# Estrogen Protects against Vaginal Transmission of Simian Immunodeficiency Virus

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**Postmenopausal women and women who use injectable, progestin-based contraceptives are at increased risk of human immunodeficiency virus (HIV) infection, suggesting that progesterone and estrogen affect HIV-1 vaginal transmission. To evaluate the individual roles of these sex hormones in vaginal transmission, ovariectomized female macaques were treated with either progesterone or estrogen followed by intravaginal inoculation with SIVmac. All 6 untreated control macaques and 5 (83%) of 6 progesterone-treated animals became infected following intravaginal SIV inoculation. Conversely, none of 6 estrogen-treated macaques was infected. Vaginal subepithelial inoculation of estrogen-treated animals resulted in infection, which shows that the block occurred at the vaginal epithelium and/or lumen. These data suggest that estrogen-deficient women are at increased risk of HIV infection, because their vaginal microenvironments are rendered more susceptible. Moreover, topical vaginal estrogen therapy may be an effective means of reducing HIV vaginal transmission in these high-risk groups.**

The World Health Organization estimates that 14.8 million women are living with human immunodeficiency virus (HIV) type 1 infection and that another 6.2 million women have died of AIDS [1]. Unprotected vaginal intercourse is the most common route through which women are infected with HIV-1 [2]. In sexually active women, the levels of estrogen and progesterone vary significantly under different natural and therapeutic conditions [3]. During the monthly reproductive cycle, estrogen levels steadily rise during the follicular phase and then fall after ovulation during the luteal phase, at which time progesterone levels rise. Women with low circulating levels of estrogen secondary to natural menopause or to therapy with depo-medroxyprogesterone acetate (DMPA) are more likely to become infected with HIV [4–7]. Specifically, use of DMPA, a long-acting progesterone-based contraceptive, is associated with depressed estrogen levels [3] and a 2–3-fold increase in the rate of HIV-1 infection [6, 7]. Additionally, postmenopausal women have very low levels of estrogen and progesterone [3] and also have a 4–8-fold increase in their chance of being infected with HIV, compared with similar premenopausal populations [4, 5]. The mechanism(s) for these increases in the rates of infection is unknown. However, because both states are associated with estrogen deficiency and only one state is associated with high progesterone levels, these findings suggest that estrogen may reduce transmission of HIV-1 across the vaginal epithelium and/or suppress viral replication after transmission has occurred.

The macaque vagina histologically resembles the human vagina and has been used to model the human vaginal epithelium [8, 9]. Atraumatic vaginal inoculation of macaques with simian immunodeficiency virus (SIV) results in passage of the virus across intact vaginal epithelium and subsequent development of systemic infection and simian AIDS [10]. To achieve the high rates of infection needed in nonhuman primate studies, we challenged the macaques with relatively high doses of pathogenic SIV. These are more rigorous conditions than might be encountered with HIV infection, in which the dose may be lower and the strains less pathogenic. In the present study, the SIV/macaque model was used to evaluate the effects of estrogen

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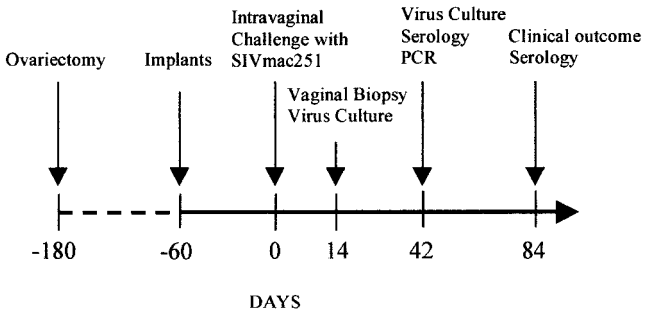


**Figure 1.** Times of ovariectomy, treatment, vaginal inoculation, and follow-up of the initial challenge. Days are shown relative to day 0, the day of intravaginal challenge with simian immunodeficiency virus (SIV) mac251. PCR, polymerase chain reaction.

and progesterone on the vaginal epithelium and to determine the rate of transmission and viral replication following intravaginal inoculation of SIV.

