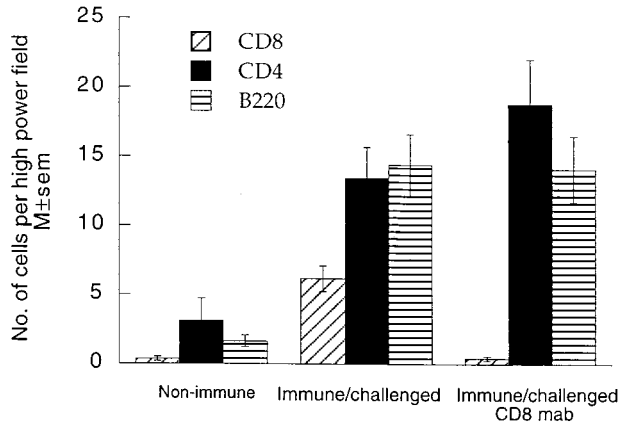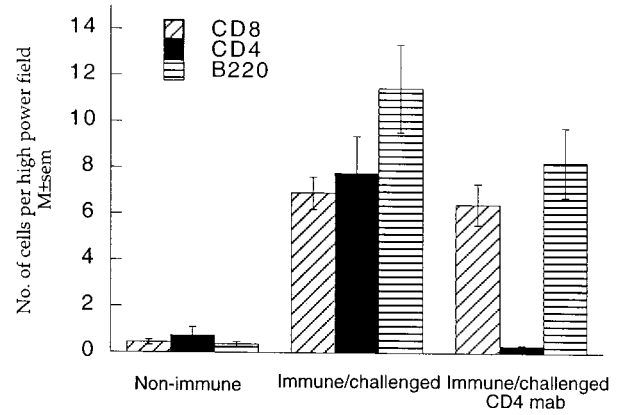

FIG. 7. Numbers of T and B lymphocytes in the vaginal mucosa of 5 non-immune mice, 10 immune/challenged mice, and 10 immune/challenged mice that were treated with MAb to deplete $CD4^+$ T cells. The $CD4^+$ T cells were stained with the MAb (clone GK1.5) that was used for T-cell depletion. To confirm these data, we also used a second MAb to stain $CD4^+$ (clone YTS191.1.2) and found 10.7 ± 2.2 $CD4^+$ T cells per high-power field in intact immune mice and 0.38 ± 0.11 in depleted mice.

blood (Table 5) and vaginas (Fig. 7) of immune mice. Nevertheless, this treatment caused only small and statistically insignificant increases in the number of mice showing infected vaginal epithelium and in the mean percentage of epithelium infected after challenge. No shed virus protein was detected in the vaginal lumen in either the intact or depleted immune group. Both intact and T-cell-depleted immune mice were substantially immune to epithelial infection and completely resisted neurological illness, while all nonimmune mice died after challenge inoculation with wild-type virus.

Immunolabeling of MHC class II antigens in vaginal epithelium. One day after vaginal challenge with HSV-2, nonimmune mice showed MHC class II antigen labeling in Langerhans cells in the vaginal epithelium and in a few dendritic cells and B lymphocytes in the stroma, but vaginal epithelial cells were unstained (Fig. 8a). All immune mice not treated with MAbs showed uniformly bright labeling of MHC class II antigen in the vaginal epithelial cells (Fig. 8b). Immune mice that were depleted of either $CD8^+$ (Fig. 8c) or $CD4^+$ T cells showed variable staining that was as bright as that seen in intact immune mice in some regions and less bright in other regions. Immune mice depleted of both $CD4^+$ and $CD8^+$ cells (Fig. 8d) or Thy-1.2^+ cells showed variable staining that was bright in some regions but much diminished or even absent in other regions.

