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Estradiol Regulates Susceptibility following Primary Exposure to Genital Herpes Simplex Virus Type 2, while Progesterone Induces Inflammation

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We report here that sex hormones modulate susceptibility to a sexually transmitted viral agent, herpes simplex virus type 2 (HSV-2), in a mouse model. Ovariectomized mice were administered either saline (control), estradiol (E_2), progesterone (P_4), or a combination of both estradiol and progesterone (E+P) and infected intravaginally with HSV-2. With an inoculation dose of 10^5 PFU, the saline- and P_4-treated mice were found to be highly susceptible to genital HSV-2 infection. Both groups had extensive pathology and high viral titers in vaginal secretions, and 100% of mice succumbed by day 4 postinfection. E_2-treated mice were protected from HSV-2 infection at the same dose and did not display any vaginal pathology or viral shedding. There was a slow progression of genital pathology in the combination hormone-treated group, along with prolonged viral shedding; 80% of animals succumbed by day 13. With lower inoculation doses of 10^3 and 10^2 PFU, 50 and 100%, respectively, of the combination hormone-treated mice survived. Localization of HSV-2 infection showed extensive infection in the vaginal epithelium of P_4- and saline-treated animals within 24 h of inoculation. E_2-treated animals were clear of infection, while the E+P-treated group had focal infection at 24 h that had progressed extensively by day 3. Infection was accompanied by persistent inflammation and infiltration of neutrophils in the P_4-treated group. An analysis of the genes in the vaginal tissue showed that inflammation in the P_4-treated group correlated with local induction of chemokines and chemokine receptors that were absent in the E_2-treated mice and in uninfected P_4-treated mice. The results show that sex hormones regulate initiation of infection and immune responses to genital HSV-2 infection.

Herpes simplex virus type 2 (HSV-2) is the major causative agent of genital herpes infections. Present statistics show that approximately one in four sexually active adults is seropositive for HSV-2, making this one of the most common viral sexually transmitted diseases (11). Following infection, HSV-2 establishes persistent infection that can reactivate and produce symptomatic or asymptomatic recurrences. Presently, there is no known cure for HSV-2 infection. The only therapy available to temporarily reduce HSV recurrences requires daily administration of antiviral drugs. An effective vaccine against HSV-2 would be an ideal choice for preventing transmission as well as recurrences (1). However, attempts to develop herpes vaccines have met with failure since the 1930s (3, 10). A recent report of another candidate HSV-2 vaccine, based on gD glycoprotein, showed a lack of efficacy, with the exception of partial protection in women who were seronegative before vaccination (22). Studies such as this reiterate the importance of examining other influences, such as gender-specific factors that may affect susceptibility and immune responses to HSV-2 infection.

The female reproductive tract is a specialized mucosal surface that has the dual tasks of facilitating the growth of an allogeneic fetus while still providing protection against potential pathogens. These diverse needs are met, at least partially, by precise regulation of immune responses in the genital tract by ovarian sex hormones, estradiol and progesterone (25). A number of clinical and epidemiological studies illustrate that sex hormones influence genital tract infections in women (21). The stage of the menstrual cycle and/or oral contraceptives are known to affect infection with candidiasis, gonorrhea, HSV-2, human immunodeficiency virus type 1 (HIV-1), and *Chlamydia* in women (2, 14, 21). In rhesus macaque models, subcutaneous implants of progesterone made the monkeys more susceptible to simian immunodeficiency virus (SIV) vaginal transmission, while estrogen was able to protect against SIV infection (15, 20). Studies in mouse models also show similar effects of hormones on sexually transmitted infections.

The effect of sex hormones in the reproductive tract appears to be tissue and pathogen specific. Mouse models of *Candida* show that mice are more susceptible to infection under the influence of estradiol (4). In our studies of a rat model of *Chlamydia*, we found that, similar to the results reported for mice, progesterone treatment led to increased susceptibility and inflammation, while estradiol appeared to protect from this sexually transmitted bacterial infection (9). Other studies of genital infection with HSV-2 have found that the susceptibility of the mice varies with the stage of the estrous cycle (5). More recently, working on a mouse model of HSV-2, we found that medroxyprogesterone acetate (Depo-Provera), a long-lasting formulation of progesterone, increased susceptibility in mice by 100-fold compared with mice that were susceptible in a normal cycle (8). Prolonged exposure to medroxyprogester-

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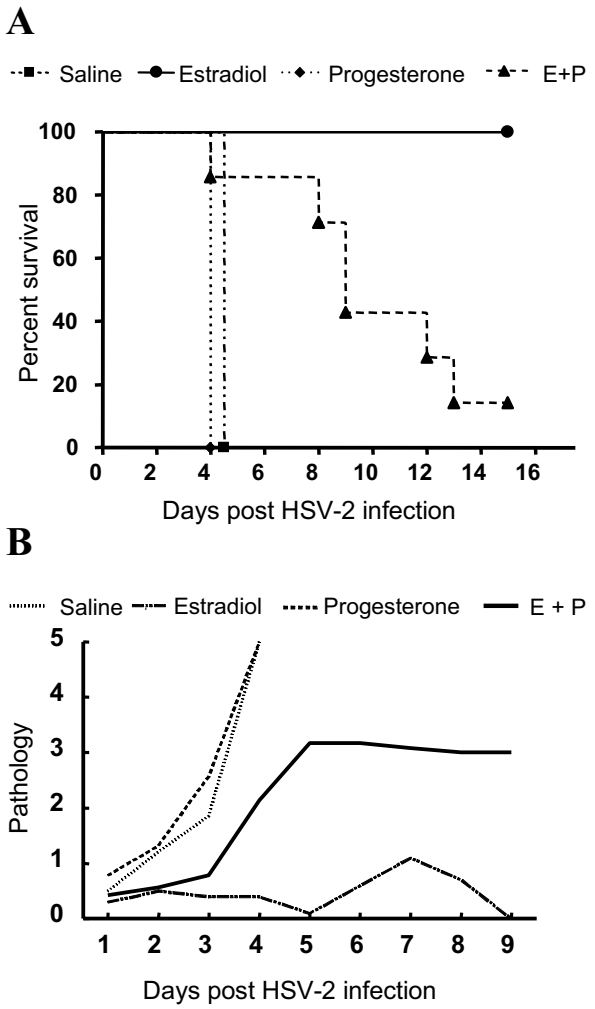

FIG. 1. Survival (A) and pathology (B) of OVX, hormone-treated mice inoculated with a high challenge dose (10^5 PFU) of HSV-2 strain 333. Mice were ovariectomized and given different combinations of hormones, as described in Materials and Methods. Following IVAG inoculation with HSV-2, vaginal pathology and survival were scored daily. Pathology scores of all of the mice in each group are shown as mean values. Each hormone group had six to eight mice per group. The experiment was repeated two times, with comparable results.

one acetate also appeared to compromise immune responses to HSV-2 in mice immunized with attenuated virus (6).