In a study reported elsewhere, we showed that progesterone therapy increases the risk of SIV vaginal transmission in menstruating female macaques [11]. Progesterone treatment was associated with a 7.7-fold increase in the rate of SIV infection and a marked thinning of the vaginal epithelium, suggesting that the effects of sex hormones on the vaginal microenvironment play an important role in conferring relative susceptibility to vaginal transmission of SIV.

To understand the individual effects of estrogen and progesterone on the vaginal epithelium and SIV vaginal transmission, female macaques were ovariectomized to remove the influence of endogenous hormone expression. After ovariectomy, macaques were treated with either progesterone or estrogen, and control animals were left untreated. The control group served as a model of HIV vaginal transmission in anovulatory or postmenopausal women, for whom vaginal atrophy is a well-described phenomenon [12].

## Materials and Methods

*Animals.* Adult female rhesus macaques, weighing 5–9 kg, were used. All animals were experimentally naive and were seronegative for D retrovirus and SIV. Each animal underwent bilateral ovariectomy by means of standard surgical techniques.

*Vaginal biopsies.* Vaginal biopsy specimens were taken with forceps from the middle of the vaginal vault. Histology specimens were fixed in formalin for 3 days and stained with hematoxylin-eosin. Samples were taken and processed simultaneously. To minimize the effect of tangential cuts, vaginal epithelial thickness was measured at the thinnest portion observed.

*Implants.* Silastic implants were prepared by K. Hermsmeyer at the Oregon Regional Primate Research Center, as described elsewhere [13]. Each estrogen implant contained 200 mg of estradiol-17$\beta$ ($E_2$). Each progesterone implant contained 400 mg of progesterone.

*Hormone measurement.* Serum was separated from clotted

**Table 1.** Hormone levels and virus isolation, serology, polymerase chain reaction (PCR), and vaginal epithelial thickness after vaginal challenge of macaques with simian immunodeficiency virus (SIV).

| Group, animal | Progesterone (ng/mL), day 0 | Estrogen (pg/mL), day 0 | Coculture | | Anti-SIV ELISA | | PCR, day 42 | Thickness[a] ($\mu$m), day 14 |
|---|---|---|---|---|---|---|---|---|
| | | | Day 14 | Day 42 | Day 42 | Day 84 | | |
| Estrogen-treated | | | | | | | | |
| AT71 | <0.10 | 761 | − | − | − | − | − | 212 |
| AT72 | <0.10 | 515 | − | − | − | − | − | 200 |
| T73 | <0.10 | 755 | − | − | − | − | − | 285 |
| T75 | <0.10 | 89 | − | − | − | − | − | 460 |
| A210 | <0.10 | 138 | − | − | − | − | − | 220 |
| I114 | <0.10 | 134 | − | − | − | − | − | 80 |
| Progesterone-treated | | | | | | | | |
| AT64 | 5.46 | <5 | + | + | + | + | ND | 2 |
| AT67 | 5.59 | <5 | + | + | + | + | ND | 3 |
| AT70 | 2.51 | <5 | + | + | + | + | ND | 2 |
| I016 | 5.16 | <5 | + | + | + | + | ND | 5 |
| I045 | 2.91 | <5 | + | + | + | + | ND | 2 |
| F056 | 4.81 | 77 | − | − | − | − | − | 5 |
| Untreated | | | | | | | | |
| AT76 | <0.10 | <5 | + | + | + | + | ND | 5 |
| AT77[b] | <0.10 | <5 | + | + | − | ND | ND | 3 |
| AT82 | <0.10 | <5 | + | + | + | + | + | 5 |
| I649 | <0.10 | <5 | + | + | + | + | ND | 3 |
| L322 | <0.10 | <5 | + | + | + | + | ND | 2 |
| P101 | <0.10 | <5 | + | + | + | + | ND | 5 |

NOTE. Limits of detection were 0.10 ng/mL for progesterone and 5 pg/mL for estrogen. ND, not determined; +, positive; −, negative.

[a] Vaginal epithelial thickness.

