JOURNAL OF VIROLOGY, Mar. 2005, p. 3117–3126 Vol. 79, No. 5
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.5.3117–3126.2005

Protection against Genital Herpes Infection in Mice Immunized under Different Hormonal Conditions Correlates with Induction of Vagina-Associated Lymphoid Tissue

Amy E. Gillgrass, Vera A. Tang, Kate M. Towarnicki, Kenneth L. Rosenthal, and Charu Kaushic*

Center for Gene Therapeutics, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Received 20 October 2004/Accepted 13 December 2004

The present study was undertaken to examine the effect of the hormonal environment on immunization with an attenuated strain of herpes simplex virus type 2 (HSV-2 TK$^-$) and subsequent protection against challenge. Ovariectomized mice were administered saline (S; control), estradiol (E_2), progesterone (P_4), or a combination of estradiol and progesterone (E+P) and immunized intravaginally (IVAG) with HSV-2 TK$^-$. Three weeks later, the immunized mice were challenged IVAG with wild-type HSV-2. Mice that were immunized following E treatment were not protected, whereas complete protection against the challenge was seen in mice from the S- and P_4-treated groups. In the P_4-treated group, 15% of mice developed chronic pathology following TK$^-$ immunization. Interestingly, about 40% of the E+P-treated mice were also protected. Upon examination of viral shedding in the vaginal secretions, it was clear that protection against challenge was dependent on the ability of the TK$^-$ virus to cause productive genital infection under different hormonal conditions. In the protected mice (the S and P groups and part of the E+P group), induced vagina-associated lymphoid tissues composed of CD11c$^+$ dendritic cells and CD3$^+$ and CD4$^+$ T cells were formed transiently in the vaginal lamina propria from day 2 to day 5 postchallenge. These aggregates were absent in the unprotected mice (the E group and part of the E+P group). Significant HSV-2-specific activation of lymphocytes was observed in the local draining lymph nodes of protected mice. This response was absent in the unprotected groups. High titers of gB-specific local immunoglobulin A (IgA) antibodies were present in the vaginal secretions of S- and P_4-treated immunized mice following HSV-2 challenge. The S-treated group of mice also had high gB-specific IgG titers. These studies show that sex hormones modify the induction of protective immune responses following IVAG immunization.

In the past two decades, the incidence of sexually transmitted infections (STIs) has grown in virtually every country in the world (2), despite the fact that in this same time period there has been a continuous increase in resources and efforts devoted to controlling these infections. Although many of the STIs do not cause mortality, they are a major source of morbidity and financial burden on health systems globally. In addition, vertical transmission of these infections from mother to infant has serious sequelae. It is widely accepted that the best strategy to control these infections on a worldwide basis would be the development of efficacious prophylactic vaccines. Despite significant efforts, this goal, for the most part, remains elusive.

Herpes simplex virus type 2 (HSV-2) infection is arguably the most common viral STI (18). A number of prophylactic and therapeutic vaccines have been designed and tested for the prevention and treatment of HSV-2 infections (16). In a recent subunit vaccine trial involving a truncated form of glycoprotein D of HSV-2, about 40% protection from disease was seen only in women who were seronegative for both HSV-1 and HSV-2 (32). This result raises two issues critical for the future success of an HSV vaccine as well as for other vaccines for STIs. The first is that while current vaccines are designed to induce systemic immunity, most sexually transmitted infections, including HSV-2, are in fact mucosal infections that are initiated in the male and female genital mucosae. To prevent sexual transmission of this virus, vaccine strategies must be designed to induce and sustain durable mucosal immune responses in the genital tract. Secondly, due consideration needs to be given to the possibility that gender-related factors may play an important role in the efficacy of these vaccines. In women, the female sex hormones estradiol and progesterone have already been shown to regulate immune responses in the reproductive tract (3, 35, 36). Therefore, it will be important to examine the effect of these hormones on STI vaccination strategies for women.

We and others have shown that estradiol and progesterone not only influence immune responses in the female genital tract but that, in fact, they also regulate susceptibility to infections (5, 14, 15, 20, 31). In previous studies, we showed that genital infection with *Chlamydia trachomatis*, a sexually transmitted bacterium, is profoundly affected by the hormonal milieu in the reproductive tract in a rat model (14, 15). More recently, we examined the effect of a long-acting progestational formulation, medroxyprogesterone acetate (Depo-Provera), on susceptibility to genital HSV-2 infection in a mouse model (12). These studies demonstrated an increased susceptibility to HSV-2 after medroxyprogesterone acetate treatment (12). In addition, we found that prolonged treatments with this hormone decreased mucosal antiviral immune responses (9). Sim-

* Corresponding author. Mailing address: Department of Pathology, MDCL 4014, McMaster University, 1200 Main St. West, Hamilton, Ontario, Canada L8N 3Z5. Phone: (905) 525-9140, ext. 22988. Fax: (905) 522-6750. E-mail: kaushic@mcmaster.ca.

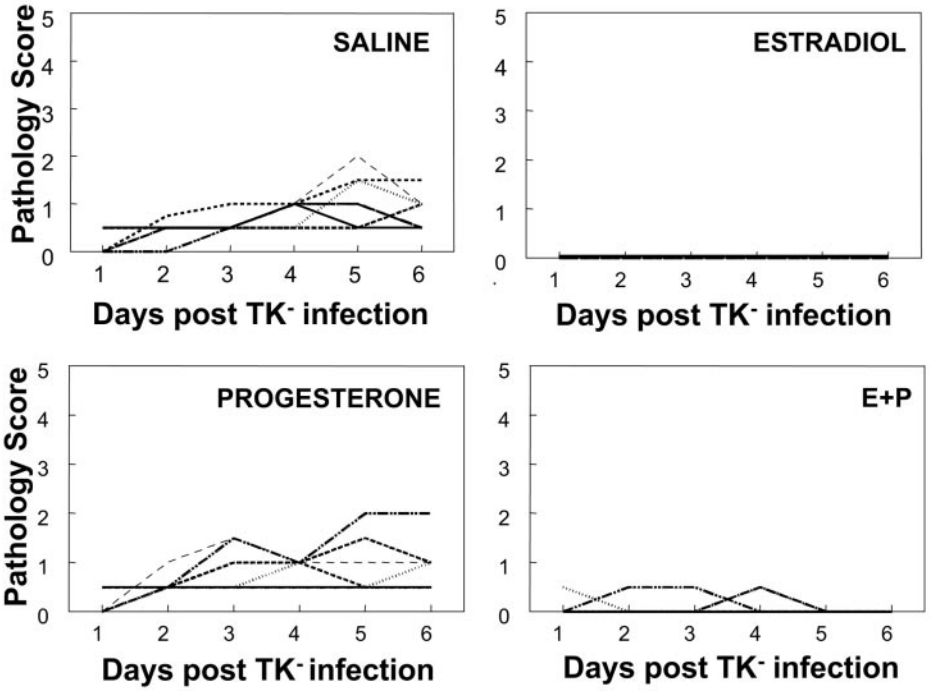

FIG. 1. Pathology of ovariectomized, hormone-treated mice after immunization with HSV-2 TK$^-$ (10^5 PFU). Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Following IVAG immunization with attenuated HSV-2 (TK$^-$), vaginal pathology was monitored daily. Pathology scores of each mouse in all four hormone groups over 6 days following immunization are shown. Pathology scores halfway between two numbers were given when a readout was transitional between two consecutive scores. Each group had six to eight mice. The results shown are representative of three separate experiments.

ilar results have been seen in monkeys. In a recent study, treatment of rhesus macaques with medroxyprogesterone acetate prior to intravaginal (IVAG) challenge with simian immunodeficiency virus mac239 abolished attenuated vaccine-induced protection (1).

