# Topical estrogen protects against SIV vaginal transmission without evidence of systemic effect

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**Background:** Accumulating data suggest that the state of the vaginal epithelium affects a woman's risk of HIV vaginal transmission and several human and non-human primate studies have shown that the rate of HIV or SIV vaginal transmission is decreased when estrogen is dominant. Systemic estrogen can protect against SIV vaginal transmission.

**Objective:** To determine the safety and efficacy of topical estrogen in preventing SIV vaginal transmission.

**Design:** The non-human primate model of HIV vaginal transmission was used to assess vaginal estriol cream in ovariectomized macaques.

**Methods:** Twelve macaques were treated intravaginally with estriol and eight with placebo cream twice a week. The vaginal and systemic effects of estriol were determined by colposcopy and serum luteinizing hormone, levels of which would decline in the presence of systemic estrogen. After 5 weeks of therapy, the animals were challenged vaginally with pathogenic $SIV_{mac251}$.

**Results:** Vaginal estriol resulted in minimal serum estriol levels and had no effect on serum luteinizing hormone levels. Vaginal epithelia cornified and thickened significantly in response to estriol therapy. One of the estriol-treated animals became infected after this single challenge, while six of the control animals became infected ($P = 0.0044$).

**Conclusions:** These data demonstrate that topical vaginal estriol can strongly protect against SIV vaginal transmission, while having no detectable systemic effect. These results support the study of topical vaginal estriol in preventing HIV vaginal transmission in at-risk women.


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**Keywords: macaques, SIV, vaginal viral transmission, topical estriol**

## Introduction

Accumulating evidence strongly suggests that the state of the vaginal epithelium influences the risk of HIV vaginal transmission. Estrogen promotes human vaginal epithelial maturation and indirectly, by enhancing epithelial cell glycogen production, makes the intravaginal environment more acidic [1]. In the setting of low estrogen, as in postmenopausal women, the human vaginal epithelium is less thick and usually becomes

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atrophic [2]. Women treated with depo-medroxyprogesterone acetate (DMPA), an estrogen antagonist, have low levels of estrogen and are at increased risk of HIV vaginal transmission [3–5]. Women, who become infected with HIV through the vaginal route while taking DMPA have more viral variants and higher viral loads than women not on a contraceptive agent [3]. Further, two studies have shown that women older than 45 years of age (postmenopausal) are at increased risk for HIV vaginal transmission compared with their younger (premenopausal) counterparts [6,7]. Finally, bacterial vaginosis, a bacterial overgrowth condition associated with vaginal inflammation and with no systemic effect, confers an increased risk to HIV vaginal transmission [8,9]. These observations can be explained by the single hypothesis that, in a setting of low estrogen, the vaginal epithelium is more easily traversed by the infecting HIV virion.

In three prior studies using the macaque model of vaginal transmission, it was established that when the vaginal epithelium is under the influence of estrogen it is highly resistant to SIV transmission [10–12]. Most recently, it has been shown that systemic estrogen protects ovariectomized macaques against vaginal transmission of $SIV_{mac}$ [11]. Moreover, the estrogen protective effect was mediated through its effects on the vaginal epithelium, since estrogen-treated animals had normal infections when SIV was inoculated either intravenously or beneath the vaginal mucosa [11]. Two of the three studies were performed with exogenous hormone administration and/or in ovariectomized macaques, which lack appreciable circulating estrogen or progesterone [10,11].

The findings from these studies suggest that estrogen therapy could be protective in sexually active postmenopausal women. Of note, 50% of all infected adults in the world are now women [13]. Over 600 million women aged 50 and older are currently living and this number is expected to increase to 1.2 billion by the year 2030 [14]. In sub-Saharan Africa and in India, women over 50 years of age represent 5.0 and 7.1% of the total population, respectively [14]. While sexual activity declines some with age, over 70% of women between 50 and 60 years of age are sexually active [15]. Therefore, postmenopausal women represent a large, unrecognized group of people who are at increased risk of HIV infection.