[b] At necropsy, AT77 had widespread SIV disease, including myelitis, severe esophagitis/gastritis, and diffuse thymic and lymph node atrophy.

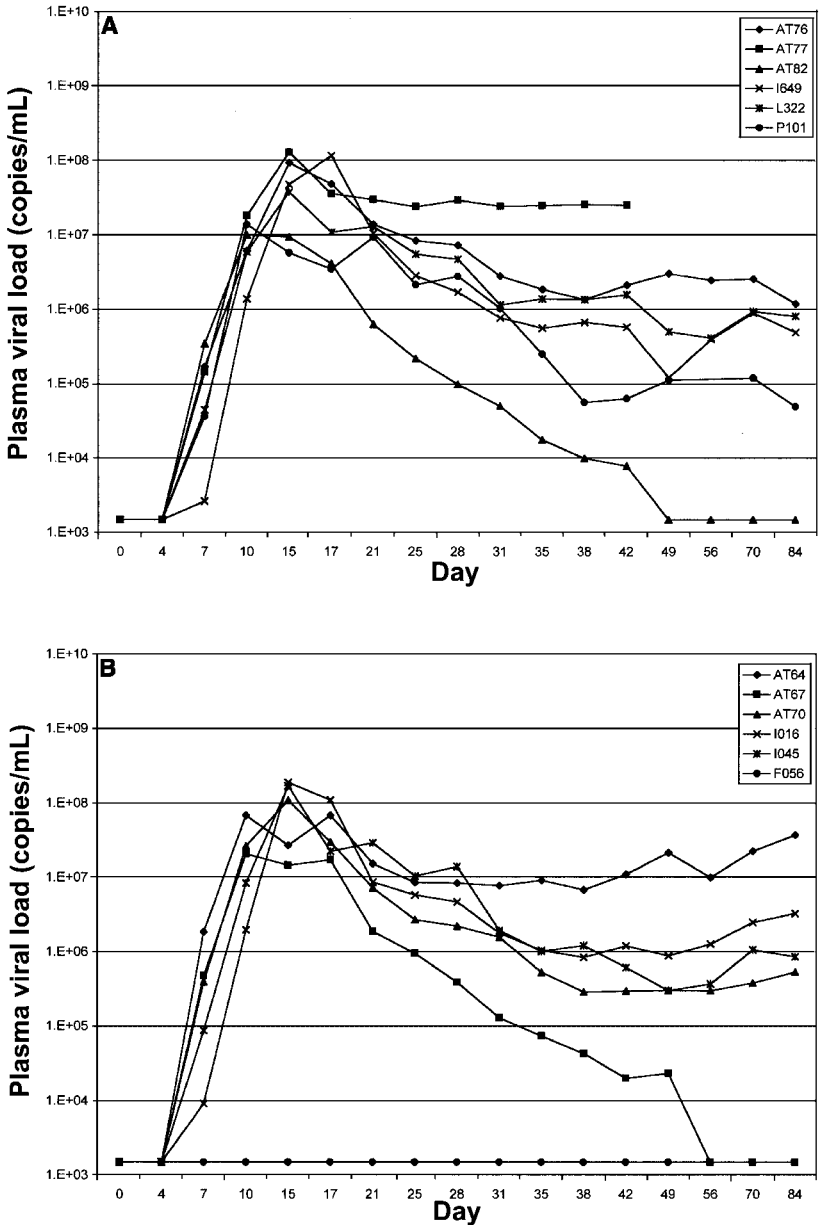


**Figure 2.** Plasma simian immunodeficiency virus (SIV) loads following initial vaginal challenge in untreated *(A),* progesterone-treated *(B),* and estrogen-treated *(C)* ovariectomized macaques. Isolated low level values in AT71 and AT75 at 1 time point each represent false-positive results, as seen in 4% of negative samples [18]. Plasma SIV loads in estrogen-treated animals after rechallenge via submucosal (AT72, A210, I114) or intravenous (AT71, AT73, AT75) routes are shown in *D*.

blood and stored at −70°C until assayed. Hormone assays were done by D. Hess at Oregon Regional Primate Research Center and used methodology described elsewhere [14]. The limits of detection for the progesterone and estrogen assays were 0.10 ng/mL and 5 pg/mL, respectively.