The present study was designed to examine the role of sex hormones in the induction of protective immune responses in mice immunized with a live attenuated strain of HSV-2 (TK$^-$). Ovariectomized mice were given estradiol, progesterone, a combination of both, or saline (control group) prior to IVAG immunization with TK$^-$ HSV-2. Mice were challenged intravaginally 3 weeks later with wild-type HSV-2. Survival and pathology were monitored and correlated with viral shedding in the vaginal secretions of the different hormone-treated immunized mice. The histopathology of the vaginal mucosa was examined, and HSV-2 infection was localized. To examine the mechanism of protection, herpes-specific T-cell responses were measured in local lymph nodes, and immune cells present in the genital tract were compared among different groups. Local humoral responses to HSV-2 in the vaginal secretions were also examined.

MATERIALS AND METHODS

Animals and hormone treatments. Inbred 8- to 10-week-old C57BL/6 mice purchased from Charles River Canada (Constant, Quebec, Canada) were used in these studies. Mouse colonies were maintained on a 12-h dark and 12-h light cycle. Ovariectomies were performed 10 to 14 days before each experiment. 17β-Estradiol and progesterone were purchased from Calbiochem (La Jolla, Calif.). Estradiol was initially dissolved in ethanol, evaporated to dryness, and then resuspended in phosphate-buffered saline (PBS). Progesterone was suspended in PBS by glass-glass homogenization. All hormones were administered by subcutaneous injection. Mice received either 500 ng of estradiol or 0.5 mg of progesterone or a combination of both in a 100-μl volume for three consecutive days. Control mice were injected with 100 μl of saline alone.

Inoculation of animals. Mice were anesthetized by injectable anesthetic (150 mg of ketamine/kg–10 mg of xylazine/ml) given intraperitoneally, placed on their backs, and inoculated IVAG with 10 μl of attenuated HSV-2 (TK$^-$) at a dose of 10^5 PFU. Three weeks later, the mice were inoculated IVAG with 10 μl of wild-type HSV-2 strain 333 at a dose of 10^5 PFU. In both cases, mice were kept on their backs under the influence of anesthesia for 45 min to 1 h to allow the inoculum to infect.

Vaginal smears and lavage fluid collection. Vaginal lavage fluid for reproductive cycle staging and plaque assays was collected by pipetting two 30-μl portions of PBS in and out of the vagina several times to give a total of 60 μl. Reproductive cycle staging was done to confirm the depletion of endogenous hormones after ovariectomy. To make vaginal smears, the fluid was smeared on glass slides and examined by light microscopy to determine the stage of the estrous cycle as described previously (37). The following classification was used for identifying the stage of the cycle: estrus, >90% cornified epithelial cells; diestrus, >75% polymorphonuclear cells; metestrus, 50% epithelial cells and 50% polymorphonuclear cells. For plaque assays, the vaginal wash fluids were frozen at −70°C.

Viral replication and pathology in the reproductive tract. Genital pathology following infection with HSV-2 was monitored daily and scored on a five-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness, swelling, and hair loss of genital and surrounding tissue; and 5, severe genital ulceration extending to surrounding tissue. Animals were sacrificed after they reached stage 4.

To assess viral shedding, vaginal washes were analyzed by plaque assay. Vero cells were grown in α-MEM (GIBCO Laboratories, Burlington, Canada) supplemented with 5% fetal bovine serum (GIBCO), 1% penicillin-streptomycin, and L-glutamine (GIBCO). For plaque assays, Vero cells were grown to confluence in 12-well plates. Samples were diluted (10^{-2} to 10^{-7}) and added to monolayers. Infected monolayers were incubated at 37°C for 2 h for viral ab-

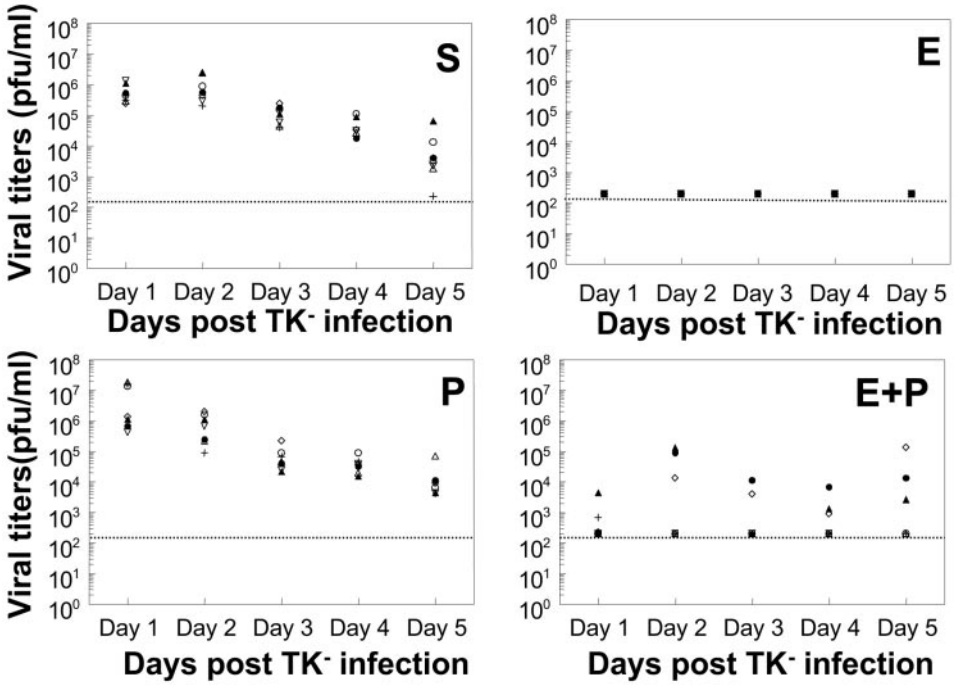

FIG. 2. Virus titers in vaginal washes of ovariectomized, hormone-treated mice after immunization with HSV-2 TK$^-$ (10^5 PFU). Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Following IVAG immunization with attenuated HSV-2 (TK$^-$), vaginal washes were collected daily and viral plaque assays were done as described in the text. Plaques were counted, and viral titers were expressed in PFU per milliliter. Each symbol represents a single animal (n = six to eight animals in each group). Dashed lines represent the lower detection limit of the assay. The results are representative of three separate experiments.

sorption. Infected monolayers were overlaid with α-MEM supplemented with 0.05% human immune serum globulin (Canadian Blood Services). Infection was allowed to occur for 48 h at 37°C. Monolayers were then fixed and stained with crystal violet, and viral plaques were counted under a light microscope. The number of PFU per milliliter was calculated by taking a plaque count for every sample and taking into account the dilution factors.

Immunohistochemistry. Genital tissues were excised from the mice and embedded in Tissue-Tek OCT compound prior to freezing in liquid nitrogen. Cryosections were cut at 7-μm thickness and kept at −70°C until use. Prior to staining, sections were placed in cold acetone for 10 min followed by air drying. Nonspecific staining was blocked by incubating sections with 5% goat serum or 0.1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. To detect HSV-2 infection, the sections were incubated with rabbit anti-HSV-2 antiserum (DAKO Corporation, Carpinteria, Calif.). To stain for T cells and CD11c, the following antibodies were used: rat anti-mouse CD4, hamster anti-mouse CD11c, hamster anti-mouse CD3, and hamster anti-mouse CD8 (all from BD Pharmingen). Incubations were carried out for 1 h at room temperature. Appropriate isotype controls were substituted for primary antibody at an equivalent concentration for control staining. Avidin-biotin coupled to alkaline phosphatase (ABC Elite kit; Vector Laboratories, Burlingame, Calif.) followed by Vector Red (alkaline phosphatase substrate kit; Vector Laboratories) was used to reveal HSV-2 localization. For the other markers, biotinylated anti-hamster or anti-rat secondary antibodies (DAKO Corporation) were used. A horseradish peroxidase Envision system (DAKO Corporation) was used for the detection of enzyme. Counterstained slides were mounted in Permount medium prior to microscopic examination.