In the earlier studies, unopposed, systemic estrogen therapy was used. Although protection against SIV vaginal transmission was achieved, estrogen therapy was associated with endometrial hyperplasia, polyps and adenomyosis [16]. The current study sought to eliminate these side effects and determine if a topical estrogen therapy could confer the same degree of protection.

## Methods

### Animals

Protocols for the animals (adult female rhesus macaques, 5–9 kg) were approved by the Tulane National Primate Research Center Institutional Animal Care and Use Committee. All animals were experimentally naive and were seronegative for D retrovirus and SIV. Each animal underwent bilateral ovariectomy using standard surgical technique.

### Vaginal creams

A commercially available preparation of estriol, Ovestin, was used. This drug has been used extensively by postmenopausal women in Europe with little or no evidence of effect outside of the vagina [17]. Estriol cream is effective in treating atrophic vaginitis, which occurs in postmenopausal women, and causes no adverse effects, including endometrial hyperplasia [17,18]. Consequently, the vaginal estriol cream should affect the vaginal epithelium without causing any systemic estrogen effect. Ovestin and its base cream were generously provided by Organon (Roseland, New Jersey, USA). Full-strength Ovestin has an estriol concentration of 1 mg/ml. Vaginal estriol creams with lower concentrations were prepared by diluting Ovestin with the appropriate amount of base cream. All creams were stored at room temperature, as recommended by the manufacturer.

### Hormone measurements

Serum was separated from clotted blood and stored at −70°C until assayed. Estradiol and luteinizing hormone (LH) were estimated by radioimmune assay as previously described [19,20]; unconjugated estriol was estimated using reagents obtained from Diagnostic Systems Laboratories (Webster, Texas, USA). In all assays, intra- and interassay percentage coefficient of variance did not exceed 10% and the limits of detection were 20 pg/ml for estradiol, 0.03 ng/ml for estriol, and 5 ng/ml for LH.

### Virus stock and challenges

The same viral stock of $SIV_{mac251}$ [2700 median tissue culture effective dose ($TCID_{50}$)/ml] was used in all challenges. The $SIV_{mac251}$ stock was titered on CEM-X-174 cells as previously described [21]. The seed stock was generously provided by Dr Ronald Desrosiers of the New England Regional Primate Research Center. On the day of intravaginal challenge, the virus stock was diluted in RPMI media to 640 $TCID_{50}$/ml. Intravaginal inoculations with 1 ml of diluted stock were performed without trauma, as previously described [21].

### Colposcopy

For each colposcopic examination, the animal was sedated according to institutional guidelines. An experi-

enced veterinarian used a standard pediatric colposcope to visualize the vagina and its epithelium.

### SIV assays

Branched DNA assays were carried out by Bayer Corporation (Emeryville, California, USA). Plasma from heparinized blood was separated and stored at −70°C until it was assayed. The lower limit of detection of the $SIV_{mac}$ viral genome was 125 copies/ml. The assay has a specificity of 96% [22].

Plasma was separated from heparinized blood and stored at −70°C until assayed. Samples were tested for anti-SIV antibodies with a commercial SIV Western blot kit, according to the manufacturer's protocol (ZeptoMetrix, Buffalo, New York, USA).

## Results

### Dosing study

The preliminary dosing study was carried out to determine the dose of intravaginal estriol that would cause an adequate estrogenic effect on the vaginal epithelium and yet have no detectable systemic effects. Ovariectomized macaques, similar to ovariectomized women, have elevated serum levels of LH. In this physiological state, serum LH levels are extremely sensitive to circulating estrogens, which, when present, inhibit LH secretion. Therefore, if an intravaginal estrogen dose enters the systemic circulation, it will result in a sharp decrease in serum LH levels. Serum LH concentration changes were monitored to determine if any of the estriol doses tested inhibited LH secretion. Lack of a negative effect on LH levels was interpreted as strong evidence that the estrogen dose tested had no systemic effects.