*Virus stock and challenges.* The same stock of SIVmac251 (2700 $TCID_{50}$/mL) was used in all challenges. The SIVmac251 stock was prepared in stimulated human peripheral blood mononuclear cell (PBMC) culture and was titered on CEM-X-174 cells, as described elsewhere [15]. The seed stock was generously provided by R. Desrosiers (New England Regional Primate Research Center). On the day of intravaginal challenge, the virus stock was diluted in RPMI medium to a concentration of 640 $TCID_{50}$/mL. Intravaginal inoculations with 1 mL of diluted stock were done without trauma, as described elsewhere [10]. Intravenous inoculations with 240 $\mu$L (640 $TCID_{50}$) of undiluted stock were also done as described

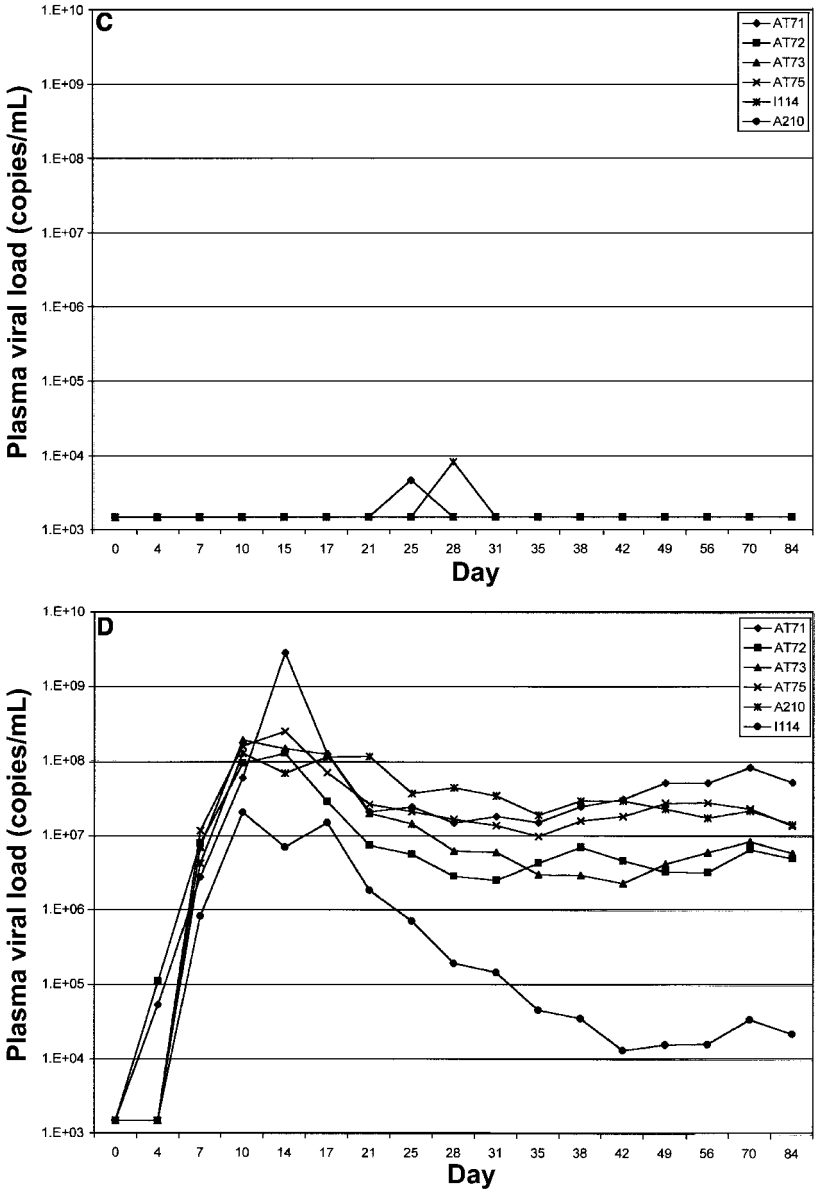


**Figure 2.** *(Continued).*

above. Vaginal submucosal inoculations were done with a 22-gauge needle and 1-mL syringe. The vaginal canal and epithelium were visualized by use of a speculum. The needle was placed into the submucosa of the lateral vaginal wall, and 240 $\mu$L of undiluted virus stock solution was injected.