ELISA for anti-HSV-2 gB IgG and IgA. HSV-2 gB-specific antibody titers were determined by an enzyme-linked immunosorbent assay (ELISA) modified from a protocol described previously (7). Briefly, Maxisorp 96-well Microwell plates (Nalge Nunc International, Rochester, N.Y.) were coated overnight with 2.5 μg of recombinant gB protein (Chiron, Emeryville, Calif.)/ml in PBS at 4°C. Plates were blocked with 2% BSA for 2 h at room temperature and loaded with 100 μl of twofold serial dilutions of samples or controls. Incubation was carried out in the dark at 4°C overnight. Plates were washed and reacted for 1 h with one of the following biotinylated antibodies: goat anti-mouse immunoglobulin G (IgG) or goat anti-mouse IgA at a 1:1,000 dilution (Pharmingen, Mississauga, Ontario, Canada). Plates were developed with extravidin-peroxidase (1:2,000 dilution) and tetramethylbenzidine. End point titers were determined and expressed as geometric mean titers. Background values were obtained by using vaginal lavage fluids from nonimmunized mice. Two times the mean background optical density value was taken as the cutoff for determining positive values.

Lymph node cell preparation and culture. Iliac lymph nodes (LN) that drain the genital tract were dissected, and a single-cell suspension was prepared by teasing the LN. Debris was allowed to settle for 2 min, and supernatant containing single cells was recovered and spun down at 500 × g for 7 to 10 min. Cells were washed with RPMI 1640 medium containing 5% FBS and plated at a density of 5×10^5 cells/well in 96-well plates. Cells were tested for HSV-2-specific proliferation by addition of gB (10 μg/ml; Chiron Inc) in triplicate cultures. Total T-cell proliferation was measured by adding T-cell mitogen, concanavalin A (ConA; 1 μg/ml), to LN from all groups. Proliferative responses were measured by the uptake of 1 μCi of [^{3}H]thymidine per well for last 18 h of a 3-day culture. Results are reported as the mean counts per minute ± the standard error of the mean from triplicate cultures. Results were analyzed by an unpaired two-tailed t test using GraphPad PRISM software. Significance was defined as P value of <0.05.

RESULTS

Pathology and virus titers following immunization with TK$^-$ HSV-2. Four groups of mice were ovariectomized, and 2 weeks later, two of the groups were treated with estradiol (E_2) or progesterone (P_4) for three consecutive days. A third group (the E+P-treated group) was treated with a combination of both hormones. The fourth group served as a control and received sham injections of saline instead of hormones. Twenty-four hours after the last injection, animals were vaccinated IVAG with 10^5 PFU of the attenuated HSV-2 (TK$^-$). Vaginal pathology was monitored, and vaginal wash fluids were collected daily to monitor viral shedding. Figure 1 shows the

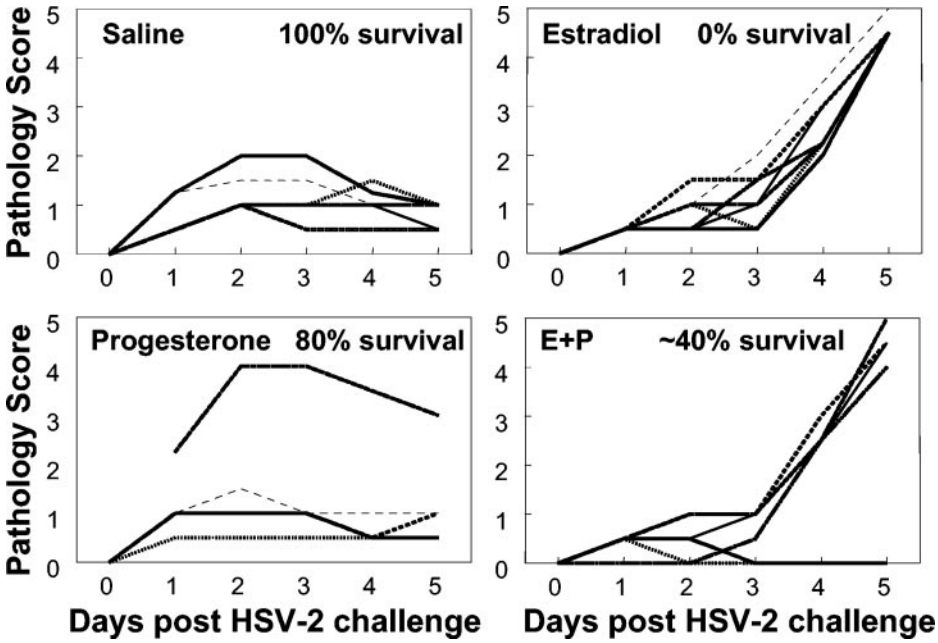

FIG. 3. Pathology and survival of ovariectomized, hormone-treated, immunized mice following challenge with wild-type HSV-2. Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Three weeks after IVAG immunization with attenuated HSV-2, mice were challenged IVAG with wild-type HSV-2 (10^5 PFU). Vaginal pathology and survival were monitored daily. Pathology scores of each mouse in all four hormone groups over 5 days following challenge are shown. Pathology scores halfway between two numbers were given when a readout was transitional between two consecutive scores. Final survival numbers are indicated for each hormone treatment group. Each group had six to eight mice. The results shown are representative of three separate experiments.

external pathology scores over 6 days postvaccination. No significant pathology was noted in the E_2 and E+P groups. Mice in P_4-treated group and control group started to show low pathology scores 24 to 48 h postvaccination. However, the pathology was limited to redness and swelling in the genital area and did not progress to the formation of ulcerated lesions typical of wild-type HSV-2 infection. In the majority of the mice in both groups, the pathology resolved in less than 10 days. In one of seven mice in the P_4-treated group, the redness and swelling persisted.

Virus titers measured in vaginal washes from the mice in the four groups up to 5 days postimmunization correlated with the pathology measurements (Fig. 2). Maximum viral shedding was seen in the P_4-treated group and in the saline-injected control group. Viral shedding was undetectable in the E_2-treated, immunized group. In the group given the combination of hormones (E+P), three out of seven mice had detectable viral shedding in their vaginal secretions after TK^- immunization.

Survival, pathology, and virus titers following challenge with wild-type HSV-2. We next examined how immunization with HSV-2 TK^-, under the influence of different hormones, could alter protection against a subsequent challenge with wild-type HSV-2. Hormone-treated, immunized mice were challenged IVAG with 10^5 PFU of wild-type HSV-2 3 weeks postimmunization. Figure 3 shows the pathology scores and survival in the four groups after challenge. All mice that were immunized under the influence of estradiol started to develop pathology 24 to 48 h after challenge. The condition of these mice continued to deteriorate; they developed genital lesions, with scores of 4 or higher on the pathology scale, and had to be sacrificed 4 to 5 days postchallenge. Mice immunized following P_4 treatment and in the absence of any hormones (S) developed minimal pathology (redness and swelling) following challenge with wild-type virus. All except one mouse in the P_4-treated group survived the challenge. The one mouse in the P_4-treated group that was sacrificed never recovered completely from the pathology it developed following TK^- immunization. Following HSV-2 challenge, it lost weight and had to be sacrificed. Interestingly, despite its condition, this mouse did not develop any genital lesions postchallenge. In subsequent experiments, in every batch, 10 to 20% of P_4-treated mice appeared to develop similar chronic pathology. Most of the mice in the P_4-treated group and a few mice in the control (no hormone, saline-treated) group also displayed "bloody" vaginal smears postchallenge, indicating a damaged epithelium. About 40% (three out of seven) of the E+P-treated, immunized mice did not develop any significant pathology and survived the challenge.

