

## Immunoglobulin G Is the Main Protective Antibody in Mouse Vaginal Secretions after Vaginal Immunization with Attenuated Herpes Simplex Virus Type 2

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**We investigated the protective role of antibodies in vaginal secretions of mice that were immune to vaginal challenge with herpes simplex virus type 2 (HSV-2). Unfractionated vaginal immunoglobulins from immune and nonimmune mice and affinity-purified immunoglobulin G (IgG) and secretory IgA (S-IgA) from immune secretions were adjusted to their concentrations in vivo. Wild-type HSV-2 was incubated in the immunoglobulin preparations for 15 min in vitro, followed by inoculation into vaginae of nonimmune mice. HSV-2 was neutralized by unfractionated antibody and purified IgG from immune secretions but not by unfractionated nonimmune antibody or by purified immune S-IgA. The protective effect of IgG in vivo was investigated by passively transferring purified serum IgG from immune and nonimmune donors to nonimmune recipients before vaginal challenge infection. Immune IgG significantly reduced the percentage of vaginal epithelium infected, concentrations of shed virus protein in the vaginal lumen, and illness scores, even though the viral antibody titers in serum and vaginal secretions of recipient mice at the time of challenge were only 29 and 8%, respectively, of those in actively immunized mice. Additionally, removal of vaginal secretions from immune mice 10 min before vaginal challenge with HSV-2 significantly increased the concentration of shed virus protein in the vaginal lumen after challenge. Collectively, the data indicate that IgG antibody in vaginal secretions of immune mice provides early protection against vaginal challenge infection, probably by neutralizing virus in the vaginal lumen. In contrast, S-IgA antibody contributed relatively little to immune protection of the vagina.**

Efforts to develop vaccines to protect women against sexually transmitted diseases would be facilitated by a better understanding of the immune mechanisms that protect the female reproductive tract against infections in animal models. A mouse model of immunity against vaginal herpes simplex virus type 2 (HSV-2) infection has been described by McDermott and coworkers (23). In this model, vaginal immunization of young, prepubertal mice with an attenuated strain of HSV-2 elicits immunity against a subsequent vaginal challenge with wild-type virus. This model has been used for numerous studies of both cellular and humoral immunity to vaginal HSV-2 infection (8, 10, 21, 22, 25, 26), but long-term studies of immunity are not possible because mice become resistant to vaginal HSV-2 infection when they reach puberty (23, 36). Previous reports have indicated that adult female mice are susceptible to vaginal HSV-2 infection during diestrus and early pregnancy (2, 45), and we have demonstrated that the age-related resistance of mice to vaginal HSV-2 infection can be overcome by pretreatment with progesterone or the long-acting progestin, Depo-Provera (DP) (36). The young mouse HSV-2 model can thus be adapted to adult mice and made suitable for long-term studies of immunity.

Immunity to reinfection by viruses is often due at least in part to antibody (40), and both young mice (21, 26) and adult mice (33) that were immunized in the vagina with attenuated HSV-2 developed virus-specific antibodies in their vaginal secretions. These antibodies might neutralize challenge virus in the vaginal lumen before it can infect vaginal epithelial cells, but a protective role of antibodies in this model is controversial. Arguments against a significant role for antibodies include

failure to detect viral antibody in vaginal secretions and lack of protection against challenge infection after passive transfer of immune serum or anti-HSV-2 monoclonal antibodies to nonimmune mice (10, 21), failure to detect specific antibodies in vaginal secretions after a parenteral immunization that produced high antibody titers in serum (21), and effective immune protection after adoptive transfer of iliac lymph node cells from immune mice to nonimmune mice (22). Also, studies of B-cell-depleted mice indicated that antibody is not important for protection against HSV-2 infection in skin (39). Conversely, Eis-Hubinger et al. (10) reported that passive transfer of monoclonal antibody to HSV glycoprotein B in mice was highly protective against vaginal challenge infection even though it was not detected in vaginal secretions. In addition, we found that immune mice that were effectively depleted of T cells by in vivo administration of monoclonal antibodies to Thy-1.2 or CD4 plus CD8 were still markedly immune to challenge infection, suggesting that antibody also contributes to immunity (37). Further studies will be needed to clarify the role of antibodies in protection against vaginal HSV-2 infection in mice.

Studies of young mice have indicated that vaginal immunization with attenuated HSV-2 elicits relatively high enzyme-linked immunosorbent assay (ELISA) titers of immunoglobulin G (IgG) viral antibody in vaginal secretions in comparison to IgA titers (21, 26). Similarly, our observations with adult immune mice demonstrated IgG viral antibody in vaginal secretions at a geometric mean ELISA titer of 6,200, whereas the titer of secretory IgA (S-IgA) viral antibody was only 1.9 (33). The apparent predominance of IgG viral antibody in the vaginal secretions of the immunized mice is surprising because at other mucosal surfaces, such as the gastrointestinal tract and upper respiratory tract, local immunization induces mainly S-

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IgA antibodies, and these antibodies are largely responsible for secretory immunity at those mucosal surfaces (18, 28, 47). It is often assumed that this is also true at other mucosal surfaces, including the female genital tract (28). At present, it remains unknown whether the ELISA titers of viral antibodies in the vaginal secretions accurately indicate the virus neutralizing activities of these antibodies. It is possible that the binding of viral S-IgA to antigen in the ELISA is inhibited by viral IgG in the secretions, and it is also possible that viral S-IgA can neutralize HSV-2 better than equal amounts of viral IgG. Direct information about the relative neutralizing effectiveness of the two antibody classes in the immune vaginal secretions would help to clarify the role of secreted antibodies in this model.

