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 (E2), progesterone (P4), 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 P4-treated groups. In the P4-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 P4 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 P4-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 mucosa. 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-
immunization with TK
combination of both, or saline (control group) prior to IVAG
Ovariectomized mice were given estradiol, progesterone, a
then resuspended in phosphate-buffered saline (PBS). Progesterone was sus-
-Calbiochem (La Jolla, Calif.). Estradiol was initially dissolved in ethanol, evaporated to dryness, and
pathology were monitored and correlated with viral shedding
vaginally 3 weeks later with wild-type HSV-2. Survival and
mechanism of protection, herpes-specific T-cell responses were
examined, and HSV-2 infection was localized. To examine the
munized mice. The histopathology of the vaginal mucosa was
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.

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 intra-
vaginally 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 im-
munized 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. 17\beta-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 sus-
pended 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- \(\mu\)l volume for three consecutive
days. Control mice were injected with 100 \(\mu\)l 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 \(\mu\)l of attenuated HSV-2 (TK \(^{−}\)) at a dose of
10^5 PFU. Three weeks later, the mice were inoculated IVAG with 10 \(\mu\)l of
wild-type HSV-2 strain 333 at a dose of 10^6 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 reproduc-
tive cycle staging and plaque assays was collected by pipetting two 30-\(\mu\)l portions of
PBS in and out of the vagina several times to give a total of 60 \(\mu\)l. Repro-
ductive 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% polymorpho-
nuclear 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) sup-
plemented with 5% fetal bovine serum (GIBCO), 1% penicillin-streptomycin,
and L-glutamine (GIBCO). For plaque assays, Vero cells were grown to conflu-
eence 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-
Viral titers (pfu/ml)

**Day 1**  **Day 2**  **Day 3**  **Day 4**  **Day 5**

Groups:
- **S**
- **E**
- **P**
- **E+P**

**Days post TK⁻ infection**

**FIG. 2.** Virus titers in vaginal washes of ovariectomized, hormone-treated mice after immunization with HSV-2 TK⁻ (10⁵ 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.

**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.

**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 (E₂) or progesterone (P₄) 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 10⁵ 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...
external pathology scores over 6 days postvaccination. No significant pathology was noted in the E2 and E+P groups. Mice in P4-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 P4-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 P4-treated group and in the saline-injected control group. Viral shedding was undetectable in the E2-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 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.

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.

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 P4-treated group and in the saline-injected control group. Viral shedding was undetectable in the E2-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. In subsequent experiments, in every batch, 10 to 20% of P4-treated mice appeared to develop similar chronic pathology. Most of the mice in the P4-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 E2-treated group had high virus titers in their vaginal secretions, none of the mice in the P4-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.
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 E2-treated TK−/H11002-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 P4-treated group, or the protected E/H11001P-treated mice (three out of seven mice) (Fig. 5E, G, and H, respectively). The P4-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 P4-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 P4-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 P4 groups and part of the E/H11001P group) showed significantly higher gB-specific proliferation than the unprotected groups (E2 and part of E+P) (P < 0.05). The gB-specific proliferation in the E+P protected group and the P4-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
in the HSV-2-specific T-cell activation in local lymph nodes in differ-
et 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 \( E/H_11001 P \)), 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 postchal-
lenge. 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 protec-
tion in ovarioectomized, hormone-treated, immunized mice with
humoral responses against HSV-2, local antibody levels in
the vaginal secretions were measured. Figure 8 shows the an-
tibody 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 compa-
rable 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 unpro-
tected group (the \( E_2 \) group and unprotected E+P) as well as
the protected E+P group did not show any significant antibod-
ies 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 im-
une responses following immunization in the genital tract.
The ability of the attenuated virus to cause a productive infec-
tion was regulated by the hormonal environment, and this
appeared to be critical in initiating immune responses. Estradiol
treatment prior to immunization caused the vaginal epi-
thelium 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.

**FIG. 5.** Histopathology and localization of infection in the vaginal tissue of ovarioectomized, hormone-treated, immunized mice after wild-type
HSV-2 challenge. Mice were ovarioectomized 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, \( \times 100 \).
This outcome correlates with previous results from our studies, where E2-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 P4-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 P4-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 P4-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-
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\(^+\)/H11001 and CD4\(^+\)/H11001 T cells surrounded by CD11c\(^+\)/H11001 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\(_2\)/H11001 P-treated, immunized group and completely absent from the E\(_2\)-treated group and the unprotected E\(_2\) group (data not shown). Similar but smaller structures were noted in the P\(_2\)- 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\(_2\)/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\(_2\)/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\(_2\)-treated groups and part of the E\(_2\)/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\(_2\), S, and part of E\(_2\)/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. 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\(_2\)/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, \(\times\)100.
Thus, CD4 T cells appear to modulate protection in all three groups. In addition to CD4⁺ T cells, most mice in the P⁺ and S-treated groups showed high titers of gB-specific local IgG and IgA. The P⁺-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