The virus titers measured after HSV-2 challenge in the vaginal washes of the four groups of mice correlated with the pathology scores (Fig. 4). While all of the mice in the E_2-treated group had high virus titers in their vaginal secretions, none of the mice in the P_4-treated group or the control group (S) had detectable viral shedding. The protected mice in the E+P group (40%) showed viral shedding in the vaginal washes until 48 h after challenge, while the mice in this group that were unprotected (60%) had high virus titers throughout. The 40% of the mice that were protected were the same ones that had high viral shedding following TK^- immunization.

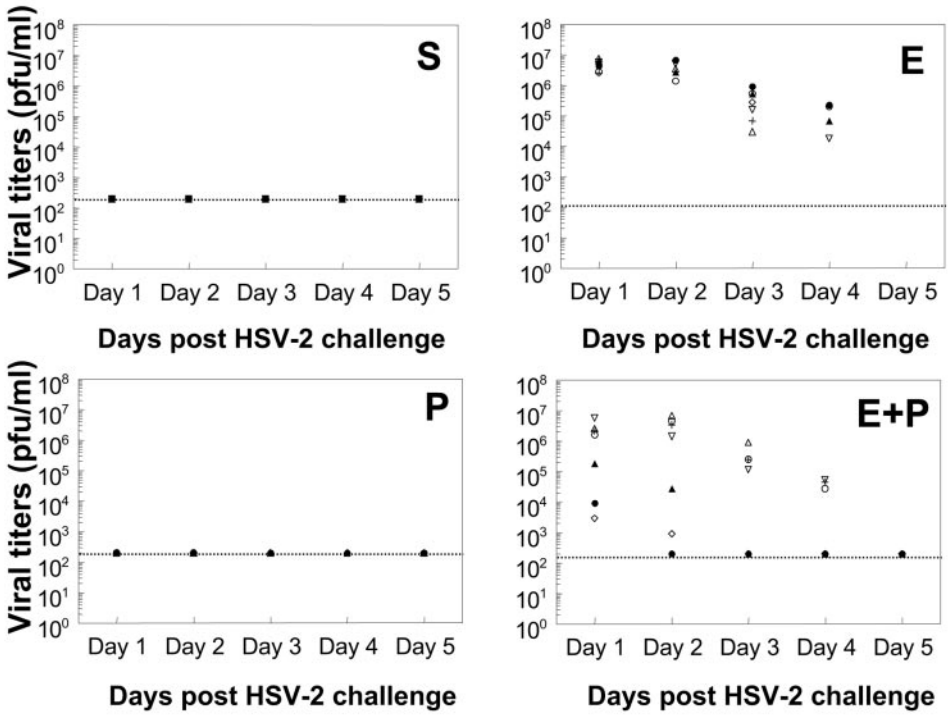

FIG. 4. Virus titers in vaginal washes of ovariectomized, hormone-treated, immunized mice after challenge with wild-type HSV-2. Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Three weeks after IVAG immunization with attenuated HSV-2, mice were challenged IVAG with wild-type HSV-2 (10^5 PFU). Vaginal washes were collected daily, and viral plaque assays were done as described in the text. Plaques were counted, and viral titers were expressed in PFU per milliliter. Each symbol represents a single animal (n = six to eight animals in each group). The dashed lines represent the lower detection limit of the assay. The results are representative of three separate experiments.

Histopathology in hormone-treated, immunized mice following challenge. Since the outcomes of IVAG HSV-2 challenge were very different in the four hormone-treated groups, the histopathology of the vaginal tissue was examined 24 h to 5 days postchallenge. The histopathology (Fig. 5A to D) and the extent of HSV-2 infection determined by immunohistochemical localization (Fig. 5E to H) on day 3 are shown. Because the mice were challenged 3 weeks after hormone treatment and immunization, there was no evidence of any lasting hormonal effect on the vaginal tissue of any of the groups. Heavy mononuclear infiltration was observed in the E_2-treated TK^--immunized group after wild-type HSV-2 challenge (Fig. 5B). Extensive localization of HSV-2 was also seen in the vaginal epithelium (Fig. 5F) of these mice, indicating the failure of immunization in these mice. The kinetics of infection and leukocytic infiltration were similar to a primary vaginal HSV-2 infection (9a). HSV-2 infection was not found by immunohistochemical localization in the control group, the P_4-treated group, or the protected E+P-treated mice (three out of seven mice) (Fig. 5E, G, and H, respectively). The P_4-treated group had the worst pathology, with extensive damage to the vaginal epithelium (Fig. 5C). Leukocytic infiltration and hyperplasia were evident in the subepithelial stroma of the P_4-treated group. The S-treated group did have some subepithelial leukocytic infiltration as well, but the epithelium was less extensively damaged than those of the P_4-treated group (Fig. 5A). The protected E+P group had the most intact epithelium, with no signs of any tissue damage (Fig. 5D).

T-cell responses in draining lymph nodes of immunized mice after challenge. In order to study the protection against wild-type virus challenge seen in some of the hormone-treated groups, we examined the local T-cell responses to HSV-2 in all four groups of mice. Cells from lymph nodes draining the genital tract (iliac lymph nodes) were examined for HSV-2-specific T-cell-proliferative responses 3 days after IVAG HSV-2 challenge. Figure 6 summarizes the proliferative responses seen in all four groups. The E+P group was split into protected and nonprotected mice. The HSV-2-specific responses were measured by in vitro stimulation with gB, a highly immunogenic HSV-2 envelope glycoprotein. All three protected groups (the S and P_4 groups and part of the E+P group) showed significantly higher gB-specific proliferation than the unprotected groups (E_2 and part of E+P) ($P < 0.05$). The gB-specific proliferation in the E+P protected group and the P_4-treated group was also significantly higher than in their respective controls (proliferation in the absence of in vitro gB challenge) ($P < 0.05$). All of the groups had comparable proliferations when they were stimulated by a T-cell mitogen (ConA), indicating a lack of immunosuppression in any of the groups.

Induced vagina-associated lymphoid tissue (iVALT) in immunized, protected mice. Based on the significant differences