The present study was initiated to examine the role of the hormonal environment in regulating susceptibility to HSV-2 and its effect on the outcome of genital infection. Ovariectomized (OVX) mice were given either estradiol (E_2) or progesterone (P_4) or a combination of both (E+P) prior to intravaginal (IVAG) infection at different doses of HSV-2 strain 333. Pathology and survival were monitored and correlated with virus titers under different hormonal conditions. The histopathology of the genital tract was examined in these mice, and the extent of infection was localized. Finally, the distribution of neutrophils and chemokine patterns were examined to characterize the type and extent of inflammation following infection under the influence of different hormones.

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. Estradiol and progesterone were purchased from Calbiochem (La Jolla, Calif.). 17β-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 1 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 of ketamine/kg–10 mg of xylazine/kg) given intraperitoneally, placed on their backs, and inoculated intravaginally with 10 μl of wild-type HSV-2 strain 333 at a high inoculation dose of 10^5 PFU/ml or low inoculation doses of 10^3 and 10^2 PFU/ml. 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. For 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 (26). Reproductive cycle staging was used to confirm the depletion of endogenous hormones after ovariectomy. The following classification was used for identifying the stage of the cycle; estrus, >90% cornified epithelial cells; diestrus, >75% polymorphonuclear cells; and 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 and 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 absorption. 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, embedded in Tissue-Tek OCT compound, and frozen 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 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 neutrophils, sections were incubated with a rat anti-mouse neutrophil antibody (Serotec, Oxford, United Kingdom). Incubations were carried out for 1 h at room temperature. Antiserum from normal rabbits was substituted for primary antibody at an equivalent concentration for control staining. The secondary antibody for HSV-2 primary was a biotinylated goat anti-rabbit antibody, while a rabbit anti-rat biotin was used with the neutrophil antibody (both from BD-Pharmingen, San Diego, Calif.). 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 antigen localization. Slides were counterstained with methyl green and mounted in Permount medium prior to microscopic examination.

Chemokine and chemokine receptors gene array. The relative expression of 67 chemokine and receptor genes were analyzed with GE Array Q series mouse chemokine and receptor array (SuperArray Inc., Bethesda, Md.) according to the manufacturer's protocol. Total RNA was isolated from vaginal tissue of OVX and E_2- or P_4-treated mice on day 3 after inoculation with 10^5 PFU of HSV-2.

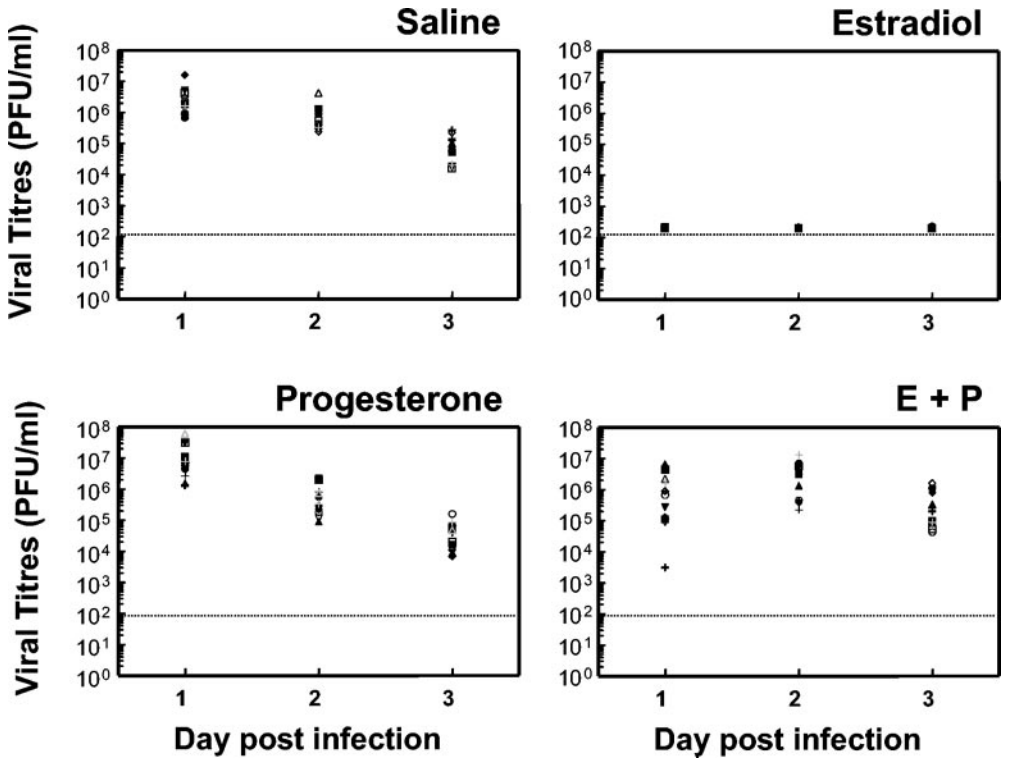

FIG. 2. Virus titers from OVX, hormone-treated mice inoculated with 10^5 PFU of HSV-2 type 333. Mice were ovariectomized and given different hormone combinations, as described in Materials and Methods. Following IVAG inoculation with HSV-2, vaginal washes were collected daily, and viral plaque assays 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 = 6 to 9 mice in each group). The dashed lines show the lower detection limit of the assay. Results are representative of two separate experiments.

