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Progesterone implants enhance SIV vaginal transmission and early virus load

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Simian immunodeficiency virus (SIV) can cross the intact vaginal epithelium to establish a systemic infection in macaques (mac). Using this SIVmac model, we found that subcutaneous progesterone implants, which could mimic hormonally based contraceptives, thinned the vaginal epithelium and enhanced SIV vaginal transmission 7.7-fold over that observed in macaques treated with placebo implants and exposed to SIV in the follicular phase of the menstrual cycle. Progesterone treatment also increased the number of SIV DNA-positive cells in the vaginal lamina propria as detected by *in situ* polymerase chain reaction analysis. Moreover, plasma viral RNA was elevated for the first three months in macaques with progesterone implants, and three of the progesterone-treated macaques developed relatively rapid disease courses. This study shows that SIV genital infection and disease course are enhanced by subcutaneous implants containing progesterone when compared with the rate of vaginal transmission in the follicular phase.

Human immunodeficiency virus, the cause of acquired immunodeficiency syndrome (AIDS), is most often transmitted to women by sexual contact. However, little is known about the influence of hormones on HIV genital transmission in the female. Hormonal cofactors may be important because progesterone, a hormone that rises in concentration during the luteal phase of the menstrual cycle and during pregnancy, induces changes in the vaginal epithelium, vaginal pH and cervical mucus of human beings[1]. Moreover, synthetic progesterone-based (progestin) contraceptives are in widespread use, but the effect on HIV transmission has been difficult to document.

Epidemiological studies give conflicting results on the effects of synthetic hormonally based contraceptives on HIV vaginal transmission. Some recent studies have found a range of sharp to no increases in the risk of transmission associated with the use of injectable and oral contraceptives[2-4] (Fontanet, A. *et al.* and Nagachinto, T. *et al.*, presented at the XIth International Conference on AIDS, 7–12 July 1996, Vancouver, BC). To address this question experimentally, we used the SIVmac animal model[5] to test if circulating progesterone, a natural hormone, affected vaginal transmission of simian immunodeficiency virus.

The SIVmac model is useful for testing potential biological cofactors because atraumatic vaginal inoculation of SIVmac results in systemic infection and AIDS (ref. 5) in this species. Various studies have previously shown that the vaginal epithelium plays a role in transmission of SIVmac. For example, the intact vaginal mucosa represents a barrier to SIVmac because about 100 to 1000 times more virus is required to establish infection in most animals by this route, compared with that required to establish infection by intravenous inoculation[5-7]. The rectal mucosa represents a similar barrier to cell-free virus[8]. A study of hysterectomized macaques with subsequent vaginal exposure to SIVmac proved that the vaginal mucosa alone is sufficient for transmission[9]. A similar observation of HIV infection was made in a woman with a congenital absence of the cervix[10]. Therefore, the vaginal epithelium of both human beings and macaques is susceptible to infection by primate lentiviruses[5,10]. Recent *in situ* polymerase chain reaction (PCR) studies identified dendritic cells in the vaginal lamina propria immediately subjacent to the epithelium as the earliest target cells for SIV infection[11]. Moreover, SIV-infected cells were found in the draining lymph nodes during the first week of infection. Thus, the vaginal epithelium is an initial

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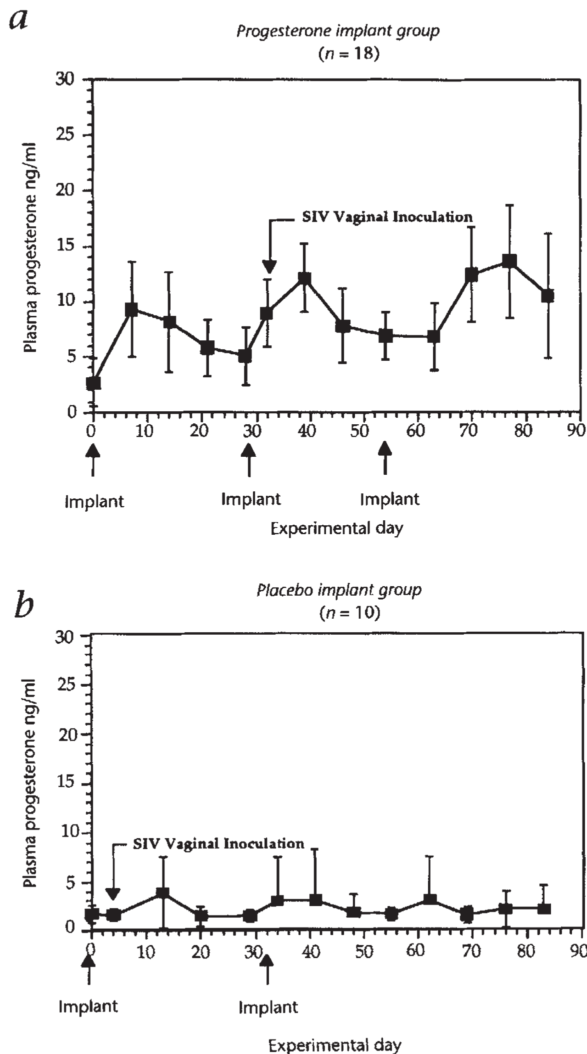