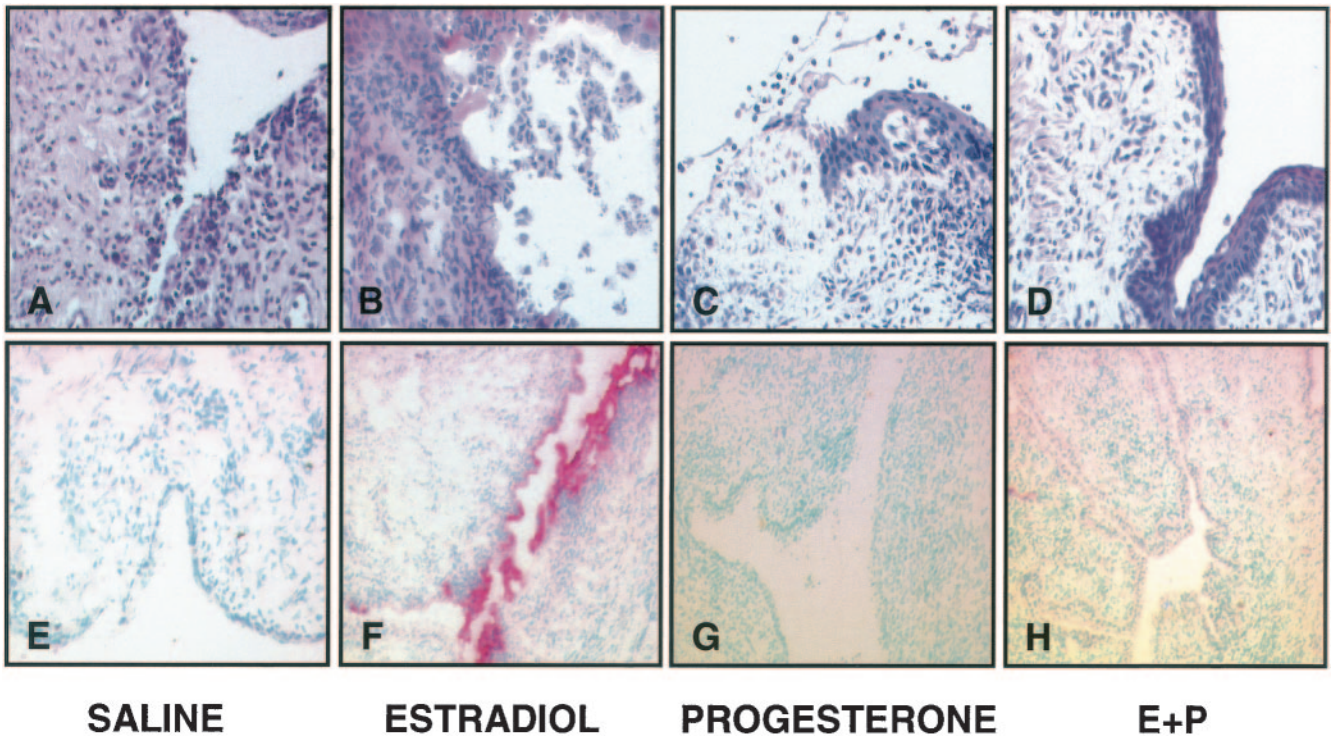

FIG. 5. Histopathology and localization of infection in the vaginal tissue of ovariectomized, hormone-treated, immunized mice after wild-type HSV-2 challenge. Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Three weeks after IVAG immunization with attenuated HSV-2, mice were challenged IVAG with wild-type HSV-2 (10^5 PFU). Mice were sacrificed 3 days postchallenge, histopathology was examined (A to D), and HSV-2 infection was localized by immunohistochemistry (E to H). Representative tissue sections are shown. Only the vaginal sections from a protected E+P mouse are shown here (D and H). Note the intact vaginal epithelium and lack of any inflammation (D) as well as the absence of any infection (H). The E_2-treated mice show acute inflammation and leukocytic infiltration (B) and extensive infection, shown in pink (F). Progesterone-treated mice show extensive epithelial damage, leukocytic infiltration in the tissue and in the lumen (C), and the absence of any HSV-2 staining (G). The saline control mice show some epithelial damage and infiltration (A) but no infection (E). Isotype controls did not show any positive staining (data not shown). Original magnification, ×100.

in the HSV-2-specific T-cell activation in local lymph nodes in different hormone treatment groups, we examined the local immune cell population in the vaginal mucosae of the four groups of mice on various days postchallenge. In all three protected groups (S, P_4, and part of E+P), the presence of transient lymphoid aggregates (LA) or iVALTs was noted. The LA were induced within 48 h after challenge with wild-type HSV-2 and diminished both in size and in frequency by day 5 postchallenge. Figure 7 shows localization on day 2 postchallenge. Immunohistochemical localization indicated that these iVALTs were composed of an outer halo of $CD11c^+$ cells and the majority of the cells in the LA were $CD3^+$ and $CD4^+$. CD8 T cells were found nonpreferentially distributed throughout the vaginal laminae propriae of all four groups. The protected E+P group had the largest number and size of iVALT (data not shown). Similar structures were found in the P_4 and S groups, although not at the same frequency and in smaller size than in the E+P group (data not shown). The unprotected groups (the E_2 group and part of the E+P group) failed to show induction of similar iVALT.

HSV-2-specific IgG and IgA levels in vaginal secretions of immunized mice following challenge. To correlate the protection in ovariectomized, hormone-treated, immunized mice with humoral responses against HSV-2, local antibody levels in the vaginal secretions were measured. Figure 8 shows the antibody titers to HSV gB measured in each group. A majority of the mice in the S and P_4 groups had high titers of gB-specific IgA. All mice immunized in the absence of hormones (the S group) also had high levels of gB-specific IgG (end point titers of >500). gB-specific IgA in the P_4-treated group was comparable to that in the S group. However, five out of six mice in this group had IgG titers lower than 1:500. Mice in the unprotected group (the E_2 group and unprotected E+P) as well as the protected E+P group did not show any significant antibodies to gB in their vaginal secretions.

DISCUSSION

The results from the present study demonstrate that sex hormones influence the induction of and the outcome of immune responses following immunization in the genital tract. The ability of the attenuated virus to cause a productive infection was regulated by the hormonal environment, and this appeared to be critical in initiating immune responses. Estradiol treatment prior to immunization caused the vaginal epithelium to become resistant to TK^- HSV-2. Consequently, upon challenge, these mice were not protected. On the other hand, the attenuated virus caused a productive infection in progesterone-treated and saline control groups; protection from viral challenge was observed in these mice 3 weeks later.

This outcome correlates with previous results from our studies, where E_2-treated mice were resistant to primary genital infection with wild-type HSV-2, whereas non-hormone-treated and progesterone-treated groups were highly susceptible (9a).

The combined results from both of these studies indicate that hormonal conditions that provide protection against primary exposure to sexually transmitted viral pathogens may be different from those that are conducive to prophylactic mucosal vaccines and subsequent protection. This outcome poses an interesting conundrum regarding the hormonal milieu that could protect against sexually transmitted infections. While estradiol treatment was advantageous in providing protection against primary exposure, this treatment group was clearly at a disadvantage in studies relying on IVAG immunization. On the other hand, progesterone-treated mice had the worst outcome in the primary exposure yet had adequate protection following challenge. Evidently, the influences of sex hormones on susceptibility and immune responses are quite complex, and different strategies may need to be considered, depending on whether the objective is prophylactic vaccine design or formulations that prevent entry of virus.

This study and many others have clearly demonstrated that mucosal immunization with live attenuated virus or viral proteins via the vaginal surface is quite effective in inducing protective immune responses against mucosal pathogens, such as HSV-2 (10, 19, 24, 29). Moreover, factors that regulate the local environment in the mucosa may have significant influence on the success of the vaccine strategy. Certainly this is evident in the case of sex hormones and genital mucosa. While immunization via other mucosal routes, such as the nasal mucosa, has been shown to be quite efficient at generating immune responses in the genital tract, it is not clear whether hormones have any influence on the induction of immune responses following nasal immunization (6, 8). In addition, whether hormones influence protection at the time of challenge needs to be examined.

The most intriguing results in these studies were from the E+P group, where immunization occurred in the presence of estradiol and progesterone. Part of this group was completely protected, while the other part behaved similarly to the estradiol-treated group, showing no protection from challenge. The contrasting outcomes correlated with an attenuated vaccine virus-shedding pattern in the two subgroups and reiterated the critical importance of productive infection by the vaccine virus. Why the same hormone treatment led to dissimilar degrees of viral infection in different mice is not clear. One possibility is that the ratios of the two hormones in this group may be at a threshold level, allowing progesterone to antagonize the estradiol effect on susceptibility in some mice and not in others. This phenomenon of situation-dependent antagonism of estradiol effects by progesterone is well recognized (11). Other examples where progesterone by itself does not appear to regulate an immune function but does clearly interfere with estradiol effects have been documented (13, 33).

While progesterone administration on its own did not appear to directly regulate susceptibility to attenuated virus, it did correlate with increased inflammation and chronic pathology upon challenge. External pathology in the P_4-treated, immunized mice following challenge was the worst among all groups, and in repeat experiments, 10 to 20% of the mice appeared to develop chronic pathology after HSV-2 TK^- immunization and had to be sacrificed. A number of studies have shown that progesterone treatment modulates inflammation and immune responses (17, 21, 28). In our own studies, after primary exposure to genital HSV-2, progesterone treatment caused increased and persistent inflammatory response (9a). Other treatment groups in the present study, including control mice (no hormone treatment), as well as part of the combination hormone-treated group, had protection comparable to that of the P_4-treated group, but the accompanying inflammatory response was absent. The induction of such a response could be a double-edged sword. Inflammatory infiltration of immune cells could potentially enhance the initiation of immune responses by secretion of chemokines, cytokines, and subsequently antiviral Th1 responses. However, in the absence of regulatory processes, these responses could lead to chronic inflammation. Uncontrolled Th1 responses have been linked to immunopathology in other sexually transmitted infections (38). Considering that 10 to 20% of mice in the P_4-treated group consistently exhibited signs of chronic pathology, this possibility needs to be examined. Results from ongoing experiments indicate that the above hypothesis is likely, since T-cell cytokine profiles from LN of P-treated mice show very high levels of the Th1 cytokine gamma interferon and an absence of interleukin-10, a key immunoregulatory cytokine, compared to other hormone groups (V. A. Tang and C. Kaushic, unpublished data).