Total RNA from five to six mice was pooled, and 5 μg of total RNA was used as a template to reverse transcribe into ^{32}P-labeled cDNA probes. The cDNA probes were hybridized to chemokine- and receptor gene-specific cDNA fragments that were spotted on the GE Array membranes. The unhybridized probe was washed off, and the amount of radioactive signal from the hybridized probe was analyzed with a STORM phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). The signal from a negative control gene (pUC18 DNA) was subtracted from the signal for all other genes. The expression of each chemokine and/or receptor was normalized to the average expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene on the same membrane and expressed as the percent GAPDH according to the following equation: chemokine and/or receptor expression = [(chemokine signal − background signal)/(GAPDH signal − background signal)] × 100.

RESULTS

Survival, pathology, and viral shedding in OVX mice inoculated with a high infectious dose of HSV-2. Four groups of mice were ovariectomized, and 2 weeks later, two of the groups were treated with E_2 or P_4 for three consecutive days. A third group was treated with a combination of both hormones (E+P) by the same regimen. The control group received sham injections of saline instead of hormones. Twenty-four hours after the last hormone injection, animals were inoculated IVAG with a high dose (10^5 PFU) of HSV-2 strain 333 wild-type virus. Vaginal pathology was monitored, and vaginal washes were collected daily to monitor viral shedding. The pathology scores and survival curves are shown in Fig. 1. Progesterone-treated mice began exhibiting vaginal pathology within 48 h. By day 4, all mice had pathology scores of 4, with extensive genital and extragenital ulceration and hair loss, and were euthanized. In contrast, six out of six animals in the estradiol group had no obvious signs of pathology over the 10 days that they were monitored, and 100% of mice survived the high-dose challenge with HSV-2. The OVX, saline-treated control mice were as susceptible as progesterone-treated mice, with a similar rapid progression of pathology. One hundred percent of the mice in the control group succumbed to infection by day 4 postinfection. Interestingly, the combination hormone-treated group exhibited slow progress in external pathology, but eventually (by day 13), six out of seven mice had to be euthanized.

Virus titers were assessed for 3 days postinfection and found to correlate well with pathology (Fig. 2). No viral shedding could be detected on any day in the vaginal washes of estradiol-treated mice inoculated IVAG with HSV-2. In contrast, all progesterone-treated mice and those in the control group had high virus titers in their vaginal washes 24 h after infection and continued to show shedding on all 3 days. Mice in the combination hormone group had variable shedding on day 1 postinfection, but by day 3 all of them had high virus titers in the vaginal washes.

Survival, pathology, and viral shedding in OVX mice inoculated with low infectious doses of HSV-2. The results of the previous experiment showed that, with the exception of mice treated with estradiol, mice in all hormone treatment groups were highly susceptible to genital HSV-2 infection. Since the

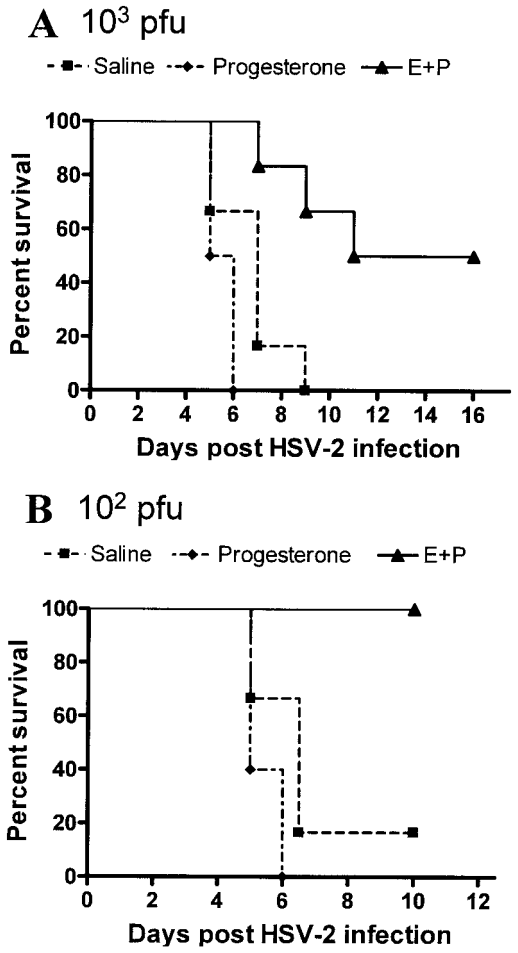

FIG. 3. Survival of OVX, hormone-treated mice inoculated with low challenge doses of 10^3 (A) and 10^2 (B) PFU of HSV-2 strain 333. Mice were ovariectomized and given different combination of hormones, as described in Materials and Methods. Following IVAG inoculation with HSV-2, vaginal pathology and survival were scored daily. Each hormone group had six to eight mice per group. The experiment was repeated two times, with comparable results.

kinetics of infection and outcome of genital HSV-2 infection are dependent on the inoculation dose of virus, mice in the three susceptible experimental groups were inoculated with lower infectious doses. The results are shown in Fig. 3. At the lower inoculation doses (10^3 and 10^2 PFU), the kinetics of infection was slower than that of the high-dose challenge (Fig. 1) in all three groups. As with the high inoculation dose, progesterone-treated mice were 100% susceptible at low doses (Fig. 3A and B). These mice reached maximum pathology scores between days 4 and 6 postinoculation, at which point they were euthanized. The control group that received saline instead of hormones exhibited a slower progression of pathology than the progesterone-treated mice. However, by day 9 after inoculation with 10^3 PFU, 100% of mice (six out of six) had to be sacrificed. At the lowest inoculation dose of 10^2 PFU, one out of six mice in the control group survived. The E+P group showed the slowest progression of pathology (data not shown). Three out of six animals (50%) in the E+P group did not progress beyond pathology scores of 2 at an inoculation dose of 10^3 PFU, and 100% of mice survived at the lowest inoculation dose.

The viral shedding pattern correlated closely with the pathology scores (Fig. 4). After inoculation with 10^3 PFU, mice in the P_4- and control saline-treated (non-hormone-treated) groups shed high levels of HSV-2 in their vaginal secretions within 24 h and maintained the shedding over the 3 days it was measured. Low virus titers were observed in vaginal washes of the E+P group 24 h postinfection, correlating with slow progress in pathology. However, by day 3 postinfection, three out of six mice had high virus titers in vaginal secretions. These were the same mice that eventually succumbed to the infection (Fig. 3). At the lowest inoculation dose (10^2 PFU), a high level of viral shedding was measured in the vaginal secretions of the saline-treated group and the progesterone-treated group. At this dose of inoculation, no detectable viral shedding was observed in the E+P group.