Fig. 1 Progesterone levels (means ± s.d.) in 18 macaques receiving implants containing 200 mg of progesterone (*a*) and 10 macaques with placebo implants containing filler only (*b*). Placebo pellets were implanted on the first or second day after the start of menses. The time of SIV vaginal inoculation is shown. Luteal phase progesterone levels can vary from about 2 to 10 ng/ml (ref. 14, 15). Plasma was separated from heparinized whole blood and stored at −70 °C until tested. Progesterone levels in plasma were determined in the Coat-A-Count progesterone kit (see the Methods section).

barrier that must be traversed before the occurrence of systemic infection. Because endogenous progesterone is known to decrease the thickness of the vaginal epithelium in women[12] and rhesus macaques[13], we tested the hypothesis that exogenously administered progesterone can enhance SIV vaginal transmission.

Protocol

Adult female rhesus macaques (*Macaca mulatta*) were divided into a treatment group ($n = 18$) and a control group ($n = 10$). Pellets containing 200 mg of progesterone were implanted subcutaneously in the intrascapular region of treated macaques every 30 days for a total of 90 days in order to maintain peripheral blood progesterone levels within the range observed in the spontaneous luteal phase of rhesus monkeys[14,15]. Placebo pellets were implanted on the first or second day of menses in the other ten macaques as a control group. All macaques were inoculated vaginally without trauma with 1 ml of virus stock containing about 640 $TCID_{50}$ (mean tissue culture infectious dose). The selection of this relatively low inoculation dose as compared with that used in our previous studies[5,6] was intentional, so that a low incidence of infection would be expected in control animals, thus permitting us to test for an enhancement effect in the progesterone-treated group. Therefore, to test the hypothesis that progesterone enhances vaginal transmission, the placebo group was inoculated 7–9 days after the onset of menses, during the follicular phase of the menstrual cycle, when endogenous progesterone levels are near their lowest[14,15] to contrast with the progesterone-treated group. The progesterone-treated group was inoculated with the same dose and stock of SIVmac 5 weeks after commencing progesterone treatment. The progesterone levels that were achieved in plasma are shown in Fig. 1. The levels in the progesterone-treated group (Fig. 1*a*) were significantly higher than in the placebo group (Fig. 1*b*), and values were within the range or slightly above that expected for macaques during the normal menstrual cycle[14,15].

Progesterone levels, SIV infection and AIDS

The results of this vaginal challenge with SIVmac251 are shown in Table 1. On the day of inoculation, average circulating progesterone levels were low in the placebo group (1.6 ± 0.7 ng/ml) and higher in the progesterone group (9.0 ± 3.0 ng/ml). SIV was subsequently isolated at multiple time points from peripheral blood mononuclear cells (PBMCs) of only 1 of 10 placebo-treated animals as compared with 14 of 18 progesterone-treated macaques. SIV infection was confirmed by anti-SIV antibody assays (Table 1) and by PCR amplification of V1-V2 DNA sequences of SIV *env* (ref. 11) from PBMCs obtained 9 or 14 days postinoculation (Fig. 2*a*). The differences in the incidence of infection between the two groups was highly significant by Fisher's exact one-tailed test ($P < 0.0008$).