*Virus isolation.* Purified rhesus PBMC were cocultured with CEM-X-174 cells, as described elsewhere [15].

*Anti-SIV ELISA.* Plasma was separated from heparinized blood and stored at −70°C until assayed. SIV antibody ELISAs were done as described elsewhere [15].

*Polymerase chain reaction (PCR).* DNA was extracted from rhesus PBMC via standard proteinase K (5′-3′, Boulder, CO) digestion [16]. Next, 150 ng of DNA (from ~30,000 cells) was added to each PCR reaction. Each animal's DNA was analyzed in quadruplicate. This nested PCR was done with an outer primer pair (Out1, TGGAGGTTCTGGAAGAACTG; Out2, CTCCAAGCTGGTACACCATA) and an inner primer pair (In1, GGAGGTTCTGGAAGAACTGA; In2, GGATGGCGATAAGCAGCTGAT) from the *tat* region of SIVmac. These primer pairs could reproducibly detect, on average, 1 copy of SIVmac template (pBK28) per reaction (data not shown). The limit of detection in this assay was 1 proviral copy/30,000 cells.

*Branched DNA (bDNA) assay.* bDNA assays were done at Bayer (Emeryville, CA) as previously described [17]. Plasma from

heparinized blood was separated and stored at −70°C until it was assayed. The lower limit of detection was 1500 copies of SIVmac genome/mL. The assay has a specificity of 96% [18].

## Results

*Animal protocol and hormone therapy.* Eighteen female macaques of reproductive age underwent bilateral ovariectomy. Each animal was randomly assigned to receive progesterone, estrogen, or no treatment. Hormone-containing implants were placed in the subcutaneous tissue on day 60 before vaginal inoculation (figure 1). Macaques in the progesterone treatment group received 1 subcutaneous 60-mm Silastic progesterone implant containing 400 mg of progesterone. Those in the estrogen treatment group received 2 subcutaneous 30-mm Silastic estrogen implants, each of which contained 200 mg of estradiol ($E_2$). The untreated animals did not receive an implant. In preliminary studies involving a separate group of macaques, these implants were shown to provide prolonged (>180 days) delivery of either hormone (data not shown). Efforts were made to achieve blood levels of progesterone of 4–8 ng/mL in the progesterone group and blood levels of $E_2$ of 250–300 pg/mL in the estrogen group at the time of SIV vaginal exposure. These values represent the natural peak of each hormone in menstruating rhesus macaques [9]. Although some variation was seen in the estrogen-treated animals, overall the implants functioned as expected (table 1). The untreated, ovariectomized animals had no detectable amounts of either progesterone or $E_2$, whereas the estrogen-treated animals had follicular phase levels of $E_2$ and no detectable progesterone. The progesterone-treated animals (with the exception of F056) had luteal levels of progesterone and no appreciable $E_2$. Animal F056 had expected levels of progesterone (4.81 ng/mL) but also had appreciable levels of estrogen (77 pg/mL).

*SIV vaginal challenge.* On day 0, each macaque was challenged intravaginally with 640 $TCID_{50}$ of pathogenic SIVmac251. The animals were then followed for a period of 12 weeks and assessed at regular intervals for evidence of SIV infection. SIV was isolated from the PBMC of all 6 untreated macaques and 5 of the 6 progesterone-treated macaques at multiple time points (days 14 and 42; table 1). In contrast, SIV was not isolated from any of the estrogen-treated macaques at any time point. Each animal from whom virus was isolated (with the exception of AT77) also developed anti-SIV antibodies by day 42. AT77, a typical rapid progressor [19], failed to seroconvert to SIV but had high virus loads as measured by bDNA assay (figure 2). AT77 developed signs of simian AIDS at 6 weeks and had widespread evidence of SIV disease at necropsy (table 1). All animals that did not have virus isolated also tested negative for an SIV antibody response on days 42 and 84 (table 1). Nested PCR with SIVmac-specific primers was done on DNA from PBMC of macaques that were negative by virus culture and serology, and results were also negative for SIVmac for each of these animals. Positive PCR results were obtained in control reactions that used PBMC from macaque AT82.