Thirteen adult female rhesus macaques underwent ovariectomy. After a healing period of over 2 months, the animals were randomly assigned to five groups. Groups 1–4 received treatment with increasing amounts of vaginal estriol: 0.025, 0.05, 0.1 and 11.0 mg/ml for groups 1, 2, 3 and 4, respectively. Group 5 was the control group receiving no estriol but treated with the base cream. A small bore feeding tube attached to a syringe was used to apply 1 ml of the appropriate cream intravaginally. Prior studies with this technique indicated that it did not cause any vaginal trauma. Treatment was given twice per week for 4 weeks.

Estriol, estradiol and LH were measured in serum samples taken from each animal at baseline and at three time points: just before each application (0 h), 1 h after application and 24 h after application. All 13 animals had estradiol levels consistently $< 20$ pg/ml and these levels did not increase during estriol intravaginal therapy (data not shown). As expected, the LH level increased in each animal following ovariectomy; the average LH level increased from $5.2 \pm 6.9$ ng/ml to $146 \pm 84$ ng/ml. Estriol levels were undetectable in all animals before therapy.

All groups were tested, but estriol was only detected in animals on the highest dose, group 4; the average 1 h estriol level was 0.25 ng/ml (Fig. 1a). By 24 h after therapy, estriol levels were again very low (average 24 h level 0.04 ng/ml) and similar to the control group values (Fig. 1b).

Changes in systemic LH levels were calculated by comparing the 1 or 24 h values with the 0 h value at each dose. Overall, there was no significant change in LH levels. For example, in the group 4 animals, which received the highest estriol dose, the average reduction in LH at 1 h was 0.8 ng/ml, compared with an increase of 1.7 ng/ml in the control animals. This difference was not statistically significant by either a t-test ($P = 0.892$) or ANOVA ($P = 0.894$). Similarly, the mean changes at 24 h were +10.0 ng/ml for group 4 and +13 ng/ml for the control animals. Again the difference was not statistically significant by t-test ($P = 0.808$) or ANOVA ($P = 0.825$). In summary, treatment with the estriol cream had no effect on circulating LH levels at either 1 or 24 h post application.

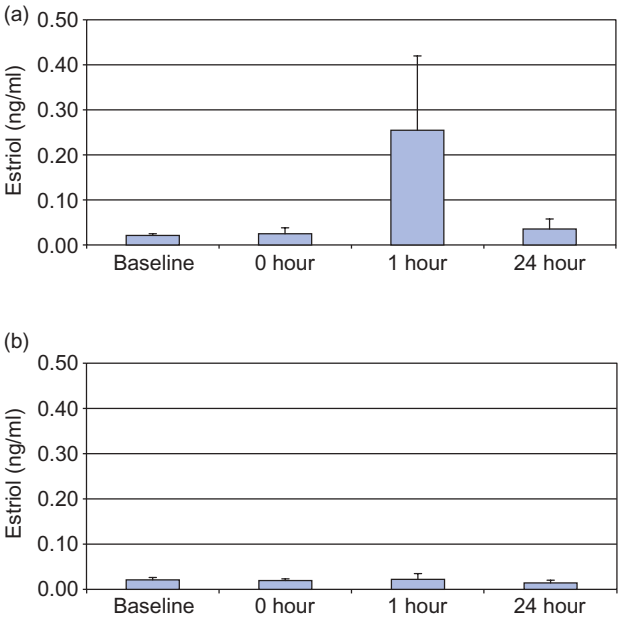


**Fig. 1. Average serum estriol for the animals receiving the highest estriol dose [group 4 (a)] and the control group (b).** Baseline estriol levels were measured from serum sampled before the initiation of the dosing study. Serum was also drawn just before each dose (0 h) and 1 and 24 h after each dose.