In the progesterone group, three macaques rapidly progressed to AIDS and were killed 38, 82 and 177 days postinoculation, respectively (Table 1). Each animal had lesions consistent with simian AIDS due to SIV infection, including lymphoid depletion, thymic atrophy and reactive systemic amyloidosis. In addition, two of the three animals (1448 and 1450) had multi-nucleated giant cell disease involving the lungs (SIV pneumonia) and brain (SIV encephalitis) and lymphoid tissues. The onset of signs of illness was more rapid than previously seen in macaques intravaginally inoculated with the same dose and stock (P.A.M., unpublished data). In the placebo group, the only SIV-infected animal remains clinically healthy, positive for antibody to SIV, and intermittently positive for virus. The 11 surviving SIV-infected macaques in the progesterone group remain SIV positive at 30 weeks postinoculation (Table 1). By using a branched DNA (bDNA) assay for quantification of SIV RNA, plasma virus load was found to be higher during the first three months of infection in all 14 progesterone-treated-infected macaques than in the one placebo-treated-infected macaque.

From previous experiments two additional SIV-infected macaques were identified retrospectively that had been exposed to the same minimal vaginal dose (640 $TCID_{50}$) during the follicular phase of their menstrual cycles as was the placebo group. Plasma from these two macaques had been frozen at −70 °C and was used for plasma RNA quantification. These macaques infected during the follicular phase also had virus loads lower than the 14 macaques infected during treatment with progesterone (Fig. 2*b*). Based on the high virus loads and the more rapid progression to AIDS, we conclude that progesterone treat-

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ment resulted in greater *in vivo* SIV replication during the early period of infection.

Changes associated with enhanced transmission of SIV

To address possible mechanisms of enhanced transmission and pathogenesis, we performed a second experiment. We implanted placebo pellets in three female rhesus macaques and progesterone pellets in three others as before. Macaques from each group were vaginally inoculated with SIV and killed 3 or 4 days later. Plasma, PBMCs, vaginal tissues, draining lymph nodes and other lymphoid tissues were examined for SIV by *in situ* PCR (ref. 11) and virus culture[6]. The structure of the entire length of the vagina and cervix from each treated and control macaque was examined by light microscopy and compared. Table 2 shows that progesterone levels fell within the range of the previous experiment (Table 1 and Fig. 1). The thickness of the stratified squamous epithelium of the vagina was measured by light microscopy and scored with a three-tier system on the basis of the number of cells that predominate in the thinnest areas. Grade 1 epithelium was 2 to 9 cells thick, grade 2 was 10 to 25 cells thick and grade 3 was ≥25 cells thick. Vaginal tissues were fixed and processed in parallel and read in a blinded fashion. All three placebo animals had thick, grade 3 vaginal epithelia. Two of three macaques (946 and 1230) in the progesterone group had uniformly thin vaginal epithelia (grade 1), and the third animal had vaginal epithelium that varied between grades 1 and 2. The two progesterone-treated macaques with grade 1 vaginal epithelium were positive by SIV isolation from plasma, PBMCs, spleen or draining lymph nodes on the day of necropsy (Table 2). From the progesterone-treated macaque with areas of grades 1 and 2 vaginal epithelium (1392), no virus was isolated from any tissues, including the internal iliac lymph nodes obtained at necropsy (Table 2). No virus was isolated from any tissues of the three placebo-treated macaques.

To assess for enhancement of the initial step of vaginal infection, we counted the number of SIV DNA-containing cells in a total of 381 low-power microscopic fields of the lamina propria immediately adjacent to the vaginal epithelium taken on the day

Table 1 SIV vaginal transmission in macaques with progesterone or placebo implants

Macaque	SIV isolation from blood mononuclear cells[a]					ELISA antibody[b]	Progesterone (ng/ml[c])
Follicular phase placebo implant	Weeks after vaginal challenge						
	1	2	3	4	34		
1234	–	–	–	–	–	–	1.7
1394	–	–	–	–	–	–	1.0
1430	–	–	–	–	–	–	1.3
1440	–	–	–	–	–	–	2.1
1444	–	–	–	–	–	–	1.3
1452	–	–	–	–	–	–	0.7
1456	–	+	+	+	–[d]	+	1.6
1458	–	–	–	–	–	–	1.4
1464	–	–	–	–	–	–	2.9
1466	–	–	–	–	–	–	1.5
						Mean ± s.d.	1.6 ± 0.7
Progesterone implant	Weeks after vaginal challenge						
	1	2	3	4	30		
1240	–	–	–	–	–	–	12.2
1388	–	+	+	+	+	+	10.1
1390	–	+	+	+	+ Dead[e] (350)	+	2.2[f]
1396	–	+	+	+	+	+	10.0
1398	–	–	–	–	–	–	6.8
1400	+	+	+	+	+	+	7.7
1402	–	–	–	–	–	–	4.3
1426	–	–	–	–	–	–	9.2
1428	+	+	+	+	+	+	8.5
1432	+	+	+	+	+	+	7.1
1434	+	+	+	+	+ Dead[e] (38)	+	10.7
1436	–	+	+	+	+	+	10.8
1438	+	+	+	+	+	+	15.4
1442	+	+	+	+	+	+	8.9
1446	–	+	+	+	+	+	8.8
1448	+	+	+	+	+ Dead[e] (84)	+ to –	12.7
1450	+	+	+	+	+ Dead[e] (177)	+	14.3
1454	+	+	+	+	+	+	8.3
						Mean ± s.d.	9.0 ± 3.0