The presence of lymphoid aggregates in protected mice in this study is a novel finding. Even though immune responses in genital herpes have been well studied, to the best of our knowledge this is the first report of genital infection triggering in-

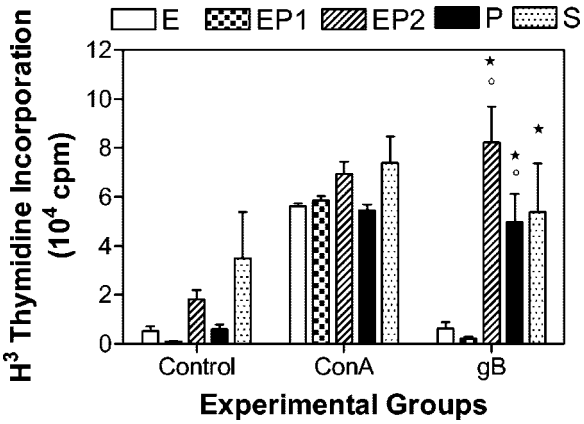

FIG. 6. Local draining lymph node proliferation in hormone-treated, immunized mice following challenge with wild-type HSV-2. Iliac lymph nodes draining the genital tract were removed 3 days after wild-type HSV-2 challenge (10^5 PFU), and cells were isolated and cultured for 48 h, as described in Materials and Methods. T-cell proliferation was measured in response to mitogen (ConA) and HSV-2 antigen (glycoprotein gB). The E+P group was divided into protected and nonprotected groups. Control cultures did not receive any stimulation. Results are shown as means ± standard errors of the mean (n = six to eight animals in the S, E_2, and P_4 groups). Data shown are representative of two separate experiments. EP1 ($n = 3$), unprotected E+P-treated animals; EP2 ($n = 4$), protected E+P-treated animals; ★, $P < 0.05$ compared to nonprotected groups (E_2 and not protected E+P); °, $P < 0.05$ compared to respective control unstimulated cultures.

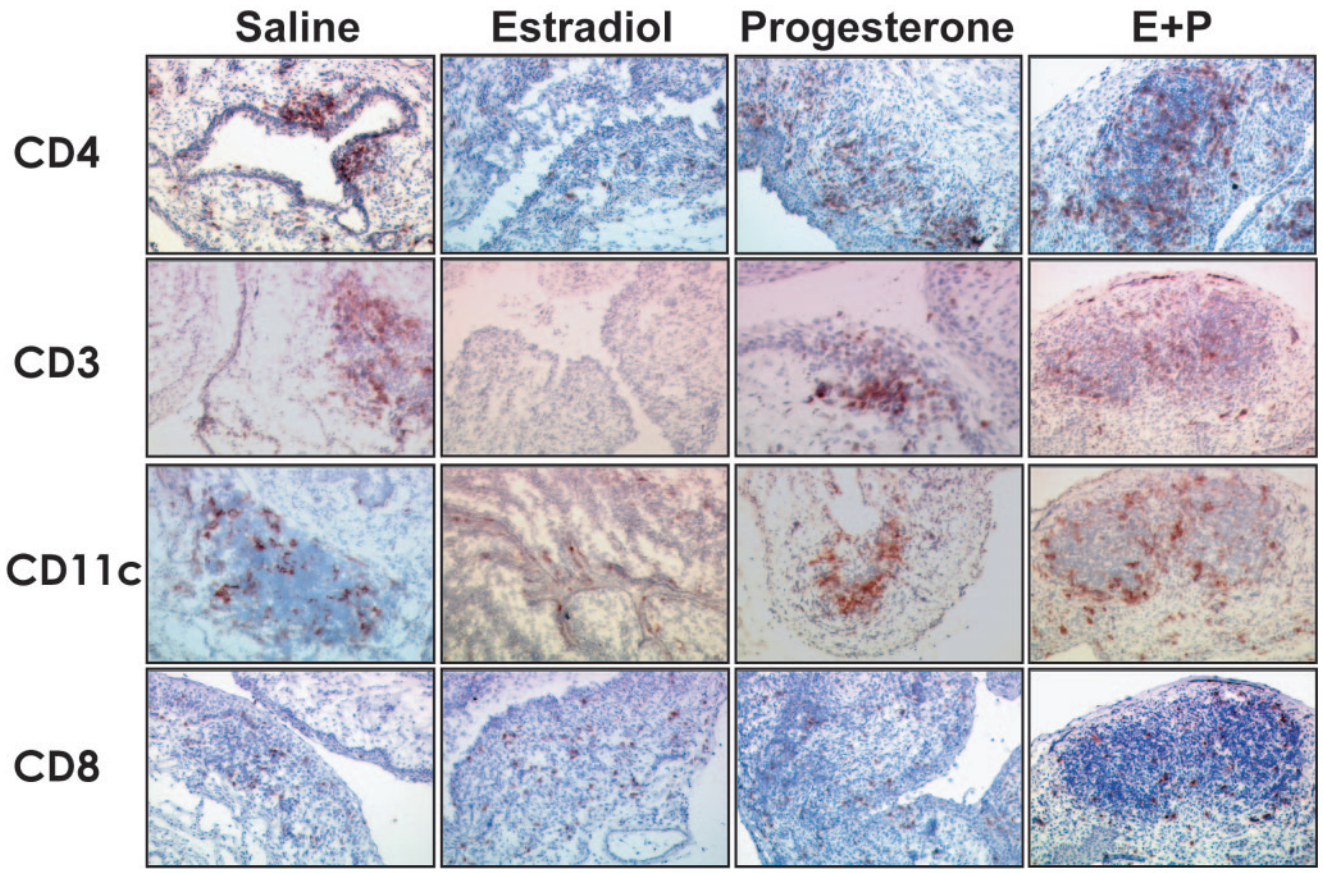

FIG. 7. iVALT in vaginal mucosae of hormone-treated, immunized mice challenged with wild-type HSV-2. Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Three weeks after IVAG immunization with attenuated HSV-2, mice were challenged IVAG with wild-type HSV-2 (10^5 PFU). Mice were sacrificed 2 to 5 days postchallenge, and immunohistochemical staining was done to localize immune cells. Representative tissue sections are shown from day 2 postchallenge. Note the large size of the iVALT in the E+P vagina compared to the size in saline- and progesterone-treated mice. The CD11c staining was seen on the periphery of the iVALTs, while $CD4^+$ and $CD3^+$ cells were localized inside as well as in close association with $CD11c^+$ cells. $CD8^+$ cells were distributed throughout the laminae propriae. Original magnification, ×100.

duction of organized lymphoid structures in the vaginal mucosa. Similar induced lymphoid structures in mucosae have recently been reported in lungs of mice lacking spleens, lymph nodes, and Peyer's patches following respiratory infection with influenza virus (27). The induced bronchus-associated lymphoid tissue in these studies had distinct B-cell follicles and T-cell areas and supported T- and B-cell proliferation. Remarkably, mice that exhibited formation of induced bronchus-associated lymphoid tissue but lacked peripheral lymphoid organs were able to clear influenza infection with better efficiency and less pathogenesis than control mice. In the present study, the lymphoid aggregates had large numbers of $CD3^+$ and $CD4^+$ T cells surrounded by $CD11c^+$ cells (a common marker for dendritic cells). They were present in immunized mice only 2 to 5 days after challenge. The transient appearance of these iVALTs coincided completely with the clearance of virus, suggesting that these structures play a critical role in protection. Notably, the iVALTs were largest and most numerous in the protected E_2+P-treated, immunized group and completely absent from the E_2-treated group and the unprotected E+P group (data not shown). Similar but smaller structures were noted in the P_4- and S-treated groups. Although the exact mechanism remains to be elucidated, the challenge with wild-type virus could attract HSV-2-specific effector and memory T cells carrying mucosal homing markers that were primed by the vaccine into the vaginal mucosa. The iVALTs could then serve as local sites for rapid expansion of these antigen-specific lymphocytes. Since the vaginal mucosae of the E+P group showed the least pathological damage among all of the hormone groups following challenge, it is interesting to speculate whether the larger size and increased number of iVALTs in the E+P group correlates with immunoregulatory $CD4^+$ T cells. We are currently investigating these possibilities.