The estrogenic effect of estriol treatment on the vaginal epithelium was examined in two ways: biopsy and colposcopy. In prior studies, it was established that estradiol therapy increased the thickness and number of cell layers in the vaginal epithelium. This effect was also observed here at each tested estriol dose. The vaginal biopsy results correlated strongly with the colposcopy findings in that a thick vaginal epithelium had a characteristic cornified appearance and a thin epithelium was very smooth (data not shown). Therefore, colposcopy was used to assess the estrogenic effect of estriol therapy. Representative colposcopy findings are shown in Fig. 2. There was some inconsistency within the vaginal epithelium in some animals. In our earlier studies using systemic hormone treatment, the vagina epithelia were uniform and were either thick or thin throughout a given subject. With the topical therapy used in these preliminary dosing studies, some animals had areas of the vaginal epithelia that indicated an estrogenic effect and other patches that were smooth. It is suspected that this was secondary to incomplete exposure of the vaginal epithelium to the estriol cream. To overcome the problem in the challenge study, the volume of estriol cream was increased to 2 ml, which produced much more consistent changes.

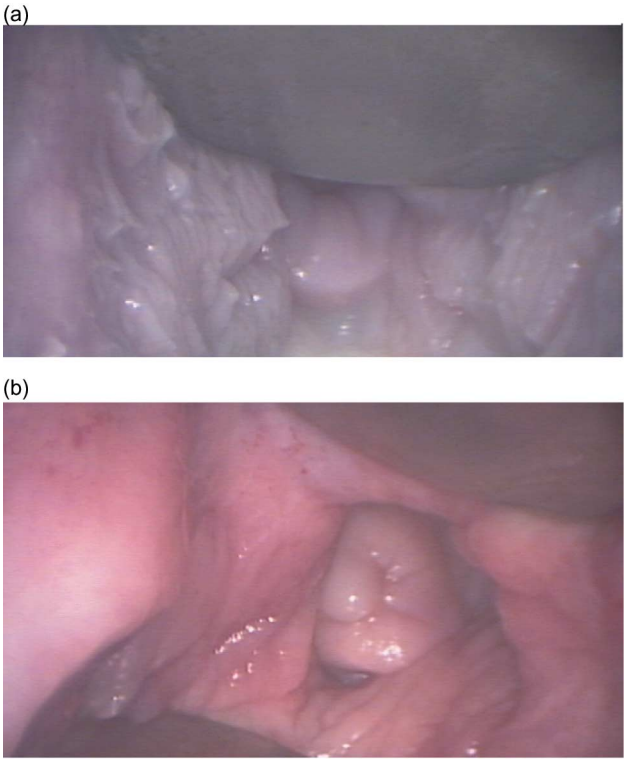


**Fig. 2. Colposcopic images of the vaginal epithelia.** The vaginal epithelia of an animal on estriol therapy (a) and an animal with base cream alone (b). Estriol therapy causes significant cornification and maturation of the epithelium. In the absence of natural or therapeutic estrogen, the macaque vaginal epithelium is smooth and pink (b). Images were taken via a colposcope and are shown at 1×. The speculum is seen in the upper right of both color images.

## Challenge study

The highest dose tested (1 mg/ml of estriol given twice per week) in the dosing study had no systemic effect and so was used as the treatment dose in the challenge study. Six animals from the previous study were used and 14 new, adult rhesus female macaques underwent ovariectomy. The animals were randomly assigned to the treatment group (12) and the placebo group (8). The appropriate cream (estriol or placebo) was given to each animal, as before, twice weekly for 5 weeks. Treatment was initiated after a 4–5 week recovery period. Colposcopy was performed at several time points (Table 1). The average LH levels during the treatment period were $151 \pm 86$ and $165 \pm 65$ ng/ml for the treatment and control groups, respectively. This difference was not statistically significant ($P = 0.16$). No estriol was detectable in the control group, as expected. In the treatment group, estriol levels fell during the course of therapy (Fig. 3). This suggested that thickening of the vaginal epithelium in response to estriol therapy resulted in a reduction in estriol absorption and, consequently, serum concentrations fell. Colposcopy revealed that the vaginal epithelium matured and cornified extensively in each of the treated animals (Table 1). Of interest, the effect of the vaginal estriol therapy on the vaginal epithelium persisted for at least 12 days after the last dose. These data show that (i) topical estriol caused maturation and cornification of the rhesus vaginal epithelium; (ii) estriol absorption was limited and caused no detectable systemic effects; and (iii) the effect of estriol treatment on the vaginal epithelium persists for $>1$ week after its cessation.