Previously, we developed a method for effective extraction of S-IgA and IgG from the vaginal secretions of progestin-treated mice and measured the concentrations of these immunoglobulin classes in the neat vaginal secretions of nonimmune and immune mice (33). In that same study, we used native polyacrylamide gel electrophoresis (PAGE) immunoblotting to demonstrate that essentially all of the IgA in vaginal secretions of these mice was in the form of 420-kDa dimeric S-IgA. In the present study, we extracted S-IgA and IgG from vaginal secretions of immune mice, purified each immunoglobulin by affinity chromatography, and concentrated it to its concentration *in vivo* in the vaginal secretions. We then investigated the relative ability of each immunoglobulin preparation to neutralize HSV-2. In addition, we purified IgG from serum of immune mice and passively transferred it to nonimmune mice, compared the resulting titers of viral IgG in vaginal secretions of the passive transfer recipients to those in actively immunized mice, and then determined whether the passively transferred IgG provided significant protection against HSV-2 challenge infection of the vaginal epithelium. Finally, we removed the vaginal secretions from immune mice immediately before inoculation of challenge virus to determine whether the absence of secreted antibodies in the vaginal lumen would lessen immunity to the challenge virus.

#### MATERIALS AND METHODS

**Animals and virus.** Female BALB/c mice were purchased from Harlan/Sprague-Dawley, Indianapolis, Ind., and were 8 to 12 weeks old at the beginning of treatment. They were housed in the SIUC Vivarium in compliance with all institutional and federal animal welfare requirements, and all experimental procedures were approved by the institutional Animal Care and Use Committee. Wild-type HSV-2 and attenuated HSV-2, a strain that contains a partial deletion of the thymidine kinase gene, HSV-2-infected Vero cell lysates, and uninfected Vero cell lysates were generously provided by Mark McDermott, McMaster University, Hamilton, Ontario, Canada (22, 23).

**Hormone treatment and immunization.** Groups of age-matched mice were injected with 0.10  $\mu$ g of estradiol benzoate (E) in peanut oil subcutaneously followed 24 h later by subcutaneous injection of 2.0 mg of DP (Upjohn Co., Kalamazoo, Mich.) diluted in phosphate-buffered saline (PBS) (E/DP-treated mice). The treated mice were susceptible to vaginal HSV-2 infection from 5 to at least 20 days after DP treatment (36). Five days after treatment with DP, half of the mice were anesthetized with tribromoethanol and immunized by intravaginal (ivag) inoculation of 20  $\mu$ l of attenuated HSV-2 at  $2.0 \times 10^6$  PFU/ml; the remaining mice were not immunized. These mice were referred to as immune and nonimmune mice, respectively. Five weeks later, all mice were again treated with E/DP. Vaginal washes were collected once daily on days 5, 6, and 7 after treatment with DP. The immunity of all immunized mice and a few nonimmune mice was then tested by ivag challenge with 20  $\mu$ l of wild-type HSV-2 at  $3.5 \times 10^6$  PFU/ml 2 days after the last vaginal washing. These mice were examined for signs of illness 8 to 14 days after challenge as described previously (36); all immunized mice remained well, but all nonimmune mice died. Serum was collected from the immune mice and the remaining nonimmune mice 14 days after the immune mice were challenged with virus. Additional nonimmune mice were treated with E/DP and used as recipients of neutralized virus or passively transferred immunoglobulins. Additional immune mice were treated with E/DP and used as donors of vaginal secretion and serum standards and to determine the effect of removal of the vaginal secretions before ivag virus challenge.

**Vaginal washes.** Vaginal secretions were collected by pipetting 50  $\mu$ l of PBS in and out of the vagina gently until a discrete clump of mucus was recovered. This usually took four to eight cycles of pipetting and required cutting the pipette tip back to a diameter of 1 to 2 mm. A second vaginal wash with 50  $\mu$ l of PBS was then done to ensure more complete recovery of the vaginal secretions; it was combined with the first wash. Vaginal washes were centrifuged at  $12,000 \times g$  for 10 min to separate the mucus from the PBS wash solution shortly after collection. The mucus and supernatant were then frozen separately at  $-20^\circ\text{C}$ . The PBS wash solution contained a cocktail of proteinase inhibitors (34).

**Extraction of immunoglobulin from vaginal mucus.** Mucus samples were thawed, weighed, and extracted twice for 2 h each time in 100  $\mu$ l of PBS per sample, with rotation at 20 rpm in a 12-ml polystyrene tube at  $4^\circ\text{C}$ . The two extracts and the original wash supernatant were pooled, made up to 300  $\mu$ l per sample, and frozen at  $-20^\circ\text{C}$  until needed. This method provided essentially complete recovery of S-IgA and presumably also IgG from the mucus (33).