Despite the similar degrees of protection in the S-treated and P_4-treated groups and part of the E+P-treated group, the immune responses that correlated with protection were not the same in the three conditions. Interestingly, the presence of CD8 cells did not correlate with protection in any of the groups, even though CD8 T cells were present, scattered in the vaginal mucosae of all mice. The protected mice from the three hormone groups (P_4, S, and part of E+P) did demonstrate formation of various sizes of iVALT, consisting predominantly of $CD3^+$ and $CD4^+$ T cells and $CD11c^+$ cells. The formation and timing of these iVALTs coincided with viral clearance.

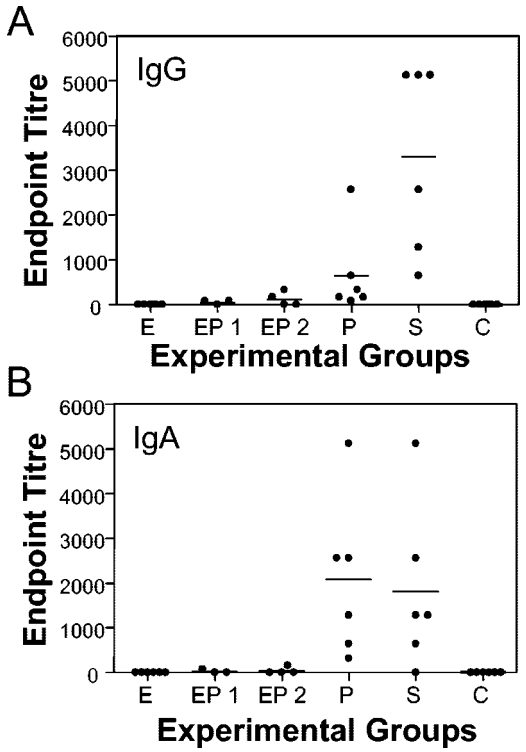

FIG. 8. HSV-2-specific antibody titers in vaginal washes of hormone-treated, immunized mice following challenge with wild-type HSV-2. Hormone-treated, immunized mice were challenged with wild-type HSV-2 (10^5 PFU). Vaginal washes were collected daily for 5 days postchallenge and pooled for each animal. HSV-2 gB-specific IgA (B) and IgG (A) antibodies were measured by ELISA, and end point titers were determined as described in Materials and Methods. E, estradiol treated; EP1, E+P treated and unprotected; EP2, E+P treated and protected; P, progesterone treated; S, saline controls; C, normal nonovariectomized mice. Each dot indicates an individual mouse. Bars show mean values for the group.

Thus, CD4 T cells appear to modulate protection in all three groups. In addition to $CD4^+$ T cells, most mice in the P_4- and S-treated groups showed high titers of gB-specific local IgG and IgA. The P_4-treated group showed a bias toward IgA secretion, since five out of six mice had gB-specific IgA end point titers of >500, while five out of six mice had IgG end point titers of <500. The protected E+P group did not show any significant HSV-2-specific antibody involvement in the local protective immune responses. More significantly, the protected E+P group had the least damage to the vaginal epithelium. Thus, hormones do appear to influence the type of protective immune responses.

Previous studies have extensively examined the role of different components of immune responses in genital HSV-2 infection. In the original model developed by McDermott et al., IVAG inoculation with wild-type HSV-2 of progesterone-treated mice caused both genital and lethal neurological disease (24). When mice were immunized with attenuated HSV-2 TK^- in this model, protective immunity was induced. In these and other studies that explored this model in more detail, local immunoglobulins as well as T-cell-mediated immune responses were shown to confer protection against challenge (7, 8, 22, 23, 26, 30). More recently, by using knockout mice and depletion studies, CD4 T cells and gamma interferon have been shown to be critical in protection against genital HSV-2 infection (10, 25, 30). However, in the majority of these studies, local and systemic immune responses were examined in intact mice exposed to attenuated or wild-type HSV-2 under the influence of exogenous progesterone. The combination of progesterone with endogenous estradiol in the intact mice likely affected immune responses. This makes it difficult to predict with accuracy the right conditions for inducing protective immune responses in mice that are under the influence of other hormonal environments.

These results emphasize the importance of taking into consideration the influence of hormones in designing therapeutic and prophylactic strategies for sexually transmitted infections. A recent study showed that following treatment with medroxyprogesterone acetate, a progesterone formulation, protection from simian immunodeficiency virus was abolished in immunized monkeys (1). The authors concluded that treatment with progesterone decreased the efficacy of a model vaccine. In other studies, a significant increase in human immunodeficiency virus type 1 DNA was detected in cervical secretions of seropositive women after starting hormonal contraception (34). Differences in human immunodeficiency virus type 1 RNA in cervical secretions have also been observed at different phases of the menstrual cycle in seropositive women (4). The present study adds to growing evidence that sexually transmitted infections in women are profoundly affected by female sex hormones and that in the future, gender-related factors will need to be considered in treatments of diseases.

ACKNOWLEDGMENTS

This work was supported by research grants to C.K. from the Institute of Gender and Health, Canadian Institutes of Health Research; the Ontario HIV Treatment Network (OHTN); and the Bickell Foundation. C.K. is a recipient of the OHTN Scholarship Award. K.L.R. is a recipient of a Career Scientist Award from the OHTN.

We thank Denis Snider for critical reading of the manuscript.

REFERENCES

1. **Abel, K., T. Rourke, D. Lu, K. Bost, M. B. McChesney, and C. J. Miller.** 2004. Abrogation of attenuated lentivirus-induced protection in rhesus macaques by administration of Depo-Provera before intravaginal challenge with simian immunodeficiency virus mac239. J. Infect. Dis. **190:**1697–1705.
2. **Aral, S. O., and K. K. Holmes.** 1999. Social and behavioral determinants of the epidemiology of STDs: industrialized and developing countries, p. 95–106. *In* K. K. Holmes, P. F. Sparling, P. A. Mardh, S. M. Lemon, et al. (ed.), Sexually transmitted diseases, 3rd ed. McGraw-Hill, New York, N.Y.
3. **Beagley, K. W., and C. M. Gockel.** 2003. Regulation of innate and adaptive immunity by female sex hormones oestradiol and progesterone. FEMS Immunol. Med. Microbiol. **38:**13–22.
4. **Benki, S., S. B. Mostad, B. A. Richardson, K. Mandaliya, J. K. Kreiss, and J. Overbaugh.** 2004. Cyclic shedding of HIV-1 RNA in cervical secretions during the menstrual cycle. J. Infect. Dis. **189:**2192–2201.
5. **Crowley, T., P. Horner, A. Hughes, J. Berry, I. Paul, and O. Caul.** 1997. Hormonal factors and laboratory detection of Chlamydia trachomatis in women: implications for screening? Int. J. STD AIDS **8:**25–31.
6. **Gallichan, W. S., T. Gurasachi, and K. L. Rosenthal.** 2001. Intranasal immunization with CpG oligonucleotides as an adjuvant dramatically increases IgA and protection against HSV-2 in the genital tract. J. Immunol. **166:**3451–3457.
7. **Gallichan, W. S., and K. L. Rosenthal.** 1996. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. J. Exp. Med. **184:**1879–1890.
8. **Gallichan, W. S., and K. L. Rosenthal.** 1998. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. J. Infect. Dis. **177:**1155–1161.
9. **Gillgrass, A. E., A. A. Ashkar, K. L. Rosenthal, and C. Kaushic.** 2003. Prolonged exposure to progesterone prevents induction of protective muco-

sal responses following intravaginal immunization with attenuated herpes simplex virus type 2. J. Virol. **77:**9845–9851.