After 5 weeks of therapy, each animal was intravaginally challenged with 640 $TCID_{50}$ $SIV_{mac251}$. Infection status was determined by serially measuring plasma SIV RNA, CD4 T cell concentration and anti-SIV antibodies. By 4 weeks after challenge, plasma SIV RNA was detected in six of the eight control group animals, and only in 1 of the 12 estriol-treated animals (Fig. 4). Serological conversion to SIV occurred in most viremic animals, and in none of those without detectable viremia. CD4 T cell concentrations fell in some, but not all, of the viremic animals, and it remained stable in all those without viremia. Animals T687 and M830 of the treatment group had one low-level positive bDNA measurement each. Since seroconversion did not occur in either animal (negative Western blots at days 56 and 70) and both had stable CD4 T cell concentrations, both readings were considered to be false positives, which have been observed before [11].

In summary, six of the eight control group animals

**Table 1. Vaginal epithelial appearance via colposcopy.**

| Animal[a] | Epithelial appearance[b] | | | | |
|---|---|---|---|---|---|
| | Day 6 | Day 8 | Day 22 | Day 38[c] | Day 50[d] |
| Treatment group (1.0 mg/ml estriol) | | | | | |
| L448 | – | – | +++ | +++ | +++ |
| N308 | – | – | +++ | +++ | +++ |
| AV87 | – | – | ++ | +++ | ++ |
| BA20 | + | ++ | +++ | +++ | +++ |
| M830 | – | – | +++ | +++ | +++ |
| L441 | – | + | + | ++ | ++ |
| T590 | – | – | +++ | +++ | +++ |
| **R334** | – | + | +++ | +++ | +++ |
| T687 | – | – | +++ | +++ | +++ |
| AV89 | – | – | +++ | +++ | +++ |
| R037 | – | – | +++ | +++ | +++ |
| J301 | – | – | +++ | +++ | ++ |
| Control group | | | | | |
| R908 | – | – | – | – | – |
| **BA19** | – | – | – | – | – |
| **T369** | – | – | – | – | – |
| **P137** | – | – | – | – | – |
| R534 | – | – | – | – | – |
| **N998** | – | – | – | – | – |
| AJ79 | – | – | – | – | – |
| **N574** | – | – | – | – | – |

[a]Infected animals are in bold type.
[b]Colposcopy grading scale: –, smooth or mildly folded epithelial tissue (Fig. 2b); +, mild folds and thickening, rarely uniform; ++, moderate thickening, mostly uniform, though some patches of smooth tissue may still be present; +++, heavy uniform thickening (Fig. 2a).
[c]Day 38 was 2 days after intravaginal challenge.
[d]Day 50 was 12 days after the last dose of intravaginal estriol.

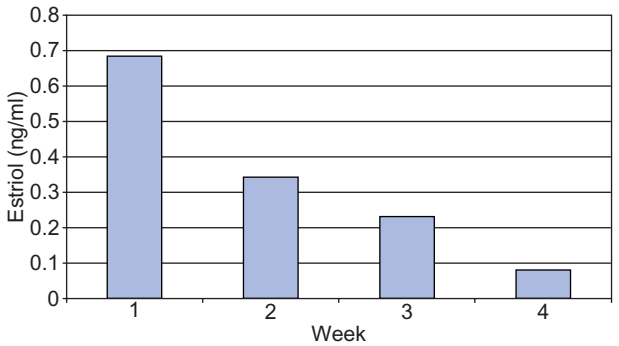


**Fig. 3. Average estriol absorption during therapy.** The average estriol levels of the treatment group decreased during the challenge study. Serum was drawn 1 h after each estriol dose and the average level is shown for each week of the therapy. $P = 0.0003$ for week 1 compared with Week 4; $P = 0.004$ for week 2 compared with week 4.

were infected after a single challenge with $SIV_{mac251}$ but only 1 of the 12 treatment group animals was infected. This difference was highly statistically significant ($P = 0.0044$, Fisher's exact test). On day 21 after infection, heteroduplex mobility analysis of results showed no significant difference in viral homogeneity between the one estriol-treated infected animal and the placebo-treated infected animals.