**Neutralization of HSV-2 with vaginal antibody.** Immunoglobulin was extracted from the vaginal mucus of immune or nonimmune mice as described above. In some experiments, the immunoglobulin-containing extracts were concentrated to the volume of the vaginal mucus that was extracted, thus providing the secreted antibody in aqueous medium at its concentration *in situ*. In other experiments, IgG and S-IgA were sequentially purified from one part of a larger volume of immunoglobulin-containing extract from immune mice. The IgG was purified by affinity chromatography on Sepharose-conjugated protein G (Calbiochem, La Jolla, Calif.), using a preelution wash with 0.50 M NaCl at pH 4.0. Its purity was confirmed by biotinylating the protein, followed by sodium dodecyl sulfate (SDS)-PAGE under reducing conditions and blotting (34). The only biotinylated polypeptides detected were heavy and light chains. The purified IgG, the remainder of the unfractionated immune vaginal extract, and a sample of unfractionated nonimmune vaginal extract were all concentrated to an IgG concentration of 100  $\mu$ g/ml, the concentration of this immunoglobulin in immune vaginal mucus *in situ* (33). The S-IgA was similarly purified by affinity chromatography on Sepharose-conjugated goat anti-mouse IgA ( $\alpha$ ) (Sigma Chemical Co., St. Louis, Mo.) as previously described (34), and its purity was confirmed as described above (34). The unfractionated portion of the immune vaginal extract, having been concentrated to an IgG concentration of 100  $\mu$ g/ml, contained S-IgA at 200  $\mu$ g/ml, which was similar to a previous measurement of 300  $\mu$ g/ml (33) and was taken as the S-IgA concentration *in situ* of these mice. The column-purified S-IgA was therefore concentrated to 200  $\mu$ g/ml. Recovery artifact was thus avoided because the purified immunoglobulin preparations were concentrated to their *in situ* concentrations. The S-IgA antiviral titers in the purified S-IgA and in the concentrated immune vaginal extract were found to be the same, indicating that the purified S-IgA retained full ability to bind HSV-2. A concentrated stock of wild-type HSV-2 was diluted in the unfractionated or purified vaginal immunoglobulin preparations, incubated for 15 min at room temperature, and then inoculated ivag into nonimmune, progestin-treated test mice.

**Passive transfer of serum immunoglobulin.** Serum was obtained from age-matched immune and nonimmune mice 14 days after the immune mice were challenged ivag with HSV-2. Whole gamma globulins ( $\gamma$ G) were obtained from these sera by precipitation with 50% saturated ammonium sulfate, and purified IgG was obtained by affinity chromatography on Sepharose-conjugated protein G (Calbiochem). The concentration of purified IgG was determined by its absorbance according to the relation  $E_{280}^{1\%} = 14.0$ . Immunoglobulins were kept frozen at  $-20^\circ\text{C}$  until they were administered to recipient mice by intraperitoneal injection in approximately 1-ml volumes.

**Measurement of S-IgA and IgG concentrations by ELISA.** Capture antibodies [goat anti-mouse IgA ( $\alpha$ ) at 10  $\mu$ g/ml and rabbit anti-mouse IgG ( $\gamma$ ) at 5  $\mu$ g/ml, both from Jackson ImmunoResearch Laboratories, West Grove, Pa.] were bound to Immulon 1 (Dynatech Laboratories, Alexandria, Va.) microtiter plate wells overnight in 0.10 M carbonate buffer at pH 9.5. After washing in PBS-0.05% Tween 20, plate wells were blocked 30 min with 0.10% skim milk powder in PBS-Tween 20. Serial twofold dilutions of samples and immunoglobulin standards in duplicate in blocking medium were then applied to the wells overnight in a humid chamber, followed next day by washing in PBS-Tween 20. Bound immunoglobulins were detected with horseradish peroxidase (HRP)-rabbit anti-mouse IgA ( $\alpha$ ) or HRP-rabbit anti-mouse IgG ( $\gamma$ ) (Jackson ImmunoResearch) in PBS-Tween, followed by washing and incubation in tetramethylbenzidine substrate. Mouse S-IgA standard was purified from milk (34). Its purity was assessed by biotinylating the protein, followed by SDS-PAGE under reducing conditions and immunoblotting. The only biotinylated polypeptides detected corresponded to secretory component, alpha chain, and light chains. The IgG standard was purchased from Sigma and was also shown to be pure by SDS-PAGE analysis under reducing conditions. The concentrations of the two standards were determined by absorbance at 280 nm, using  $E_{280}^{1\%} = 14$ .

**Specific viral antibodies.** Microtiter plate wells (Falcon Pro-Bind no. 3915; Becton-Dickinson and Co., Lincoln Park, N.J.) were filled with 100  $\mu$ l of UV-inactivated lysate of HSV-2-infected Vero cells in carbonate buffer at pH 9.5, covered with sealing film, centrifuged at 2,700 rpm for 2 h in a Beckman GS-6R centrifuge, and then incubated overnight at  $4^\circ\text{C}$ . On the next day, plate wells were washed with PBS-0.05% Tween 20 and blocked 30 min with 0.10% skim milk powder in PBS-Tween 20. Serial twofold dilutions of samples in blocking medium were then placed in the wells and incubated overnight in a humid chamber. After washing in PBS-Tween, the wells received HRP-rabbit anti-

mouse IgA ( $\alpha$ ) or HRP-rabbit anti-mouse IgG ( $\gamma$ ) (Jackson Immunoresearch) in PBS-Tween for 2 h, followed by washing and introduction of tetramethylbenzidine substrate. Reactions were stopped with 1.0 M sulfuric acid, and absorbances were measured at 450 nm. The sample antibody titer was defined as the reciprocal of the sample dilution at which the absorbance declined to 0.30 above the background absorbance of nonimmune samples at the same dilution (0.05 or less). Control experiments demonstrated that the ELISA reactions were all at background levels when immune and nonimmune samples were incubated on lysates of uninfected Vero cells.