Histopathology in OVX, hormone-treated mice prior to and following infection. To examine the effect of hormone treatment and infection in the genital tract, the histopathology of vaginal tissue in hormone-treated mice was examined 24 h (Fig. 5A to D) and 3 days after infection with 10^5 PFU of HSV-2 (Fig. 5E to H). Control, noninfected mice were also examined 3 days after hormone treatment (Fig. 5I to L). The histology of vaginal tissue from hormone-treated uninfected mice shows that with this dose regimen, both estradiol and progesterone effects could be distinguished clearly in treated mice compared to OVX, saline-treated controls. Following infection, the non-hormone-treated control group (OVX, saline treated, infected) showed moderate inflammation and leukocytic infiltration in the tissue and lumen (Fig. 5A). By day 3, there was evidence of epithelial damage. In comparison, the vaginae of saline-treated, noninfected mice displayed normal characteristics of OVX genital tracts, with thin epithelial linings and no signs of inflammation or tissue damage (Fig. 5I). In the progesterone-treated, infected group, there was extensive infiltration and inflammation within 24 h (Fig. 5C), and by day 3 postinfection, the epithelial lining was extensively ulcerated and the inflammation was still visible (Fig. 5G). The noninfected, P_4-treated mice showed a thinning of the vaginal epithelium corresponding with progesterone treatment of OVX mice, without any inflammation (Fig. 5K). The E_2-treated, infected animals displayed no sign of any inflammation or pathology 24 h postinoculation (Fig. 5B). The keratinized epithelial lining was very prominent in these mice. By day 3 postinoculation, the epithelium had lost the keratin lining, and leukocytic infiltration was observed in the vaginae of both infected E_2-treated mice and noninfected E_2-treated controls (Fig. 5F and J). There were no other signs of inflammation or tissue damage. The E+P group did not show any significant infiltration or inflammation 24 h postinoculation (Fig. 5D). However, nominal intraluminal leukocytic infiltration was obvious on day 3 postinfection (Fig. 5H). Focal damage to the epithelium was also evident at this time point. The histology in the noninfected E+P group showed normal pseudostratified squamous epithelium without keratinization.

Localization of HSV-2 infection in OVX, hormone-treated mice. In order to understand the kinetics and extent of HSV-2 infection in the genital tracts of OVX hormone-treated mice, HSV-2 was localized in the vaginal tissue 24 h and 3 days after

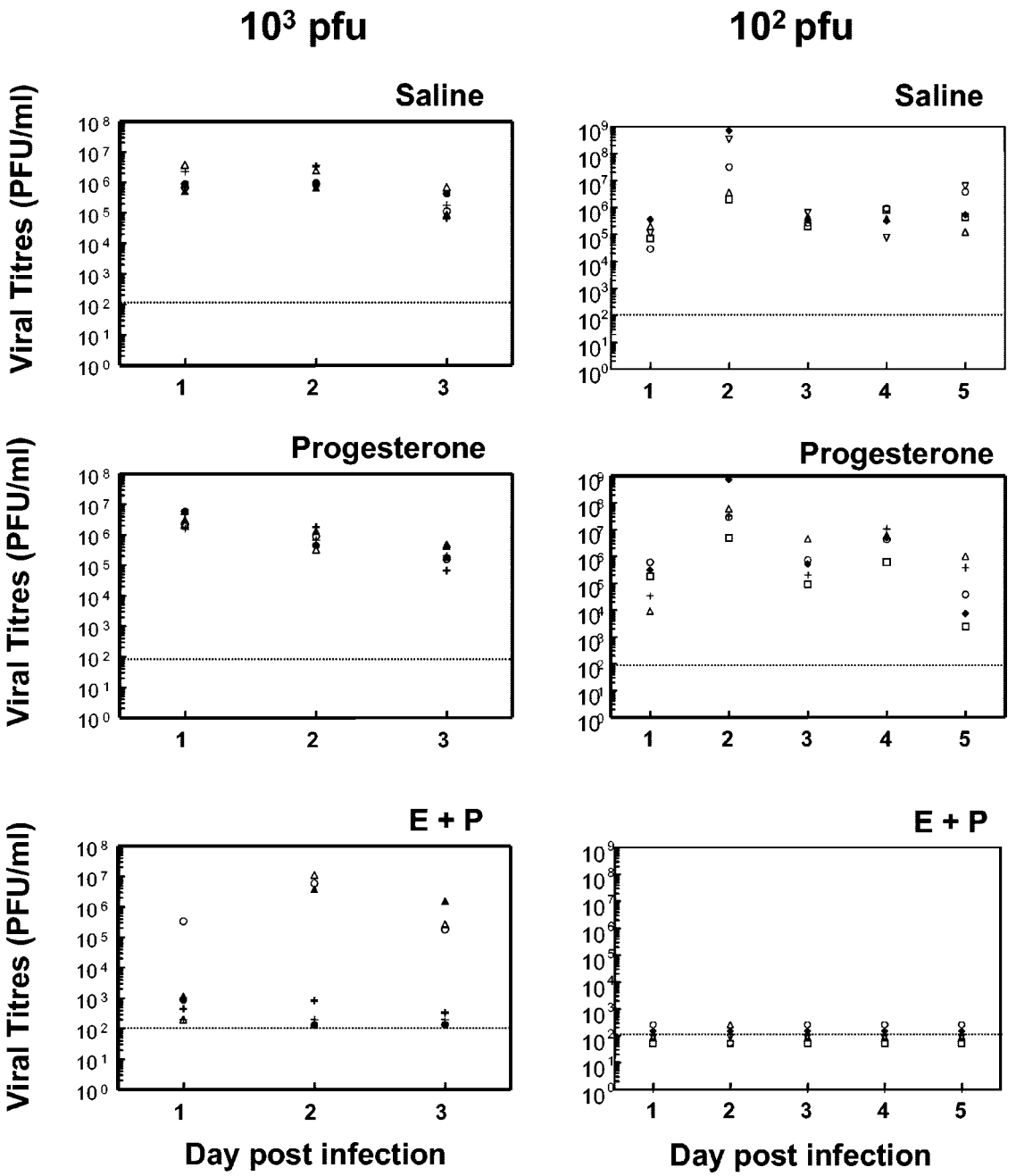

FIG. 4. Virus titers from OVX, hormone-treated mice inoculated with low challenge doses (10^3 and 10^2 PFU) of HSV-2 type 333. Mice were ovariectomized and given different hormone combinations, as described in Materials and Methods. Following IVAG inoculation with HSV-2, vaginal washes were collected 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 = 6 to 9 mice in each group). The dashed lines show the lower detection limit of the assay. Results are representative of two separate experiments.