9a. **Gillgrass, A. E., S. A. Fernandez, K. L. Rosenthal, and C. Kaushic.** 2005. Estradiol regulates susceptibility following primary exposure to genital herpes simplex virus type 2, while progesterone induces inflammation. J. Virol. **79:**3107–3116.

10. **Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson.** 2001. Protective vaccination against genital herpes simplex virus, type 2 infection in mice is associated with a rapid induction of local IFN-gamma-dependent-RANTES production following a vaginal viral challenge. Am. J. Reprod. Immunol. **46:**420–424.

11. **Katzenellenbogen, B. S.** 2000. Mechanisms of action and cross-talk between estrogen receptor and progesterone receptor pathways. J. Soc. Gynecol. Investig. **7:**S33–S37.

12. **Kaushic, C., A. A. Ashkar, L. A. Reid, and K. L. Rosenthal.** 2003. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. J. Virol. **77:**4558–4565.

13. **Kaushic, C., E. Frauendorf, and C. R. Wira.** 1997. Polymeric immunoglobulin A receptor in the rodent female reproductive tract: influence of estradiol in the vagina and differential expression of messenger ribonucleic acid during estrous cycle. Biol. Reprod. **57:**958–966.

14. **Kaushic, C., A. D. Murdin, B. J. Underdown, and C. R. Wira.** 1998. *Chlamydia trachomatis* infection in the female reproductive tract of the rat: influence of progesterone on infectivity and immune response. Infect. Immun. **66:**893–898.

15. **Kaushic, C., F. Zhou, A. D. Murdin, and C. R. Wira.** 2000. Effect of estradiol and progesterone on susceptibility and early immune responses to *Chlamydia trachomatis* infection in the female reproductive tract. Infect. Immun. **68:** 4207–4216.

16. **Koelle, D. M., and L. Corey.** 2003. Recent progress in herpes simplex virus immunobiology and vaccine research. Clin. Microbiol. Rev. **16:**96–113.

17. **Kuhn, L., L. Denny, A. E. Pollack, and T. C. Wright.** 1999. Prevalence of visible disruption of cervical epithelium and cervical ectopy in African women using Depo-Provera. Contraception **59:**363–367.

18. **Kuklin, N. A., M. Daheshia, S. Chun, and B. T. Rouse.** 1998. Role of mucosal immunity in herpes simplex virus infection. J. Immunol. **160:**5998–6003.

19. **Kwant, A., and K. L. Rosenthal.** 2004. Intravaginal immunization with a viral subunit protein plus CpG oligonucleotides induces protective immunity against HSV-2. Vaccine **22:**23–24.

20. **Martin, H. L., Jr., P. M. Nyange, B. A. Richardson, L. Lavreys, K. Mandaliya, D. J. Jackson, J. O. Ndinya-Achola, and J. Kreiss.** 1998. Hormonal contraception, sexually transmitted diseases, and risk of heterosexual transmission of human immunodeficiency virus type 1. J. Infect. Dis. **178:**1053–1059.

21. **Marx, P. A., A. I. Spira, A. Gettie, et al.** 1996. Progesterone implants enhance SIV vaginal transmission and early virus load. Nat. Med. **2:**1084–1089.

22. **McDermott, M. R., L. J. Brais, and M. J. Evelegh.** 1990. Mucosal and systemic antiviral antibodies in mice inoculated intravaginally with herpes simplex virus type 2. J. Gen. Virol. **71:**1497–1504.

23. **McDermott, M. R., C. H. Goldsmith, K. L. Rosenthal, and L. J. Brais.** 1989. T lymphocytes in genital lymph nodes protect mice from intravaginal infection with herpes simplex virus type 2. J. Infect. Dis. **159:**460–466.

24. **McDermott, M. R., B. J. Smiley, L. J. Brais, H. E. Rudzroga, and J. Bienenstock.** 1984. Immunity in the female genital tract after intravaginal vaccination of mice with an attenuated strain of herpes simplex virus type 2. J. Virol. **51:**747–753.

25. **Milligan, G. N., and D. I. Bernstein.** 1997. Interferon-gamma enhances resolution of herpes simplex virus type 2 infection of the murine genital tract. Virology **229:**259–268.

26. **Milligan, G. N., D. I. Bernstein, and N. Bourne.** 1998. T lymphocytes are required for protection of the vaginal mucosa and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. J. Immunol. **160:** 6093–6100.

27. **Moyron-Quiroz, J. E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund, and T. D. Randall.** 2004. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nat. Med. **10:**927–934.

28. **Nohmi, T., S. Abe, K. Dobashi, S. Tansho, and H. Yamaguchi.** 1995. Suppression of anti-Candida activity of murine neutrophils by progesterone in vitro: a possible mechanism in pregnant women's vulnerability to vaginal candidiasis. Microbiol. Immunol. **39:**405–409.

29. **Parr, E. L., and M. B. Parr.** 1999. Immune responses and protection against vaginal infection after nasal or vaginal immunization with attenuated herpes simplex virus type-2. Immunology **98:**639–645.

30. **Parr, M. B., and E. L. Parr.** 2003. Vaginal immunity in the HSV-2 mouse model. Int. Rev. Immunol. **22:**43–63.

31. **Sonnex, G.** 1998. Influence of ovarian hormones on urogenital infection. Sex. Transm. Infect. **74:**11–19.

32. **Stanberry, L. R., S. L. Spruance, A. L. Cunningham, D. L. Bernstein, A. Mindel, S. Sacks, S. Tyring, F. Y. Aoki, M. Sloui, M. Denis, P. Vandepapeliere, G. Dubin, and the GlaxoSmithKline Herpes Vaccine Efficacy Study Group.** 2002. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. N. Engl. J. Med. **347:**1652–1661.

33. **Tibbetts, T. A., O. M. Conneely, and B. W. O'Malley.** 1999. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol. Reprod. **60:**1158–1165.

34. **Wang, C. C., R. S. McClelland, J. Overbaugh, M. Reilly, D. D. Panteleeff, K. Mandaliya, B. Chohan, L. Lavreys, J. Ndinya-Achola, and J. K. Kreiss.** 2004. The effect of hormonal contraception on genital tract shedding of HIV-1. AIDS **18:**205–209.

35. **Wira, C. R., and C. Kaushic.** 1996. Mucosal immunity in the female reproductive tract: effect of sex hormones on immune recognition and responses, p. 375–386. *In* H. Kiyono, P. L. Ogra, and J. R. McGhee (ed.), Mucosal vaccines: new trends in immunization. Academic Press, New York, N.Y.

36. **Wira, C. R., C. Kaushic, and J. Richardson.** 1999. Role of sex hormones and cytokines in regulating mucosal immune system in the female reproductive tract, p. 1449–1461. *In* P. L. Ogra, J. Mestecky, et al. (ed.), Mucosal immunology. Academic Press, New York, N.Y.

37. **Wira, C. R., and R. M. Rossoll.** 1995. Antigen presenting cells in the female reproductive tract: influence of estrous cycle on antigen presentation by uterine epithelial and stromal cells. Endocrinology **136:**4526–4534.

38. **Yang, X.** 2003. Role of cytokines in Chlamydia trachomatis protective immunity and immunopathology. Curr. Pharm. Des. **9:**67–73.