## Discussion

This study extends our previous studies to examine topical estrogen treatment. In ovariectomized rhesus macaques, topical estrogen protected against SIV vaginal transmission through its effects on the vaginal epithelium. More importantly, this protection was achieved without detectable systemic estrogenic effect. Estriol was used because it is a weaker estrogen than estradiol and effective in treating atrophic vaginitis. Topical estradiol has systemic effects in postmenopausal women [23]. Serum estriol levels became measurable only in the highest dose tested. However, these serum levels were low and transient and they decreased over time on therapy, suggesting that estriol absorption decreased as the vaginal epithelia cornified. Topical vaginal estriol therapy had no effect on macaque LH levels at either 1 or 24 h after treatment. There was no evidence suggesting that estriol was converted to estradiol. Topical estriol caused maturation and thickening of the vaginal epithelium to the same degree that systemic estradiol had in our previous studies, while the base compound had no effect on the vaginal epithelium. Estriol-treated animals were strongly protected against SIV vaginal transmission (8.3% infection rate) compared with animals treated with base cream alone (75% infection rate). This level of protection is again very similar to that afforded by systemic estradiol [11].

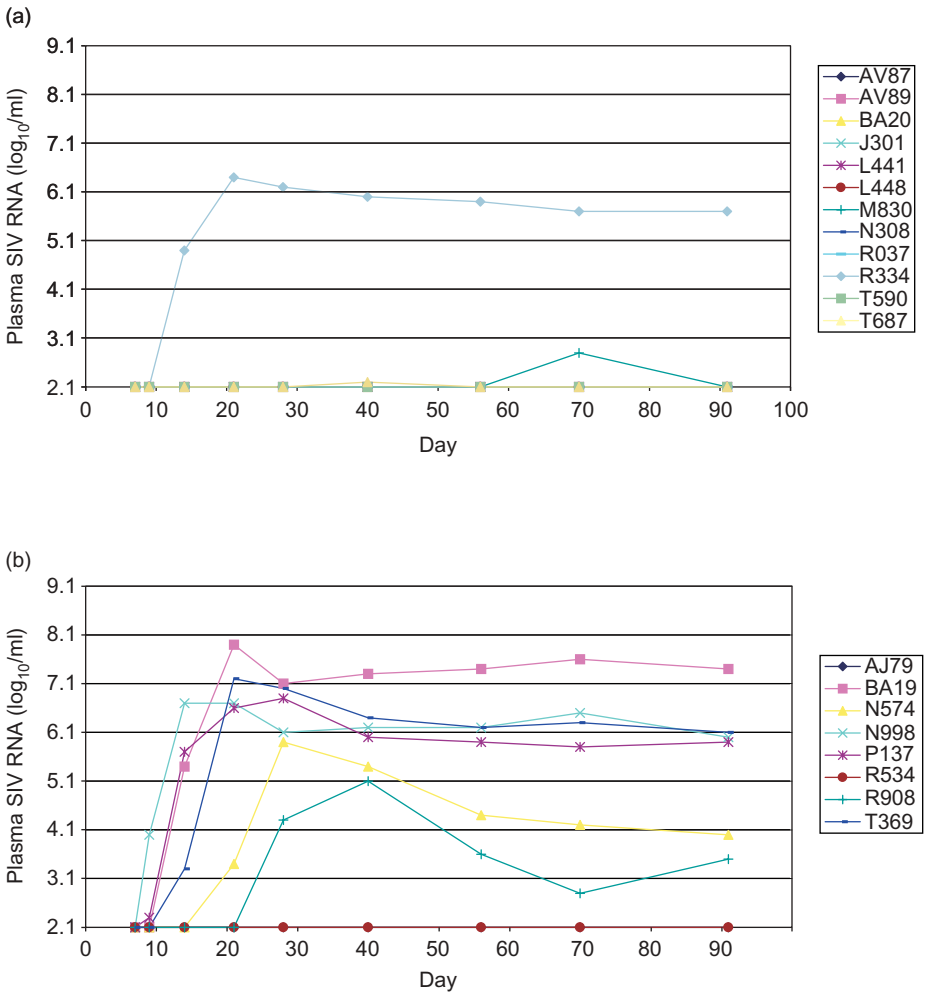


**Fig. 4. Plasma viremia.** SIV plasma viral load is plotted versus day post-challenge for the treatment group (a) and the control group (b) animals.

Recent data in women have strongly suggested that our findings in macaques have relevance to HIV transmission in women. First, women on DMPA have suppressed estradiol levels and also have a two- to three-fold increased rate of HIV infection [3–5]. This finding is similar to our finding that systemic progesterone increased susceptibility to SIV vaginal transmission in macaques that have a menstrual cycle [10]. Second, women over the age of 45 years are at increased risk of acquiring HIV through heterosexual contact [6,7]. In this and a prior study, we have shown that ovariectomized macaques on no therapy are much more susceptible to SIV vaginal transmission and that systemic or topical estrogen therapy greatly reduces that susceptibility. Third, a recent study by Overbaugh and colleagues [3] confirmed that women on oral contraceptive pills or DMPA are at increased risk of infection and that women on DMPA at the time of HIV infection had significantly higher viral loads, a higher rate of having multiple viral variants and more rapid CD4 T cell