inoculation (Fig. 6). Twenty-four hours after IVAG HSV-2 inoculation, extensive infection was observed in the vaginae of saline- and P_4-treated mice (Fig. 6A and C). Both of the groups showed localization all along the epithelium, and in the P_4-treated mice, there was positive staining within the extensive leukocytic infiltration observed in the lumen. HSV-2-specific staining was completely absent from all estradiol-treated mice at both time points (Fig. 6B and F). At 24 h postinoculation, the E+P group had bright staining corresponding with focal infection in the vaginal epithelium (Fig. 6D). Three days after inoculation, there was much more extensive infection in the E+P group, as shown by bright staining all along the epithelium (Fig. 6H). Interestingly, less staining was observed in both saline- and P_4-treated mice at the 3-day time point than at the 24-h time point (Fig. 6E and G), possibly because of the extensive damage to the epithelial layer and migration of the

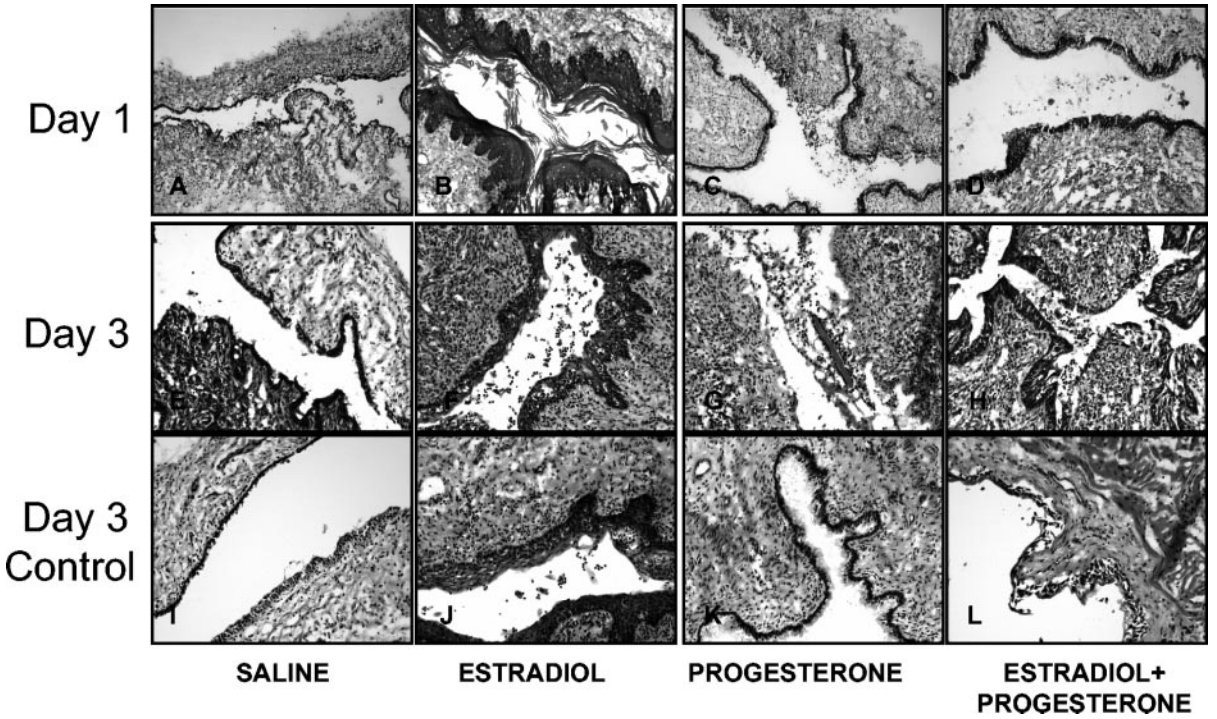

FIG. 5. Histopathology of vaginal tissue of OVX, hormone-treated mice inoculated IVAG with HSV-2. Mice were sacrificed either 24 h postinfection (A to D) or 3 days postinfection (E to H). Control noninfected mice that received hormones were also examined on the same day as the mice examined 3 days postinfection (I to L). Note the thin epithelium in saline-treated (A, E, and I) and progesterone-treated (C, G, and K) mice. Progesterone-treated mice also have acute inflammation and heavy leukocytic infiltration in the lumen. Also note the thickened epithelium in the estradiol-treated mice (B, F, and J), denoting the effect of the hormone, including keratinization of the superficial layer (B). Original magnification, ×100.

infection to extragenital areas. Again, estradiol-treated mice had no signs of infection 3 days postinoculation, as evidenced by the lack of any positive staining for HSV-2.

Characterization of inflammation following IVAG infection in OVX, hormone-treated mice. To characterize the inflammatory response seen in the hormone-treated mice infected with HSV-2, vaginal tissue sections were stained with a neutrophil antibody. In saline-treated infected mice, positive staining was observed predominantly in the blood vessel endothelial lining and subepithelial lamina propria 24 h postinfection (Fig. 7A). No staining for neutrophils was detected in estradiol-treated mice at this time point (Fig. 7B). A large number of neutrophils were seen in the vaginal lamina propria in the progesterone-treated mice 24 h after infection (Fig. 7C). There was also extensive staining in the lumen, indicating that neutrophils were the predominant cells in the leukocytic infiltrate seen in the lumen of these mice. Compared to progesterone-treated mice, the E+P group had a small number of positive cells in the vaginal tissue at 24 h postinfection (Fig. 7D). By day 3 postinfection (Fig. 7E to H), there were very few neutrophils in the vaginal tissue of saline-treated, infected mice, whereas large numbers of neutrophils were still present in the lumen of the infected vaginae of P_4-treated mice (Fig. 7E and G). A few positive cells were detected in the tissues from E+P-treated mice (Fig. 7H). Interestingly, the E_2-treated mice had a considerable number of neutrophils distributed throughout the vaginal epithelium on day 3 after hormone treatment, for both infected as well as noninfected mice (Fig. 7F and J). The other control groups with just hormone treatment in the absence of infection did not show any neutrophil staining.