**Serum and vaginal secretion standards.** Ten progestin-treated mice were immunized by inoculation of attenuated HSV-2 into the vagina. Immune serum and vaginal mucus were collected 6 weeks later. Equal volumes of the 10 serum samples were diluted 1/100 in PBS-0.1% bovine serum albumin, combined, aliquoted, and frozen at  $-70^{\circ}\text{C}$  for later use as immune serum standards. Immunoglobulin was extracted from the vaginal mucus samples as described above, after which equal volumes of the extracts were combined, aliquoted, and frozen for later use as immune vaginal secretion standards.

Specific antibody titers in serum and vaginal secretions of experimental mice, as a fraction of the titers in the standards from immune mice, were measured as follows: the  $\log_2$  geometric mean titers of the experimental group and replicates of the standard were measured to the nearest 10th of a dilution on the same microtiter plate, using an absorbance of 1.0, which was in the steepest part of the dilution curve, as the titer endpoint; the difference between these two means was denoted  $d$ , and the mean concentration of specific antibody in the samples as a percentage of the standard was calculated as  $100\%/2^d$ . We assume that titer differences were due to concentration differences and not to affinity differences because all viral antibodies in these studies were elicited in the same way, i.e., by vaginal immunization with attenuated HSV-2.

**Illness scores.** Illness was indicated by ruffled fur, arched backs, feeble movements, paralysis of one or both hindlimbs, and a swollen red vulva. Illness usually, but not always, led to death or euthanasia. 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 from 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. Mice that never showed signs of illness were scored 0.0.

**Shed virus proteins in the vaginal lumen.** Shed virus proteins were measured by ELISA in extracts of vaginal mucus that was collected 72 h after vaginal challenge with wild-type virus. The method was similar to that recently described by Franco and Greenberg (12). Vaginal mucus was collected as described above and frozen. When thawed, the mucus was extracted in 100  $\mu\text{l}$  of PBS per sample for 2 h at  $4^{\circ}\text{C}$  with rotation in a 12-ml polystyrene tube. The extract was combined with the original vaginal wash supernatant, brought to pH 9.5 by addition of 18  $\mu\text{l}$  of 1.0 M carbonate buffer, and made up to a volume of 180  $\mu\text{l}$  (water was absorbed by the mucus during extraction). The vaginal mucus extracts were then serially diluted threefold in 0.10 M carbonate buffer in a Falcon Pro-Bind flat-bottom microtiter plate. Plates were covered with sealing film, centrifuged at 2,700 rpm for 2 h in a Beckman GS-6R centrifuge at  $4^{\circ}\text{C}$  and then incubated overnight at  $4^{\circ}\text{C}$ . Plate wells were then washed with PBS-0.05% Tween 20, blocked with 0.10% skim milk powder in PBS-Tween 20, and incubated in rabbit anti-HSV-2 (Dako Corp., Carpinteria, Calif.) at 1/2,000 in blocking medium for 2 h. After being washed in PBS-Tween 20, wells were incubated 2 h in HRP-donkey anti-rabbit IgG (Jackson Immunoresearch) 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. Titer was defined as the reciprocal of the dilution at which the absorbance declined to 0.30 above the background absorbance with noninfected vaginal extracts at the same dilution (0.05 or less).  $\log_3$  geometric mean titers and their standard errors were calculated for each group and were used to determine the statistical significance of differences between groups. Geometric mean titers are also presented because they indicate more clearly the relative concentrations of shed viral proteins in the groups.

**Quantitation of epithelial infection.** Vaginae were fixed and processed for immunolabeling of HSV-2 as previously described (36). The percentage of vaginal epithelium infected by HSV-2 was determined by using an image analysis system. A high-resolution RGB color camera with integration (AIC-O-VI 470, Hyper HAD CCD; Optronic Engineering, Goleta, Calif.) was attached to a fluorescence microscope (Olympus). Computerized image analysis was performed with a MacIntosh computer (Quadra 840 AV) equipped with an LG-3 frame grabber (Scion Corp., Frederick, Md.) and using the NIH Image 1.55 program (Wayne Rasband, National Institutes of Health). The lengths of HSV-2-labeled segments of vagina were measured in four histological sections sampled from four areas of each vagina. Thereafter, the coverslips were removed and the sections were stained with hematoxylin and eosin. These stained sections were used to measure the total length of vaginal epithelium in each section. The percentage of vaginal epithelium that was HSV-2-infected was then calculated.

## RESULTS

**Neutralization of HSV-2 by unfractionated vaginal secretions.** Vaginal mucus was collected from immune and nonim-

TABLE 1. Neutralization of HSV-2 by vaginal secretions

Dilution of virus <sup>a</sup>	Incubation time <sup>b</sup> (min)	Log <sub>3</sub> shed virus protein GMT $\pm$ SEM <sup>c</sup>		Mean illness score <sup>d</sup> $\pm$ SEM	
		Immune	Nonimmune	Immune	Nonimmune
1/50	5	1.8 $\pm$ 1.1 (7.2)	6.2 $\pm$ 0.3 (908)	0.8 $\pm$ 0.5	3.0 $\pm$ 0.0
1/20	15	0.6 $\pm$ 0.4 (1.9)	6.5 $\pm$ 0.6 (1,300)	0.0 $\pm$ 0.0	2.4 $\pm$ 0.4
1/10	15	0.0 $\pm$ 0.0 (1.0)	7.0 $\pm$ 0.7 (2,200)	0.0 $\pm$ 0.0	3.0 $\pm$ 0.0

<sup>a</sup> Wild-type HSV-2 at  $3.5 \times 10^7$  PFU/ml was diluted in immune or nonimmune vaginal secretions.