depletion. In the macaque vaginal transmission model, we too observed that increased viral variation was associated with high viral loads and rapid disease [24]. In summary, the human data and the data derived in the macaque model support the hypothesis that the vaginal epithelium is a natural and important barrier against HIV infection in women and that hormonal alterations of this barrier can either enhance (estrogen) or decrease (progesterone) its protective effects.

Together, the combined record of estriol safety in women, our data, and data on risk factors of HIV vaginal transmission support the use of estriol vaginal cream in women who have low levels of estrogen, to reduce their risk of heterosexual transmission. While many investigators have focused on the development of topical vaginal microbicides, most microbicides have to be used near or at the time(s) of the sexual encounter(s). The effects of topical estriol, by comparison, are

long lasting and were still seen in all animals 12 days after the last dose of estriol. Consequently, estriol topical therapy in hypoestrogenic women who are at risk for HIV infection could be administered two to three times per week and remote from the sexual encounter. This feature should dramatically increase overall adherence and subsequent protection. Topical estriol therapy may also be used in conjunction with an effective microbicide to maximize protection rates.

Most women infected with HIV are premenopausal and have estrogen levels that rise and fall during the menstrual cycle [1]. While no study on women has addressed this issue, it is possible that the majority of HIV vaginal transmission occurs during the luteal phase of menses, when estrogens are lower and progesterone, which acts as an antagonist of estrogen, is high. In a preliminary study on macaques, luteal phase was associated with an increased rate (50%) of vaginal SIV transmission compared with the follicular phase (14%) [12]. In premenopausal women, any estrogen therapy that had systemic effects could be viewed with some concern. We now know that topical, vaginal estriol therapy at 1 mg/ml has no systemic effect and, therefore, could be used in macaques with a menstrual cycle without affecting hypothalamic–pituitary–ovarian interaction. Future studies will examine the protective effect of estriol therapy against SIV vaginal challenge during the luteal phase of menses. If estriol protects in such macaques and has no effect on the endocrine system or ovulation, topical estriol should be studied as a means to reduce the risk of HIV transmission in premenopausal women.

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