In the infected macaques, plasma virus was detected on day 7 and rose to a peak within the first 21 days, followed by a decline to a plateau from days 21 to 42 (figure 2). This pattern of viral replication did not differ significantly between the infected macaques in the untreated group and those in the progesterone treatment group. In long-term follow-up, no differences were seen in the rate of disease progression (data not shown) between the infected macaques in the progesterone-treated or the untreated groups after 12 months. The estrogen-treated macaques and the 1 uninfected progesterone-treated macaque had plasma virus load measurements consistently below the limit of detection of the bDNA assay, and they remained clinically healthy.

*Vaginal biopsy and pH.* To determine the effects of hormone treatment on the vaginal epithelium, biopsies were done on day 14 after vaginal challenge. Day 14 was chosen so that the biopsy site would not affect SIV transmission. Biopsy samples were taken, using forceps, from the middle third of the lateral vaginal wall, and samples were coded to blind researchers to the treatment status of each animal before measurement of the vaginal epithelial thickness. When the code was opened, the results revealed that all progesterone-treated and untreated macaques had thin vaginal epithelia ($\leqslant 5$ $\mu$m; table 1). In contrast, 5 of the 6 estrogen-treated macaques had thick vaginal epithelia ($\geqslant 200$ $\mu$m), and the remaining macaque in this group had a moderately thick vaginal biopsy sample (80 $\mu$m). The vaginal epithelial thickness for all macaques in the progesterone-treated and untreated groups averaged 3.2 and 3.8 mm, respectively, whereas the average thickness for the estrogen-treated group was 243 $\mu$m. Representative examples of vaginal biopsy samples are shown in figure 3. Vaginal pH was determined via intravaginal placement of pH paper. The estrogen-treated animals had a lower vaginal pH (average, 7.7), whereas the progesterone-treated and untreated animals had an average vaginal pH of 8.4 and 8.0, respectively, which is within the pH range at which retroviruses are stable [20].

*SIV rechallenge.* Occult infection of each estrogen-treated animal with SIV was ruled out by a repeat virus-isolation attempt and an anti-SIV serology assay on day 290, both of which yielded negative results for all animals (data not shown). Subsequently, macaques in the estrogen treatment group were rechallenged with 640 $TCID_{50}$ of SIVmac251 (same virus stock) by either intravenous inoculation ($n = 3$) or direct injection into the vaginal submucosa ($n = 3$). The latter route of inoculation allowed access to the vaginal submucosa but bypassed the vaginal epithelium and microenvironment. Estrogen levels on the day of rechallenge were similar to those from the initial challenge (table 2). On the day of rechallenge, each animal underwent repeat vaginal epithelial biopsy, which again showed pronounced thickening for all animals (table 2). In contrast to our