[a]SIV was isolated from blood mononuclear cells of vaginally exposed macaques, as previously described[6]. Mononuclear cells (2–3 × 10^7) from macaques with progesterone or placebo treatment were cocultured with 5 × 10^6 CEM ×174 indicator cells. Culture supernatants were tested weekly for p27 antigen, as described previously[6]. Negative cultures were tested for eight consecutive weeks before being discarded.
[b]Assay by ELISA for SIV antibody was performed on plasma on the day of vaginal inoculation, monthly for 3 months and at 8 months postinoculation as reported[6]. All infected macaques were positive for SIV antibody by month 1 or 2 and remained so except for 1448, a rapid progressor to AIDS, which was antibody positive by day 60, but was negative on the day of death.
[c]Plasma progesterone levels on day of inoculation
[d]In week 44, macaque 1456 was retested and was negative for SIV by culture. This macaque remains healthy and SIV antibody positive.
[e]Day of killing is in parentheses.
[f]Progesterone levels from 1390 were high throughout pellet treatment but dipped on the day of vaginal challenge (data not shown).

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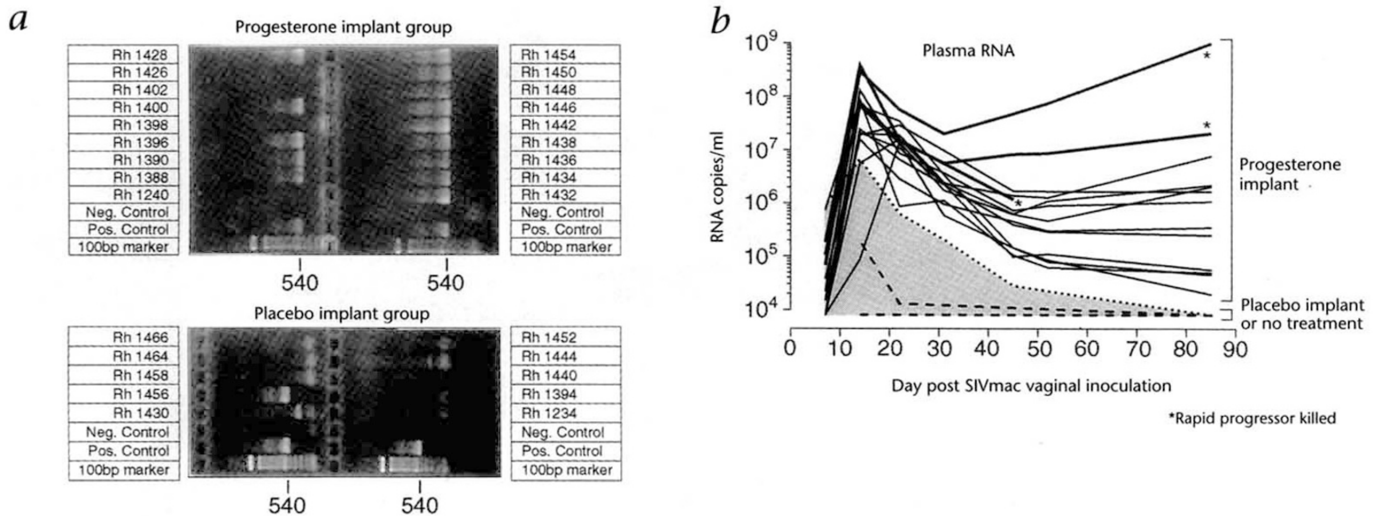