DISCUSSION

Immunization of mice in the vagina with attenuated HSV-2 elicits immunity to subsequent vaginal infection by wild-type virus. This immunity greatly reduces infection of the vaginal epithelium as early as 24 h after vaginal challenge (16). The possible role of T cells in this early immunity against epithelial infection was investigated in the present study. Depletion of Thy-1.2^+ cells from immune mice in vivo by injection of a MAb shortly before vaginal challenge with HSV-2 decreased immunity to infection of the vaginal epithelium in comparison to nondepleted immune mice. Similarly, combined depletion of $CD4^+$ and $CD8^+$ T cells and depletion of $CD8^+$ cells alone also reduced immunity to vaginal epithelial infection. Decreased immunity was evidenced by the increased numbers of T-cell-depleted mice whose vaginal epithelia were infected after challenge, by the increased percentage of the epithelium that was infected, and by increased shed virus protein concentrations in the vaginal lumen. These observations indicate that vaginal immunization with attenuated HSV-2 elicits immune T cells that play a significant role in resistance to reinfection of the vaginal epithelium by wild-type HSV-2. This finding is contrary to results of Fidel et al. (4) concerning vaginal immu-

TABLE 5. Effects of depletion of CD4 lymphocytes on immunity to vaginal HSV-2 infection

Mice[a]	Depleted	Mean % of cells ± SEM in blood[b]		No. of infected mice[c]	Mean % of vaginal epithelium infected ± SEM	Shed virus protein[d]	IgG anti-HSV-2[e]	Illness score
		CD4	CD8					
Nonimmune	No			10/10	11 ± 5.1	4,200	1.0	3.0
Immune	No	50 ± 2.3	8.4 ± 1.5	1/20	0.25 ± 0.1	1.0	560	0.0
	Yes	0	13 ± 1.2	3/20[f]	0.54 ± 0.2[g]	1.0[h]	420	0.0