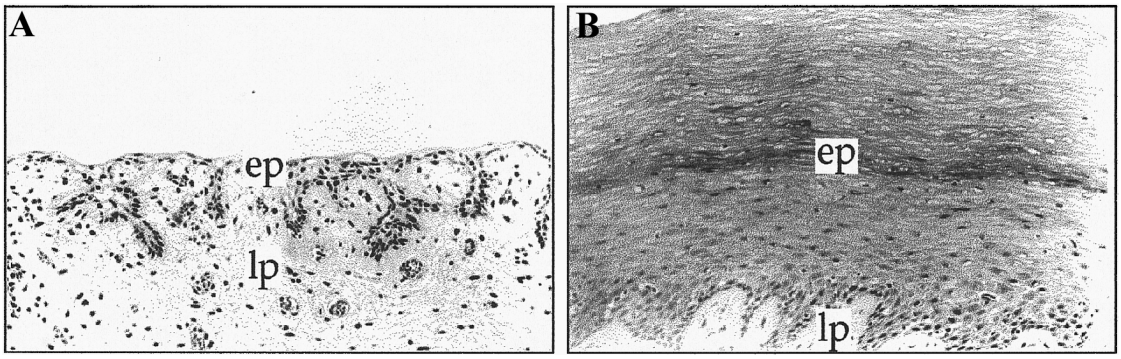


**Figure 3.** Examples of vaginal epithelial biopsy samples from animal AT82 *(A;* 5 $\mu$m) and animal A210 *(B;* 220 $\mu$m). Vaginal epithelial thickness was determined by measuring each epithelium. Epithelial layers (ep) are above lamina propria (lp).

earlier results in which estrogen-treated macaques resisted nontraumatic vaginal SIV infection, all 6 macaques had virus isolated on days 14 and 42 after direct injection of the virus into the vaginal submucosa or intravenous inoculation. Additionally, all 6 were positive for anti–SIV antibody on days 42 and 84 (table 2). Moreover, plasma viremia in the rechallenged macaques was similar to that in the untreated and progesterone-treated infected animals (figure 2).

## Discussion

We found that the rate of infection of the estrogen-treated group after intravaginal challenge was 0, which was significantly different from that of the progesterone-treated group ($P = .015$) and that of the untreated control group ($P = .002$; both comparisons by Fisher's exact test). However, all estrogen-treated animals became infected with SIV after submucosal or intravenous inoculation. Once infected with SIV, estrogen-treated macaques developed plasma virus loads that were similar to those in untreated macaques. Therefore, estrogen therapy does not appear to effect SIV replication within the vaginal submucosa or throughout the lymphatic tissues. Rather, these observations are consistent with the hypothesis that estrogen protects against SIV vaginal transmission through its influence on the vaginal microenvironment. Additionally, progesterone, which has been shown to inhibit certain strains of HIV-1 in vitro [21], had no obvious effect on SIV replication, as assessed by plasma virus load.

The data from this study are consistent with previous observations in cycling macaques. Animals undergoing progesterone therapy, which suppresses and antagonizes estrogen, had a much higher rate of infection after intravaginal inoculation than did untreated cycling macaques [11]. Furthermore, macaques challenged intravaginally with SIV in the luteal (progesterone-dominant) phase of the menstrual cycle had a higher infection rate than did those challenged during the follicular (estrogen-dominant) phase [22]. Taken together, these results confirm the low infection rate observed after intravaginal challenge during an estrogen-dominant state. Conversely, progesterone therapy increased the rate of infection in cycling macaques, presumably because of the antagonism of endogenous estrogen production and thinning of the vaginal epithelium. In the untreated, anovulatory state, the macaque vaginal epithelium atrophies in the absence of estrogen, and a high rate of vaginal transmission is seen.

Of particular interest, we noted in this study and another study reported elsewhere [11] that vaginal epithelial thickness inversely correlates with susceptibility to infection via intravaginal challenge. Vaginal epithelial thickening seen in the estrogen-treated animals may be causally linked with resistance to SIV infection or may simply be a marker of the dominant effect of estrogen on the vaginal microenvironment. After intravaginal challenge, macaques can be infected through the cervical epithelium [23], which is simple columnar and does not change in the presence or absence of estrogen [24]. Therefore, vaginal epithelial thickening alone may not explain the observed protection. Rather, the vaginal microenvironment may be more resistant to SIV when it is primarily under the influence of estrogen. Although estrogen therapy did not change the macaques' vaginal pH in this study, estrogen, in addition to its effects on the vaginal epithelium, is known to increase cervical mucus production, decrease cervical ectopy, and alter vaginal bacterial flora [24, 25]. The combined effects of estrogen on the vagina may contribute to reducing the probability of infection.