Given the extent of inflammation and neutrophil infiltration following HSV-2 inoculation, we examined the chemokine and chemokine receptor profile in the genital tracts of E_2- and P_4-treated mice prior to and following infection. The chemokine and chemokine receptor patterns seen in vaginal mucosae of E_2-treated mice were similar in the presence and in the absence of infection (data not shown). Among the chemokines that showed significant levels of expression were CXCL5 and CXCL7, CCL 21a, and MIP-2 (data not shown). P_4 treatment by itself did not appear to induce chemokines and chemokine receptors. However, infection following P_4 treatment led to the induction of transcription of a number of chemokines and their receptors. Among the chemokines that exhibited significant increases were an array of C-C and C-X-C chemokines, including RANTES, MIP-2, MCP, LIX, and IP-10. A few chemokine receptors, including CCR-7, Ltb4r2, and TAP binding protein mRNA, were also upregulated postinfection. Table 1 summarizes the genes which were seen in vaginal tissue of mice infected with HSV-2 under the influence of progesterone.

DISCUSSION

In order to examine the effect of reproductive hormones on susceptibility and immune responses to genital HSV-2 infection, we used a mouse model where the endogenous source of hormones was removed. We then reconstituted the mice with

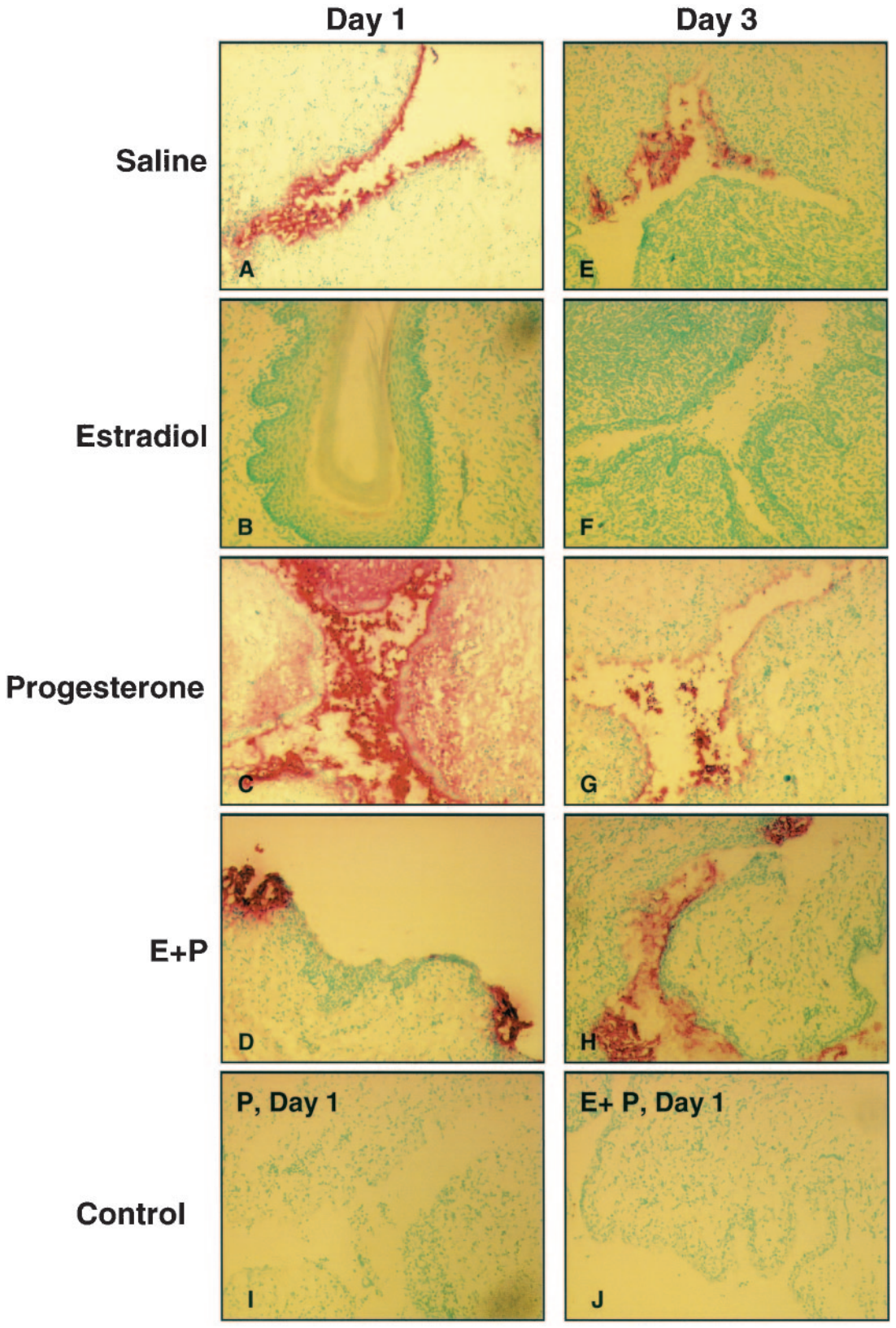

FIG. 6. Localization of infection in the vaginal tissue of OVX, hormone-treated mice infected with HSV-2. A polyclonal rabbit serum was used to detect HSV-2-specific staining, as described in Materials and Methods. Representative tissue sections from each hormone group are shown for day 1 postinfection (A to D) and day 3 postinfection (E to H). Positive staining (pink) in the vaginal epithelium was seen in saline-treated mice (A and E) and progesterone-treated mice (C and G). The E+P group had focal infection at 24 h postinfection (D) and more extensive infection at day 3 (H). No HSV-2 staining was observed in estradiol-treated mice. Isotype controls for day 1 progesterone (I) and E+P (J) are also shown. Original magnification, ×100.