Fig. 2 SIV proviral DNA and plasma RNA in progesterone- and placebo-implanted macaques during the first 90 days after atraumatic vaginal exposure to SIVmac. *a*, The 540-bp *env* DNA fragments were amplified from plasma as reported[11] from each macaque in which SIV was isolated from PBMCs, but not from SIV-exposed macaques that were negative for SIV isolation in Table 1. The SIV DNA was amplified from 1 of 10 placebo-treated macaques (1456) as compared with 14 of 18 progesterone-implanted macaques. *b*, All 14 SIV-infected macaques in the progesterone group (solid lines) had a higher virus load in plasma as measured by the bDNA assay throughout the first 90 days as compared with SIV-positive macaques exposed during the follicular phase of the menstrual cycle (shaded area and dotted lines). Asterisks denote three macaques killed with simian AIDS within 6 months of infection.

of necropsy (Table 2). The mean number of SIV DNA-positive cells per low-power field was 14.1 (range 5 to 22) in the progesterone group and 2.1 (range 1 to 3) in the placebo group (Table 2). Representative results from two macaques, one from each treatment group, killed on day 3 are shown in Fig. 3. The vaginal epithelium from macaque 946 was grade 1, with as few as two to three epithelial cells separating the lumen of the vagina from the lamina propria (Fig. 3*a*). In marked contrast, the vaginal epithelium from macaque 1204 in the placebo group was grade 3, with ≥25 cells in thickness (Fig. 3*d*). Macaque 946 had numerous SIV DNA-positive cells, including a positive cell in the vaginal epithelium (Fig. 3*b*), as compared with a typical field from macaque 1204 (placebo group), showing only two positive cells (Fig. 3*e*). Many of the positive cells showed dendritic cell form and structure as previously reported[11]. Numerous MHC-II DR-positive cells were present in the vaginal mucosa of both macaques (Fig. 3, *c* and *f*). Therefore, it appears that ample targets were present in both groups and that progesterone did not induce observable changes in the number of susceptible target cells. Collectively, these findings point to the thinning of the vaginal epithelium as being an important component for the enhanced vaginal transmission of SIV. However, other progesterone-induced mechanisms may come into play, including other changes in the cervical or vaginal epithelium that were undetected, changes in the immune system and possibly even direct enhancement of viral replication.

Discussion

Whether steroid hormones affect transmission of HIV has been an important question that has been difficult to answer in human studies. Both estrogen and progesterone have effects on the female genital tract that may affect HIV transmission. Progesterone thins the vaginal epithelium, increases the vaginal pH, reduces the amount of cervical mucus and increases its viscosity, whereas estrogen produces the opposite effects[1]. To date studies have not distinguished between the consequences of virus exposure in the follicular and luteal phases, and designing such studies in women would be highly problematic. Epidemiological studies regarding oral contraceptive use and HIV transmission are often confounded by the use of more than one contraceptive during the interval studied. Other differences between study groups may also confound interpretation. For example, it has been suggested that women who choose hormonal contraceptives may be less likely to use barrier contraceptives to prevent STD infection, making studies difficult to interpret. Animal models, therefore, are uniquely valuable because they allow for controlled experimental studies when comparable data are unavailable in humans.

In the rhesus macaque model we have shown that progesterone treatment of rhesus macaques results in atrophy of the vaginal epithelium and increased incidence of systemic SIV infection after intravaginal SIV exposure. Compared with the genital tract of progesterone-treated animals, the normal female follicular-phase genital tract represents a barrier to SIV infection. Although not all potential mechanisms for the progesterone-mediated enhancement of transmission were addressed in our study, the correlation between enhanced SIV transmission and the decreased thickness of the vaginal epithelium was striking. A thin vaginal epithelium may allow more virions to move through the epithelium, or susceptible target cells such as Langerhans dendritic cells may be more exposed in a thinner epithelium, potentiating their infection and spread of virus into the lamina propria to T cells[9,16].

The finding that progesterone enhances infectivity of a sexually transmitted virus is not without precedent. Mice in diestrus, when the vagina is atropic, are more susceptible to vaginal herpes simplex virus type 2 (HSV-2) infection than those in estrous when the vagina is thick[17]. Similarly, oophorectomized mice are susceptible to vaginal HSV-2 inoculation, whereas treatment with estradiol increases their resistance to infection. Finally, Depo-Provera, an injected synthetic progesterone-based contraceptive, enhanced HSV-2 transmission in mice compared with Depo-Estradiol, which blocked transmission[18].