<sup>b</sup> Virus was incubated in immune or nonimmune vaginal secretions for the indicated times at room temperature and then inoculated into the vaginae of nonimmune mice.

<sup>c</sup> Shed virus protein in the vaginae of nonimmune mice was measured 72 h after inoculation of virus that was incubated in immune or nonimmune vaginal secretions, and  $\log_3$  geometric mean titers (GMTs) and GMTs (in parentheses) were determined. The  $\log_3$  GMTs were significantly lower in the immune groups in each experiment ( $P < 0.005$ ).

<sup>d</sup> Illness scores were significantly lower in the immune groups in each experiment ( $P < 0.005$ ).

mune mice. The immunoglobulins and other soluble components of the mucus were extracted into PBS and then concentrated back to the original volume of the mucus so that their concentrations were equal to those in the vaginal mucus *in vivo*. Wild-type HSV-2 was diluted in the concentrated mucus extracts and incubated for 5 or 15 min *in vitro* before inoculation into the vaginae of nonimmune test mice. The results of three experiments are shown in Table 1. Test mice that received virus that was diluted in nonimmune vaginal secretions had high concentrations of shed virus proteins in their vaginae 72 h later and maximum or near-maximum illness scores 8 to 14 days later, whereas little or no virus protein was detected in vaginae and little or no illness was observed in test mice whose virus was diluted in immune vaginal secretions. The differences between immune and nonimmune groups were highly significant in each case. Vaginal mucus is replaced in less than 24 h (33), so it is unlikely that any antibody or virus from the original inoculum remained in the vaginae of the nonimmune test mice 72 h later to interfere with ELISA measurement of viral protein. The shed virus protein titers in the lumen thus indicate virus replication in the vaginal epithelium. The mean illness score caused by a 10,000-fold dilution of the virus in PBS (0.40) was significantly higher than that caused by a 10-fold dilution of virus that was incubated for 15 min in immune vaginal secretions (0.0,  $P < 0.05$ ), indicating that about 99.9% of the virus was neutralized by the immune secretions. Although the three virus dilutions and incubation times that we used do not provide dose-response information and some variability between experiments is apparent, the results clearly demonstrate that wild-type HSV-2 at concentrations that were 1,000-fold higher than the minimum needed to cause lethal illness in nonimmune mice was effectively neutralized by unfractionated vaginal secretion from immune mice at its concentration *in situ* in the vagina but not by secretion from nonimmune mice.

**Neutralization of HSV-2 by purified IgG and S-IgA from immune secretions.** To establish that neutralization of HSV-2 by unfractionated immune vaginal secretions was due to antibodies, and to clarify whether IgG or S-IgA was more important for neutralization, we extracted immunoglobulins from additional vaginal secretions. The nonimmune extract and a part of the immune extract were reserved, while IgG and S-IgA were purified sequentially from the remainder of the immune extract by affinity chromatography. All immune immunoglobulin preparations were thus derived from the same original

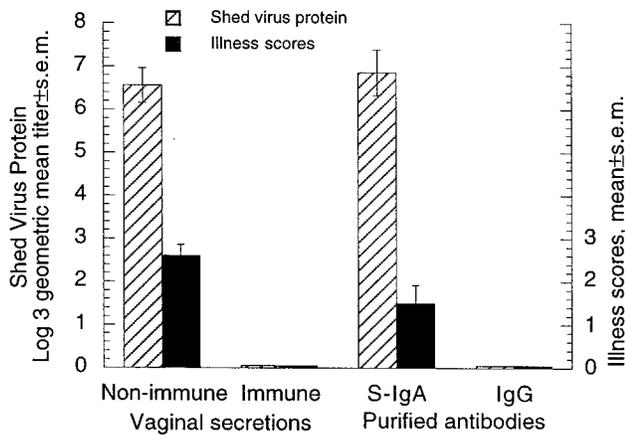


FIG. 1. Neutralization of HSV-2 by vaginal secretions and purified antibodies. Wild-type HSV-2 at  $2.0 \times 10^5$  PFU/ml was incubated for 15 min in vitro in unfractionated vaginal secretions from immune or nonimmune mice or in purified IgG or S-IgA from the immune vaginal secretions. The IgG and/or S-IgA concentrations in the four preparations were adjusted to their concentrations in situ in immune vaginal secretions. Unfractionated vaginal secretions from immune mice and purified IgG from the immune vaginal secretions effectively neutralized HSV-2, whereas unfractionated nonimmune vaginal secretions and purified S-IgA from immune secretions had little if any effect.