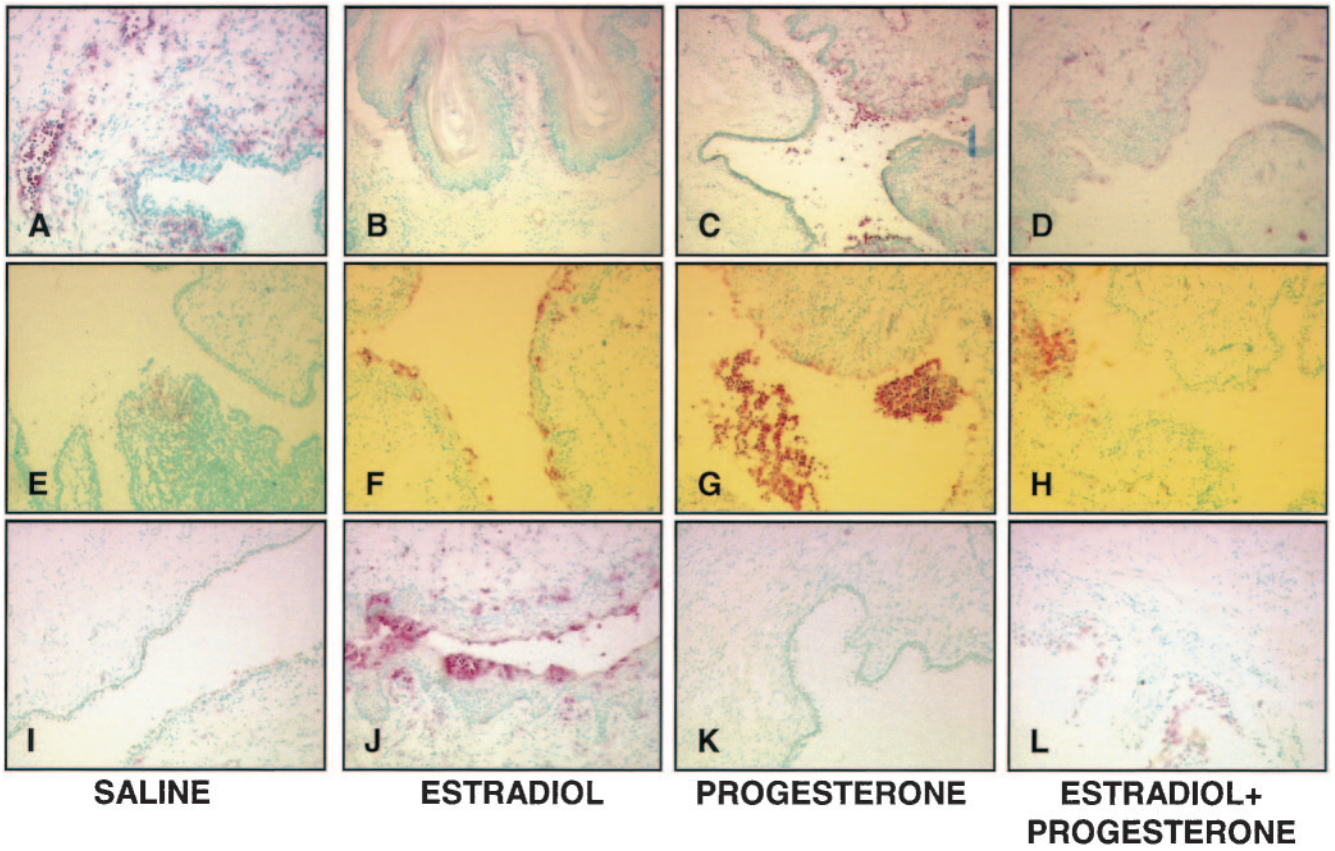

FIG. 7. Localization of neutrophils in vaginal tissue of OVX, hormone-treated mice infected with HSV-2. A rat anti-mouse neutrophil antibody was used to detect specific staining, as described in Materials and Methods. Mice were sacrificed either 24 h postinfection (A to D) or 3 days postinfection (E to H). Control noninfected mice that received hormones were also examined on the same day as the mice examined 3 days postinfection (I to L). Positive staining (pink) is seen in the endothelium of saline-treated mice on day 1 (A) and mostly following infection of progesterone-treated mice (C and G). Significant numbers of neutrophils are also seen in the superficial layers of vaginal epithelium 3 days after E_2 treatment was stopped in both infected and noninfected tissue (F and J). Original magnification, ×100.

physiological doses of either E_2 or P_4 or a combination of both. These mice were infected IVAG with HSV-2 to examine how the hormonal environment altered susceptibility. We found that in the presence of E_2, mice were not susceptible to genital HSV-2 infection, as shown by the absence of pathology and viral shedding in their vaginal secretions. In the absence of any hormones, mice were highly susceptible to vaginal infection with HSV-2. P_4 treatment in these mice did not appear to alter their susceptibility significantly at a high inoculation dose (10^5 PFU). At lower inoculation doses of HSV-2, the infection in P_4-treated mice progressed faster than in the non-hormone-treated group, indicating that P_4 was possibly exacerbating the infection. At the lowest inoculation dose (10^2 PFU), 100% of P_4-treated mice died, while some survival was seen in the control saline group. The P_4-treated mice had extensive inflammatory response, characterized by a persistent and heavy leukocytic infiltration into the vaginal tissue and lumen. This persistent inflammatory response was absent in animals infected under other hormone conditions and in non-hormone-treated controls. The presence of inflammation coincided with the induction of a number of chemokines and receptors in the vaginal tracts of P_4-treated, infected mice. The E+P group had intermediate susceptibility at the high inoculation doses, characterized by focal infection that spread slowly. At lower inoculation doses, increased protection was seen in this group, indicating the dominating protective effect of estradiol.