[a] Ten nonimmune, 20 immune, and 20 immune depleted mice were used. In each group, half of the mice were used for epithelial infection and the other half were used to measure shed virus protein titers.
[b] Measured by flow cytometry 1 day after challenge inoculation.
[c] Detected by staining of vaginal epithelium or virus protein in vaginal lumen.
[d] Geometric mean shed virus protein titers in vaginal secretions 3 days after virus challenge.
[e] Geometric mean specific viral IgG titers in vaginal secretions 1 day before virus challenge.
[f] Not significantly larger than values in nondepleted immune mice by chi-square test ($P = 0.3$).
[g] Not significantly larger than values in nondepleted immune mice by one-tailed Student's t test ($P = 0.5$).
[h] No shed virus protein detected in either depleted or nondepleted immune mice.
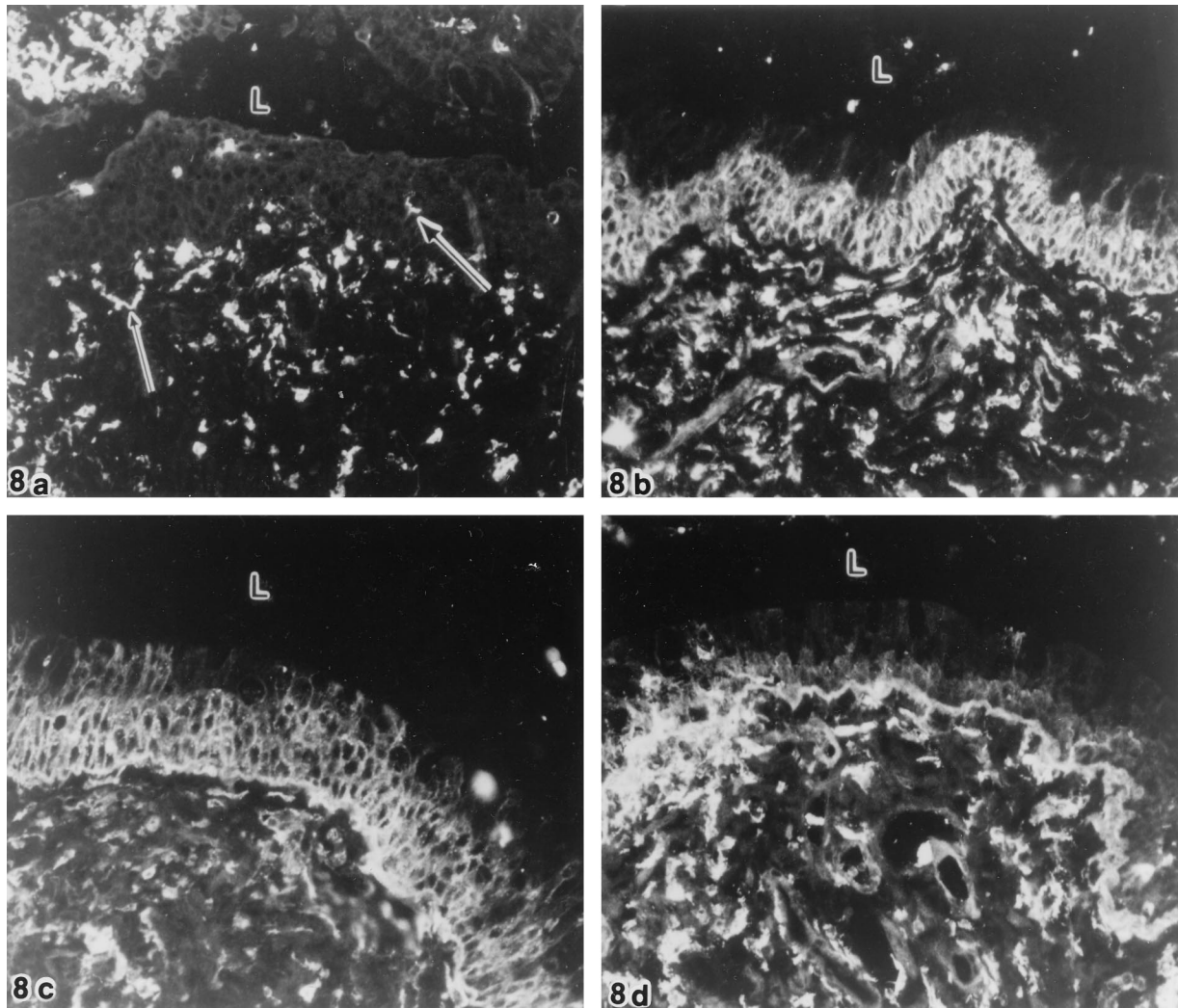
FIG. 8. Fluorescence micrographs showing immunolabeling of MHC class II antigens in vaginal mucosa 1 day after vaginal challenge with HSV-2. (a) Nonimmune mice showed labeling in dendritic cells in the epithelium (large arrow) and stroma (small arrow), but the vaginal epithelial cells were negative. (b) Immune/challenged mice showed bright staining in the vaginal epithelium. (c) Immune/challenged mice depleted of $CD8^+$ or $CD4^+$ T cells showed bright labeling in some regions of the epithelium and less bright staining in other regions. (d) Immune/challenged mice depleted of both $CD8^+$ and $CD4^+$ T cells or $Thy-1^+$ T cells showed variable labeling in the vaginal epithelium within the same section of vagina, some regions being bright, some moderate, and some not labeled. L, lumen. Magnification in all panels, $\times 190$.

nity to *Candida albicans* in mice but is consistent with results of Rank et al. (18) regarding the role of T cells in vaginal immunity to chlamydiae in guinea pigs.

By conventional measures, the in vivo depletion of T cells achieved in the present study was effective. Flow cytometric data indicated that T-cell depletion in blood of immune mice was complete or nearly complete, and counts of immunostained T cells in sections indicated that T-cell depletion was also nearly complete in the vaginal mucosa. However, we observed variable staining of MHC class II antigens in the vaginal epithelium of all T-cell-depleted mice after virus challenge, including some areas of bright staining. Since upregulation of MHC class II antigens in the vaginal epithelium at 24 h after challenge is presumably due to gamma interferon secretion by memory T cells (16), it appears that some memory T cells were still present in the vaginas of T-cell-depleted mice. This evidence of failure to obtain complete elimination of functional T cells from the vagina suggests than the contribution of T cells

to immunity against vaginal challenge by HSV-2 may be greater than we observed in the present study.