source. Before use, the purified immune IgG and the unfractionated extracts of immune and nonimmune vaginal secretions were concentrated to an IgG concentration of 100  $\mu$ g/ml, the concentration of this immunoglobulin in situ in immune vaginal secretions (33). The purified immune S-IgA was concentrated to 200  $\mu$ g/ml, which was the concentration of this immunoglobulin in the unfractionated extract of immune secretions after adjustment to an IgG concentration of 100  $\mu$ g/ml. Wild-type HSV-2 was diluted in each immunoglobulin preparation, incubated briefly in vitro, and then inoculated into the vaginae of nonimmune test mice (Fig. 1). Incubation of HSV-2 in unfractionated immune vaginal secretions completely neutralized the virus, whereas incubation in nonimmune vaginal secretions did not neutralize the virus, as expected from the results of Table 1. Incubation of HSV-2 in the purified IgG from immune vaginal secretions also neutralized the virus; no shed virus protein and no illness were detected in any of the test mice. In contrast, incubation of HSV-2 in the purified S-IgA from immune vaginal secretions had little if any neutralizing effect on the virus. The geometric mean shed virus protein titer in this group was 1,870 and was not significantly different from that of the nonimmune group ( $P = 0.66$ ), although the illness score in the purified S-IgA group was lower than that in the nonimmune group, and this difference was marginally significant ( $P = 0.04$ ). The neutralization experiment of Fig. 1 was repeated with a 10-fold-higher concentration of challenge virus,  $2.0 \times 10^6$  PFU/ml. Purified S-IgA again failed to neutralize the virus, whereas purified IgG caused significant ( $P < 0.05$ ) reductions in both the geometric mean shed virus protein titer (from 1,130 to 10) and the illness score (from  $3.0 \pm 0.0$  to  $1.1 \pm 0.4$ ) in comparison to nonimmune vaginal secretions. The results of these experiments indicate that the virus-neutralizing effect of immune vaginal secretions is due almost entirely to virus-specific IgG.

**Passive transfer of  $\gamma$ G or purified IgG.** To determine whether virus-specific IgG alone can protect against vaginal HSV-2 infection in vivo, we passively transferred  $\gamma$ G or purified IgG from serum of immune or nonimmune donors into nonimmune recipients 2 days after treatment with E/DP and challenged the recipients ivag with wild-type HSV-2 72 h later.

$\gamma$ G from 7.0 ml of pooled immune serum having an IgG anti-HSV-2 titer of 24,000 was divided equally among six recipients, and nonimmune  $\gamma$ G from five donors was injected into four recipients. Specific viral IgG was detected 48 h later in vaginal secretions of the mice that received immune  $\gamma$ G, but its geometric mean titer was only  $3.0\% \pm 0.8\%$  of that in the vaginal secretion standard prepared from immune mice. Figure 2 shows log<sub>3</sub> geometric mean shed virus protein titers and mean illness scores in the recipient mice. Despite the low titer of virus-specific IgG achieved in vaginal secretions of recipient mice, immune  $\gamma$ G significantly reduced the geometric mean shed virus protein titer in the vaginal lumen from 1,260 to 12.5 ( $P = 0.0054$ ) and reduced the mean illness score from  $2.5 \pm 0.50$  to  $0.33 \pm 0.33$  ( $P = 0.0054$ ). Since specific antibody was present in the vaginal lumen, some part of the reduced shed virus protein titer might be due to a blocking effect of that antibody in the ELISA measurement of viral protein. Our neutralization results indicate that the antibody should also be expected to reduce virus titers measured by plaque assay.

Purified IgG from pooled immune serum with IgG anti-HSV-2 titer of 125,000 was injected into five mice at 2.0 mg each, and the same amount of purified IgG from nonimmune serum was administered to five additional mice (Fig. 2). The geometric mean viral IgG titer in vaginal secretions of recipient mice at 48 h after transfer of immune IgG was only  $3.8\% \pm 1.4\%$  of that in the immune vaginal secretion standard. Nevertheless, the group receiving immune IgG had a significantly lower geometric mean shed virus protein titer in the vagina (480 versus 7,010,  $P = 0.012$ ) and a significantly lower illness score ( $0.40 \pm 0.24$  versus  $2.4 \pm 0.58$ ,  $P = 0.015$ ) in comparison to the mice that received nonimmune IgG.

The purified immune IgG described above was next administered to 13 mice at 3.25 mg each, and the same amount of nonimmune IgG was injected into 8 control mice 2 days after treatment with E/DP. Nine immune mice and all control mice were challenged ivag with wild-type virus 72 h later. Vaginal secretions were collected from the remaining four immune mice 72 h later to provide measurements of IgG anti-HSV-2 titers in the vagina at the moment of vaginal challenge. The challenged mice were sacrificed 30 h later for measurement of epithelial infection in the vagina, and serum was collected at

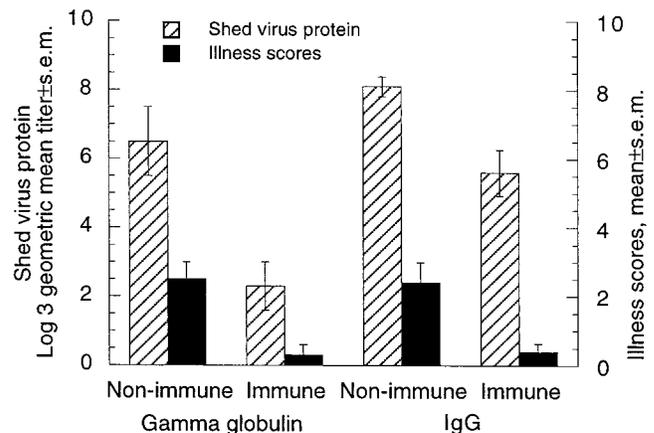


FIG. 2. Passive transfer of  $\gamma$ G or purified IgG.  $\gamma$ G or purified IgG from the serum of immune or nonimmune donors was passively transferred to nonimmune recipient mice. The recipients were challenged ivag 72 h later with  $20 \mu$ l of wild-type HSV-2 at  $3.5 \times 10^6$  PFU/ml. Shed virus protein titers were measured in the vaginae of recipient mice 72 h after challenge, and illness scores were recorded 8 to 14 days after challenge. Recipients of immune  $\gamma$ G or immune IgG had significantly lower shed virus protein titers and illness scores.