Postmenopausal women commonly have atrophic vaginitis, which is caused by estrogen deficiency [26]. This condition is associated with a dry, friable, thin vaginal epithelium, which bleeds after minimal trauma [12, 27]. Atrophic vaginitis is successfully treated with local or systemic estrogen replacement therapy, which results in thickening of the vaginal epithelium and lowering of the intravaginal pH [28–30]. The vaginal epithelium in untreated ovariectomized macaques is similar to that seen in atrophic vaginitis. In studying ovariectomized macaques receiving no hormone therapy, we have modeled HIV vaginal transmission in women with atrophic vaginitis. Our findings suggest that women with atrophic vaginitis may be at increased

**Table 2.** Hormone levels, virus isolation, serology, and vaginal epithelial thickness for estrogen-treated animals after rechallenge (day 0) with simian immunodeficiency virus (SIV) via intravenous or submucosal routes.

| Animal | Progesterone (ng/mL), day 0 | Estrogen (pg/mL), day 0 | Coculture | | Anti-SIV ELISA | | Thickness[a] ($\mu$m), day 0 |
|---|---|---|---|---|---|---|---|
| | | | Day 14 | Day 42 | Day 42 | Day 84 | |
| AT71 | <0.10 | 505 | + | + | + | + | 157 |
| AT72 | <0.10 | 897 | + | + | + | + | 183 |
| AT73 | <0.10 | 289 | + | + | + | + | 248 |
| AT75 | <0.10 | 168 | + | + | + | + | 505 |
| A210 | <0.10 | 209 | + | + | + | + | 228 |
| I114 | <0.10 | 491 | + | + | + | + | 168 |

NOTE. Limits of detection were 0.10 ng/mL for progesterone and 5 pg/mL for estrogen. +, positive.

[a] Vaginal epithelial thickness.

risk for contracting HIV through vaginal intercourse. Two studies support this hypothesis. In a study of male-to-female transmission in women married to men infected with HIV-2, women ⩾45 years old were 8 times more likely to become infected than were those <45 years old [5]. In a European study, increased age of the woman (⩾45 years) was found to confer a similar, increased risk for male-to-female HIV-1 transmission [4].

Although specific data on the number of postmenopausal women who are at risk are not available, >600 million women aged ⩾50 are currently living, and this number is expected to increase to 1.2 billion by the year 2030 [31]. In sub-Saharan Africa and in India, women >50 years of age represent 5.0% and 7.1% of the total population, respectively [31]. Although sexual activity declines some with age, >70% of women 50–60 years old are sexually active [32]. Therefore, postmenopausal women appear to represent a large, unrecognized group of people who are at increased risk of HIV infection. In light of our current findings, human trials that examine the effects of estrogen therapy on HIV vaginal transmission in postmenopausal women are warranted.

Women who use long-acting, progestin-based contraceptives, such as DMPA, have low levels of estrogen. Worldwide, 65 million women use such agents, and DMPA is one of the most common forms of contraception used in countries, such as Thailand, where HIV incidence is high [33–35]. Although earlier studies have yielded conflicting results, 2 recent prospective studies have established that women who use DMPA have a 2- to 3-fold increase in risk of HIV-1 infection [6, 7]. The mechanism(s) by which DMPA confers increased susceptibility to HIV-1 infection is not known. However, the data from the present study suggest that DMPA may increase susceptibility to HIV vaginal transmission through its antiestrogenic effects on the vaginal microenvironment. The hypothesis that the vaginal microenvironment contributes to the likelihood of HIV vaginal transmission is supported by another recent observation. Specifically, bacterial vaginosis, a bacterial overgrowth condition with no systemic effect, confers an increased risk of HIV vaginal transmission [36, 37]. Therefore, it appears that certain conditions that affect the vaginal microenvironment can increase the risk of HIV vaginal transmission.

In summary, estrogen therapy in the macaque model effectively blocked vaginal transmission of SIV. This block against infection occurred in the vagina but not in the submucosa or systemic tissues. Estrogen-deficient states are associated with high vaginal transmission of SIV in this model and with increased vaginal HIV transmission in women. Neither progesterone nor estrogen appeared to have any impact on systemic SIV replication or rate of disease. A safe, inexpensive therapy, such as vaginally delivered estrogen, could have a profound impact on the AIDS epidemic. In postmenopausal women and women taking DMPA who routinely engage in high-risk vaginal sex, estrogen therapy may reduce their susceptibility to vaginal transmission and should be evaluated.

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