Although the SIV/rhesus macaque model is relevant to HIV transmission and pathogenesis[5], it is not known how precisely

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Table 2 Correlation between progesterone plasma levels, thickness of vaginal epithelium and SIV infection at 3 and 4 days after vaginal exposure

Follicular placebo implant					Progesterone implant				
Macaque	Progesterone level[a]	Vaginal epithelial thickness[b]	Virus isolation	SIV DNA cells/field (total fields)[c]	Macaque	Progesterone level[a]	Vaginal epithelial thickness[b]	Virus isolation	SIV DNA cells/field (total fields)[c]
1204	1.4	≥25 cells (grade 3)	negative	2.8 (232)	946	13.8	<10 cells (grade 1)	PBMCs plasma iliac node	14 (16)
1216	1.1	≥25 cells (grade 3)	negative	2.2 (50)	1230	14.6	<10 cells (grade 1)	PBMCs spleen iliac node	21.2 (46)
1208	1.2	≥25 cells (grade 3)	negative	1.4 (11)	1392	13.8	<10–24 cells (grade 1 and 2)	negative	7.2 (26)

[a]Progesterone levels (nanograms/milliliter) at the time of inoculation were done as for those shown in Fig. 1.
[b]The thickness of the vaginal stratified squamous epithelium was measured by light microscopy and scored with a three-tier system, based on the number of cells predominating in the thinnest area. The number of cells at the thinnest part of the vaginal epithelium was determined by observing the epithelial thickness along the entire length of the vaginal stratified squamous epithelium. Grade 1, 2–9 cells thick; grade 2, 10–24 cells thick; grade 3, ≥25 cells thick.
[c]The number of SIV DNA-positive cells was counted in a total of 381 low-power fields. One low-power field was 1.5 mm in diameter. Multiple tissue sections were examined for animals 1204, 1216, 1230 and 1392; one tissue section each was examined for 1208 and 946.

the minimally infectious dose mimics semen-mediated transmission of HIV to women. The amount of HIV that is in semen has not been extensively studied; however, some studies have shown that $>10^4$ genomes per milliliter of semen are detected by reverse transcriptase PCR (RT-PCR)[19]. More recent studies have shown that up to about 100 infectious units per milliliter of semen are found in infected men (D. Anderson, personal communication). However, this estimate may be low because of the difficulties of culturing semen. Therefore, a minimally infectious macaque vaginal dose of about 640 $TCID_{50}$ is not an exceedingly large dose of SIV when compared with HIV in several milliliters of human semen, according to recent studies. The effects of seminal plasma on vaginal transmission were not addressed in this study.

The finding that exogenous progesterone increased the efficiency of vaginal SIV transmission suggests that women may be at greater risk of HIV infection from vaginal intercourse when exposed to high levels of endogenous or exogenous progesterone. However, the magnitude of this potential risk in women cannot be predicted by this model. The results of these macaque studies point to a need for two types of studies in humans. The first is to test the effect of progestins on vaginal epithelium. The second is to conduct large prospective and case-control epidemiological studies to define whether or not synthetic progestins have any effect on HIV transmission in women.