this time for measurement of IgG anti-HSV-2 titers. Virus infection of the vaginal epithelium was significantly less in recipients of immune IgG than in recipients of nonimmune IgG ( $3.1\% \pm 0.90\%$  versus  $8.2\% \pm 1.4\%$ ,  $P = 0.0085$ ). Specific viral IgG in vaginal secretions of four mice that received immune IgG was, at the moment of virus challenge,  $7.9\% \pm 2.3\%$  of the immune vaginal secretion standard, while IgG viral antibody in serum when the mice were killed was  $29\% \pm 7.8\%$  of the immune serum standard.

Collectively, the passive transfer data indicate that purified immune IgG alone significantly protected against vaginal HSV-2 infection, as evidenced by reduced infection of the vaginal epithelium 30 h after vaginal challenge and reduced illness 8 to 14 days later. The reduced shed virus protein concentrations in the vaginal lumen 72 h after challenge are consistent with the other data, but some part of the reduction might have been due to the presence of antibody in the vagina. The protection afforded by IgG was observed even though the titers of viral antibody in serum and especially in the vaginal secretions of recipient mice were much lower than the corresponding titers in immune mice 6 weeks after attenuated virus infection.

**Role of vaginal secretions in immunity to HSV-2.** The neutralization and passive transfer data indicate that virus-specific IgG in vaginal secretions of immune mice can reduce challenge infection of the vaginal epithelium by wild-type HSV-2. This implies that removal of the vaginal secretions from immune mice shortly before virus challenge should reduce immunity. To test this prediction, we immunized mice in the vagina with attenuated HSV-2 and 6 weeks later divided them into two groups. In one group the vaginal mucus secretions were washed out with PBS, and 10 min later both groups were challenged ivag with wild-type virus at  $3.5 \times 10^6$  PFU/ml. Shed virus protein titers in the vaginal lumen were measured 48 h later in both groups as an indication of virus infection in the adjacent epithelium. Since the vaginal mucus is replaced in less than 24 h (33), vaginal antibody titers in the washout group should have recovered to the level of the nonwashout group after 48 h, so if vaginal antibody influences shed virus protein measurements, the influence should have been the same in both groups. Differences in shed virus protein titers should therefore reflect differences in virus replication in the epithelium. The  $\log_3$  geometric mean titer of shed virus protein was significantly higher in the mucus washout group than in the control group ( $3.5 \pm 0.71$  versus  $0.96 \pm 0.63$ ,  $P = 0.02$ ). The geometric mean titers (47 and 2.9, respectively) indicate that the mean concentration of shed virus protein in the vaginal lumen was 16-fold higher in the washout group than in the control group. This result supports a conclusion that secreted IgG in the vaginal lumen of immune mice plays a significant role in vivo in protecting the epithelium against a challenge infection by wild-type HSV-2.

## DISCUSSION

The possibility that specific antibodies in genital tract secretions from immune females can neutralize sexually transmitted disease pathogens has received little direct investigation since early studies by Coughlan and Skinner (5). They reported that HSV-2 was neutralized by 16-h incubation in vitro in cervico-vaginal secretions from women who had previously been infected by this virus. In the present study, we observed high levels of vaginal infection by HSV-2 when the challenge virus was mixed with unfractionated, nonimmune vaginal secretions or with purified S-IgA from immune vaginal secretions before inoculation into vaginae of nonimmune test mice. In contrast,

little or no infection of the vaginal epithelium was observed in test mice when the challenge virus was mixed with unfractionated, immune vaginal secretions or with purified IgG from immune secretions. Since the vaginal secretions and purified antibodies were used at their concentrations in vivo and they neutralized virus that was about 1,000-fold more concentrated than the minimum needed to cause lethal illness in nonimmune mice, it is probable that the IgG viral antibody in vaginal secretions of immune mice can neutralize a substantial portion of a challenge virus inoculum before it infects the epithelium. Complement was not present during incubation of challenge virus with purified antibodies in vitro, but it may be present in vaginal secretions in vivo (35) and might have played a role in IgG-mediated virus neutralization after inoculation of the challenge virus into vaginae of test mice. The neutralization results are in accord with the viral antibody titers measured by ELISA in immune vaginal secretions, where IgG was present at a geometric mean titer of 6,200, in comparison to a titer of only 1.9 for S-IgA (33). The present results also demonstrated that the low ELISA titer of viral S-IgA in immune vaginal secretions was not due to competition with viral IgG because affinity-purified S-IgA from these secretions had the same titer as the unfractionated secretions when both were adjusted to the same S-IgA concentration.