A salient result from the present study is that E_2, in the absence of any other hormonal influence, made mice resistant to vaginal infection with HSV-2. Previous studies of intact mice show that medroxyprogesterone acetate treatment increased susceptibility to genital HSV-2 infection, while mice were refractory to infection following Depo-estradiol (2 μg/mous; The Upjohn Co., Kalamazoo, Mich.) treatment (18). However, in these studies the exogenous hormones were injected in non-OVX mice, superimposing their effect on the circulating hormone levels and making it difficult to determine the effect of individual hormones. Both estradiol and progesterone regulate the other's receptors and antagonize the biological effects of each other (7, 23). It is therefore critical to examine the outcome of each hormone directly before combining or superimposing their effects. In the present study, we examined the effect of estradiol and progesterone on their own and in combination by using physiological doses of the hormones. The results showed that when it was administered alone, estradiol made the mice resistant to genital infection with HSV-2. With the combination of estradiol and progesterone used in this

TABLE 1. Genes regulated in vaginal tissue of mice infected with HSV-2 under the influence of progesterone[a]

Gene class	Gene	Description	Regulation
Small inducible cytokines	Scya5 (RANTES)	Chemokine (C-C motif) ligand 5	↑
	Scya6 (C10)	Chemokine (C-C motif) ligand 6	↑
	Scya7 (MCP-3, MARC)	Chemokine (C-C motif) ligand 7	↑
	Scya21a (6Ckine/SLC/exodus-2)	Chemokine (C-C motif) ligand 21a (serine)	↑
	Scyb2 (MIP-2)	Chemokine (C-X-C) ligand 2	↑
	Scyb5 (LIX/GCP-2/ENA78)	Chemokine (C-X-C) ligand 5	↑
	Scyb10 (IP-10)	Chemokine (C-X-C) ligand 10	↑
	Scyb11 (eotaxin)	Chemokine (C-X-C) ligand 11	↑
	Scyb15	Chemokine (C-X-C) ligand 15	↑
Chemokine receptors	Cmkbr7 (CCR-7)	Chemokine (C-C motif) receptor 7	↑
	Ltb4r2	Leukotriene B4 receptor 2	↑
	Tapbp	TAP binding protein	↑
Other related genes	SDF-1a	Chemokine (C-X-C motif) ligand 12	↓
	Epo	Erythropoietin	↑

[a] Chemokine and chemokine receptor genes were analyzed by microgene array analysis. OVX mice were treated with E_2 or P_4 and inoculated IVAG with 10^5 PFU of HSV-2 or left untreated. Three days later, total RNA was extracted from vaginal tissue and reverse transcribed to cDNA and labeled with [^{32}P]dCTP. Labeled cDNA was hybridized to GE Array membranes, and signals were analyzed with a phosphorimager, as described in Materials and Methods. The signals of the chemokine and receptor genes were normalized to a housekeeping gene (GAPDH) and expressed as the percent GAPDH. ↑, upregulation; ↓, downregulation. Any genes that were expressed ≤20% GAPDH were not included in the analysis. The data summarized in this table is from two separate experiments.

study, the estradiol effect was dominant on susceptibility when virus was present in low numbers. However, in the presence of large amounts of virus, this protective effect was overcome.

The mechanism by which E_2 made mice nonsusceptible is not clear. One well-accepted mechanism is that during estrus and under the influence of estradiol, the vaginal epithelium is several layers thick and keratinized in superficial layers, making it impermeable to viral entry (18). While this is a plausible explanation that may be true when mice are solely under the influence of estradiol, there may be additional factors that affect susceptibility. So far, the presence of HSV-2 has not been examined in the absence of "productive" infection. It is possible that the virus does enter the vaginal epithelium in estradiol-treated mice. In this case, estradiol may influence factors present in the epithelium or the surrounding tissue to exert antiviral effects that could limit or even terminate infection in the epithelium. The second possibility is that the entry of HSV-2 into the genital epithelium could be modified by the expression of viral receptors that may be hormonally regulated. Support for this possibility comes from a recent study, where nectin-1-δ, one of the HSV-2 receptors, was not expressed in mouse vaginae at estrus, when estradiol levels are high and mice are known to be resistant to genital HSV-2 (13). These other possibilities need to be explored to fully understand the mechanism by which E_2 regulates susceptibility.

In this study, progesterone by itself did not appear to have a significant role in modulating susceptibility. Only at lower inoculation doses was there an indication that P_4-treated mice may be more susceptible than non-hormone-treated controls. However, P_4-treated mice did have significantly increased inflammation following infection at all challenge doses. Previously, we have seen similar proinflammatory effects of progesterone in genital infection with *Chlamydia trachomatis* in a rat model (8). Progesterone therefore appears to have a role in inducing inflammation and possibly immune responses following infection in the genital tract. Neutrophils were the predominant cell type constituting the inflammatory infiltrate following infection in P_4-treated mice. Neutrophils are known to play an important role in inflammatory and innate immune responses (17). They have been shown to be involved in protection against HSV challenge in the vaginal mucosa (16). In the present study, however, the presence of neutrophils did not correlate with protection. This result indicates that they may be playing a different role. Neutrophils have been shown to be a cellular source of chemokine production that can orchestrate sequential recruitment of other immune cells (19). There is also evidence that in respiratory infections, they augment epithelial damage induced by viral infection and contribute to pathophysiology (24). Indeed, the main consequence of progesterone treatment in our studies appeared to be persistent inflammation, signifying that neutrophils may be contributing to epithelial damage. However, given the wide array of chemokines seen postinfection in P_4-treated mice, it is likely that they also participate in coincident induction of innate and adaptive immune responses. In fact, this is supported by our more recent experiments, where we immunized mice under P_4 influence with an attenuated HSV-2 strain and saw excellent protection against wild-type challenge (6a). Interestingly, although these mice were protected, they did exhibit symptoms of chronic pathology.

The present model should provide valuable insights into the mechanism of hormone regulation of immune responses to sexually transmitted viral infections in general and HSV-2 in particular. Clinical studies have clearly documented the effect of hormones on susceptibility to viral sexually transmitted infections, including HSV-2 and HIV, in women. Experimental work in monkey models provided clear evidence that hormones influence infection by SIV. Progesterone implants enhanced SIV vaginal transmission and viral loads, while estradiol provided protection from infection (15, 20). More recent studies have shown that HIV-infected women showed enhanced viral shedding in their cervicovaginal secretions following hormonal contraceptive treatments (12). These studies emphasize the importance of understanding the role of hormones

in susceptibility to sexually transmitted viral agents. This knowledge is critical for developing better prophylactic and therapeutic strategies against these infections in women.

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