The data presented here do not effectively determine which T-cell subset is most important in immunity to vaginal challenge by HSV-2. While $CD8^+$ cell depletion appeared to reduce immunity more than $CD4^+$ cell depletion, these depletions were not compared in the same experiment, and comparisons between experiments would be less accurate. Also, the MHC class II antigen staining in mice that were depleted of both $CD4^+$ and $CD8^+$ cells indicated that memory T-cell depletion was incomplete, and it is possible that the $CD8^+$ cell depletion was more effective than the $CD4^+$ cell depletion. Moreover, in vivo depletion of $CD4^+$ cells in BALB/c mice with MAb GK1.5, as used in the present study, has been reported to act selectively against resting cells and to spare activated memory cells (2). Previous studies of immunity to HSV-2 infection in a variety of mouse models have indicated that either $CD8^+$ cells (20) or $CD4^+$ cells (6) can play a role in

protection and that experimental conditions strongly influence which T-cell subset is more protective (19). During clearance of a primary vaginal HSV-2 infection in nonimmune mice, $CD4^+$ cells appeared to be more important than $CD8^+$ cells (10), but this would not necessarily be the case during resistance to reinfection in immune mice.

Immune mice that were depleted of T cells at the time of vaginal challenge remained highly resistant to virus infection of the vaginal epithelium, and they did not become ill due to HSV-2 infection of the nervous system. The immunity to epithelial infection was at least partly due to IgG viral antibody in vaginal secretions. We have previously shown that purified IgG from vaginal secretions of immune mice, at its concentration in vivo, effectively neutralized HSV-2 concentrations that were 1,000-fold higher than the minimum needed to cause lethal illness in nonimmune mice (15). Collectively, previous and present data indicate that IgG viral antibody and immune T cells both contribute to immunity against reinfection of vaginal epithelium by HSV-2. It is difficult to estimate which of the two forms of immunity is the more important in protection, largely because T-cell immunity was not entirely eliminated in the present study. We suggest that viral antibody in vaginal secretions neutralizes challenge virus in the vaginal lumen before it can infect the epithelium and that it plays a major role in immunity to low-dose viral challenge, whereas memory T cells help to clear virus from infected epithelial cells when the dose of challenge virus exceeds the neutralizing capacity of locally secreted antibody. Since in the present study we wanted to maximize the effects of T-cell immunity, we used a relatively high dose of wild-type HSV-2 for vaginal challenge, 10^7 PFU/ml, which is about 3,000-fold higher than the minimum needed for lethal infection in nonimmune mice.

The data obtained in the present study clearly illustrate the strength of the immunity that is stimulated in mice by vaginal immunization with attenuated HSV-2. In mice that were immunized only once, and with a challenge virus dose that was 3,000-fold higher than the minimum needed to cause lethal illness in nonimmune mice, the percentage of the vaginal epithelium that was infected 24 h after challenge was 1.2, 2.0, 2.2, or 7.7% of that in nonimmune mice, and shed virus protein in the vaginal lumen 72 h after challenge was detected in only 1 of 38 immune mice. Viral antibody titers in mice that were immunized only once were markedly increased by boosting (11, 14), indicating that primary immunity was not maximal. The adult mouse model of immunity to vaginal HSV-2 infection, using attenuated HSV-2 for vaginal immunization, thus provides an excellent benchmark against which to compare other vaccines and vaccination procedures that are intended to protect against HSV-2 infection of the female genital tract. Such comparisons, together with further studies of immune mechanisms in the model, may facilitate development of vaccines against human sexually transmitted viral diseases.

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