To further investigate whether immune IgG can provide significant protection against vaginal HSV-2 infection in vivo, we passively transferred purified polyclonal IgG from immune mouse serum to nonimmune test mice 3 days before vaginal challenge with wild-type virus. The passively transferred IgG protected recipient mice even though the titers of virus-specific IgG achieved in their serum and vaginal secretions were low compared to those in mice that were immunized with attenuated virus. It is of interest that McDermott et al. (21) were unable to detect a monoclonal HSV-2 antibody in vaginal secretions after passive transfer to nonimmune mice, and the recipient mice were not protected against challenge infection. Similarly, Eis-Hubinger et al. (10) did not detect HSV-1 antibody in vaginal secretions or protection after passive transfer of polyclonal serum antibody. Passively transferred monoclonal antibody to HSV glycoprotein B was also not detectable in vaginal secretions, but it nevertheless protected the recipients against vaginal infection by HSV-1 (10). Our data quantitate the surprisingly low levels of passively transferred IgG that reach the vaginal secretions of recipient mice, relative to levels present in serum of the same mice and relative to levels present in the vaginal secretions of immune mice. Our ability to achieve and detect these low levels of viral IgG in the vaginal secretions of passive transfer mice may relate to the use of progestin-treated mice, larger amounts of specific IgG administered to recipient mice, more thorough extraction of immunoglobulin from the vaginal mucus, or other factors.

To further test the hypothesis that specific antibodies in vaginal secretions of immune mice protect the vaginal epithelium against challenge infection by HSV-2 in vivo, we removed the vaginal mucus from a group of immune mice 10 min before vaginal inoculation of challenge virus. Shed virus protein concentrations in the vaginal lumen of these mice 48 h later were 16-fold higher than in immune mice whose vaginal secretions were not removed before challenge. Taken together, the neutralization, passive transfer, and washout data indicate that virus-specific IgG in vaginal secretions of immune mice significantly reduces infection of the vaginal epithelium by challenge virus, whereas S-IgA contributes relatively little to this protection.

It is widely accepted that local immunization at mucosal surfaces elicits mainly secretory IgA antibody, which then plays

the major role in immune protection at those mucosal surfaces (18, 28, 47). This paradigm applies well to most of the gastrointestinal tract and upper respiratory tract, and it is often assumed that it applies as well to other mucosal tissues such as the female genital tract (28). It is therefore of interest to consider why local immunization in the vagina with attenuated HSV-2 elicited mainly IgG viral antibody and this IgG provided the main humoral protection against challenge infection. There is agreement that the specific antibody response after vaginal infection by attenuated HSV-2, as measured by ELISA titers, is almost entirely IgG (21, 26, 33). This predominant IgG response is consistent with our notion that the vagina is a poor inductive site for IgA responses because several important components of the mucosal immune system that are involved in the induction of IgA responses are absent or poorly represented in the female genital mucosa (38). This line of reasoning suggests that the IgG-based secretory immunity that is stimulated by vaginal immunization with attenuated HSV-2 may be relatively weak, and that immunization at an IgA inductive site would produce stronger immunity. Indeed, it is widely assumed that an S-IgA response will be required to produce optimum immunity in the female genital tract (4, 6, 8, 9, 15–17, 19, 24, 31, 41, 43, 44), and many laboratories have studied immunization in the intestine (1, 3, 11, 16, 27, 30, 32, 42, 49), nasopharynx (7, 13–15, 29, 44), and pelvis (20, 46, 48) at least partly with a view toward induction of S-IgA responses in the female genital tract. Actually, however, the mucosal immunity elicited in mice by vaginal immunization with attenuated HSV-2 is quite strong, even in primary immunity. Replicating virus was observed in 3 to 11% of the vaginal epithelium 24 h after HSV-2 was inoculated into vaginae of nonimmune mice, whereas virus was detectable in only 0.08 to 0.25% of the epithelium in immune mice at this time (37). Also, shed virus protein titers in the range of 5,000 to 6,000 were observed in the vagina 72 h after nonimmune mice were inoculated with HSV-2, whereas no virus protein was detected in vaginae of immune mice at this time (37). The dose of challenge virus used in these studies was about 1,000-fold higher than is needed to cause lethal infection in nonimmune mice; thus, vigorous immunity was needed to suppress the challenge infection so completely. Few data are available on immunity to vaginal HSV-2 infection in mice that were immunized at an IgA-inductive site such as the nasopharynx, but direct comparisons of such immunity to that induced by vaginal immunization would be of considerable interest. In one study, mice that were immunized intranasally with adenovirus that carried the gene for HSV glycoprotein B had high concentrations of HSV-2 in the vaginal lumen following vaginal challenge, and they were only partially protected against illness (14). The immunity of the nasally immunized mice thus appears to have been weaker than that of mice immunized vaginally with attenuated HSV-2, but the adenovirus vaccine may not have replicated well in the nasopharyngeal mucosa, and it expressed only one HSV glycoprotein. Cogent reservations have been expressed about the strength of IgA immunity in remote sites after immunization at inductive sites (28).

The titer of viral S-IgA antibody in vaginal secretions might increase after vaginal boosting more than the IgG titer and thus increase the relative neutralizing effectiveness and importance of S-IgA in secondary immunity. Indeed, Milligan and Bernstein (26) have reported that S-IgA titers increased more than IgG titers in young mice whose primary immunity to HSV-2 was boosted 3 weeks later by vaginal challenge. Our preliminary measurements confirm this observation and indicate that vaginal boosting 6 weeks after primary immunization of adult mice caused a 27-fold increase in S-IgA viral titers in

the vagina 8 weeks later while at the same time increasing IgG titers only 3-fold. Nevertheless, the ELISA titer of viral IgG in the vagina 8 weeks after boosting was still 350-fold higher than the S-IgA titer, and although we have not yet done neutralization studies with these mice, it seems likely that virus neutralization by IgG in the vaginal secretions of boosted mice would still exceed that of the S-IgA.

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