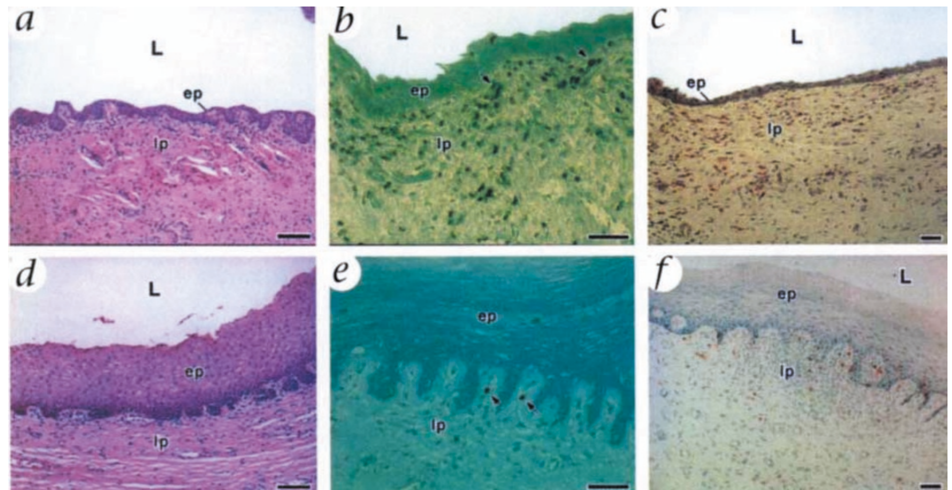

Fig. 3 Effect of progesterone implants on vaginal epithelial thickness, the number of SIV DNA-positive cells and MHC class II DR-positive cells in the lamina propria 3 days postinoculation. L, lumen of the vagina; ep, epithelium, lp, lamina propria. Vaginal epithelia of progesterone-implanted macaque 946 (*a*) and placebo-implanted macaque 1204 (*d*) were stained with hematoxylin and eosin. Marked thinning of the vaginal epithelium was found in macaque 946 as compared with the normal follicular phase vaginal epithelium in *d*. Upon *in situ* PCR for SIVmac *gag* DNA, macaque 946 showed a relatively high number of cells positive for SIV DNA [two such areas are marked by arrows, all of which were adjacent to the epithelium (*b*)]. Many of the infected cells had a form and structure consistent with that of dendritic cells[11]. In contrast, macaque 1204 had only two SIV DNA-positive cells (arrows) (*e*). Immunohistochemistry for DR antigen-bearing cells in the lamina propria revealed that both macaque 946 (*c*) and 1204 (*f*) had high numbers of MHC-bearing cells in that area. Scale bar, 100 μm.

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Methods

Macaques. Protocols for animals (adult female macaques, 6–8 kg) used in this study were reviewed and approved by the LEMSIP institutional animal care and use committee. For all procedures, animals were lightly anesthetized with 10 mg/kg ketamine HCl. All macaques were experimentally naive and had no prior exposure to SIV or type D retrovirus, and all were negative for SIV and type D retrovirus antibody before the start of experiments.

Subcutaneous implants. Macaques were implanted subcutaneously on the dorsum between the scapulas with 90-day-release pellets containing either 200 mg progesterone or pellet filler (Innovative Research of America, Toledo, Ohio). Our preliminary studies with these pellets showed that progesterone levels fell significantly after 30 days, requiring a second and third implant at 30 and 60 days of the experiment adjacent to the site of the initial implant.

SIV vaginal titrations and inoculations. The parental stock was SIVmac251 (3.2×10^3 $TCID_{50}$) and was prepared as described[6]. Preliminary studies were conducted to establish the minimally infectious dose of the SIVmac251 stock by vaginal exposure. Undiluted stock and 1:5 and 1:10 dilutions were tested by vaginal inoculation at random during the menstrual cycle, except that no inoculations were done during the period of menses. It was shown that a 1:5 dilution, 640 $TCID_{50}$, infected less than half of the macaques (4 out of 11) when inoculated at random during the menstrual cycle. Another study showed that 640 $TCID_{50}$ infected only one of seven macaques when all were exposed in the follicular phase of their menstrual cycle, when endogenous levels of progesterone were relatively low. Therefore, the stock was diluted 1:5 in medium and placed atraumatically into the vagina of all 34 macaques in these studies (Tables 1 and 2) with an 8 French pediatric feeding tube attached to a syringe barrel. Macaques were maintained in immobilized state, with the perineum slightly elevated, for approximately 15 min after vaginal exposure to maintain exposure of the inoculum to the vaginal tissue.

Enzyme-linked immunosorbent assay. SIV antibody assays were performed by ELISA on plasma on the day of vaginal inoculation, monthly for 3 months and at 8 months postinoculation as reported[6].

SIVmac quantitative genome assay. Quantitative assays for the measurement of SIV RNA were performed at Chiron Corp. (Emeryville, California) using a branched DNA (bDNA) signal amplification assay for SIV similar to the Quantiplex HIV-RNA assay previously reported[20]. In the SIV bDNA assay, target probes were designed to hybridize with the *pol* region of SIVmac251 and SIVmac239. SIV RNA in plasma samples were quantified by comparison with purified and *in vitro* transcribed SIVmac *pol* RNA method as described[21]. The lower quantification limit of this assay was 10,000 SIV RNA equivalents per milliliter. SIV RNA associated with viral particles was measured in material pelleted from 1 ml heparinized plasma samples (23,500*g* for 1 h at 4 °C).

Progesterone assay. Plasma was separated from heparinized whole blood and stored at −70 °C until tested. Progesterone levels in plasma were determined in the Coat-A-Count progesterone kit (DPC, Los Angeles, California) as recommended by the manufacturer. Assays were conducted at BioQual, Inc. (Rockville, Maryland).

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