Intravaginal Inoculation of Rhesus Macaques with Cell-Free Simian Immunodeficiency Virus Results in Persistent or Transient Viremia

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The simian immunodeficiency virus (SIV)-rhesus macaque model of heterosexual human immunodeficiency virus transmission consists of atraumatic application of cell-free SIVmac onto the intact vaginal mucosa of mature female rhesus macaques. This procedure results in systemic infection, and eventually infected animals develop the clinical signs and pathologic changes of simian AIDS. To achieve 100% transmission with the virus stocks used to date, multiple intravaginal inoculations are required. The current titration study utilized two stocks of SIVmac and demonstrated that a single intravaginal dose of cell-free SIV can reliably produce infection in rhesus macaques. This study also demonstrated that some animals intravaginally inoculated with cell-free SIVmac develop transient viremia characterized by a limited ability to isolate virus from peripheral blood mononuclear cells and lymph node mononuclear cells and no seroconversion to SIV antigen. SIV could be isolated from the peripheral lymph nodes of transiently viremic animals only during periods of viremia and not at times when SIV was not detected in circulating mononuclear cells. Thus, peripheral lymphoid tissues were not reservoirs of infection in the transiently viremic animals. Taken together, these results suggest either that the SIV infection was cleared in the transiently viremic animals or that SIV infection is limited to a compartment of the genital mucosal immune system that cannot be assessed by monitoring SIV infection in peripheral blood mononuclear cells and peripheral lymphoid tissue.

The simian immunodeficiency virus (SIV)-rhesus macaque model of heterosexual human immunodeficiency virus (HIV) transmission (18, 20) has been used to explore the mechanisms of mucosal HIV transmission (21–23) and to test the ability of vaccines to prevent vaginal transmission of SIV (14, 34). The model consists of atraumatic application of cell-free SIVmac onto the intact vaginal mucosa of mature female rhesus macaques (20). This procedure results in systemic infection, and in the initial studies, infected animals eventually developed the clinical signs and pathologic changes characteristic of simian AIDS (SAIDS) (20). When a well-characterized SIVmac virus stock is used, a single intravaginal application of a virus inoculum of 10^3 50% tissue culture-infective doses (TCID₅₀) results in infection of approximately 67% of inoculated animals (19). To achieve 100% transmission with this virus stock, multiple intravaginal inoculations are required (19). Challenge studies designed to test the ability of vaccines or topical microbicides to prevent vaginal transmission of SIV could be greatly simplified if a single 100% infectious intravaginal dose of cell-free SIV were available. An objective of the current study was to identify a dose of cell-free SIVmac capable of consistently infecting animals following a single intravaginal inoculation. A number of cofactors to sexual HIV transmission have been identified through epidemiologic studies, including vaginal irritation produced by nonoxynol-9-

containing spermicides (2). Thus, we also sought to identify a dose of SIV which did not produce infection in animals inoculated intravaginally. This dose could then be used to test the ability of cofactors or chemical irritants to enhance vaginal transmission of SIV.

Cell-free HIV and cell-associated HIV are rapidly inactivated in vitro at acidic pHs (26). Cell-free SIV is also inactivated in vitro at low pHs (18a). In women, the vaginal pH is highly variable among individuals and can vary throughout the menstrual cycle. A recent study (24) found that in sexually active, fertile women without gynecological abnormalities the vaginal pHs ranged from 3.4 to 6.9. The vaginal pHs were highest (6.0 to 7.0) during menstruation and decreased during the intermenstrual period, with no uniform pattern. In some women, pH values throughout the menstrual cycle remained fairly constant and in others the vaginal pH dropped by as much as 2.1 pH units in the intermenstrual period. The pH of the rhesus macaque vagina is also low, although it is generally somewhat less acidic than the pH of the human vagina (17).

The pH of seminal plasma ranges from 7 to 8, and after ejaculation the pH of the human vagina rises to 7.0 or higher (8). Following infusion of 0.25 ml of human seminal plasma, the acidic pH of the rhesus macaque vagina is also neutralized (18a). Cell-free HIV and SIV can survive for extended periods under these conditions. On the basis of the above observations, the presence of seminal plasma in the vagina at the time of inoculation may lower the dose of SIV required for genital transmission of the virus. This study attempted to determine if addition of seminal plasma to the SIV inoculum could increase the efficiency of vaginal SIV transmission. By using this ap-

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proach, we identified a 100% genital animal infectious dose with a single inoculation of cell-free SIV. In addition, the results of this study indicate that intravaginal inoculation with low doses of SIV can result in transiently viremic and seronegative animals. These observations may provide insight into heterosexual transmission of HIV.

MATERIALS AND METHODS

Animals. All of the animals used in this study were colony-bred, multiparous, female rhesus macaques (*Macaca mulatta*) from the California Regional Primate Research Center. The animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. When necessary, the animals were immobilized with 10 mg of ketamine HCl (Parke-Davis, Morris Plains, N.J.) per kg injected intramuscularly. The investigators adhered to the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Resource Council. Prior to use, the animals were negative for antibodies to HIV-2, SIV, type D retrovirus, and STLV-1.

Viruses. Two different SIV stocks were used in the studies described here. The stock used in study 1 consisted of SIVmac251 grown in human peripheral blood mononuclear cells (huPBMC) and contained 10^3 TCID₅₀/ml. The stock used in study 2 consisted of SIVmac251 grown in rhesus macaque peripheral blood mononuclear cells (rhPBMC) and contained 10^5 TCID₅₀/ml. The SIVmac used to produce both stocks was obtained from Ronald Desrosiers of the New England Primate Research Center. This stock has been shown to cause persistent viremia and eventual disease in rhesus macaques at the New England Primate Research Center (5). The cell-free virus stocks were stored at -135°C without cryopreservatives. Aliquots of the SIV stocks were thawed at 37°C , and appropriate dilutions were made with sterile phosphate-buffered saline and then used immediately for animal inoculations.

Human seminal plasma (huSP). huSP was collected from healthy volunteers after 2 to 3 days of abstinence, and the samples were subjected to routine semen analysis. Physical and biochemical characteristics (pH, cellularity, and viscosity) were within the normal range. The number of round cells never exceeded 10^6 /ml, and semen bacterial cultures were always negative, indicating that the donors were free from accessory gland infections. Semen samples were allowed to liquefy for 30 min at 37°C . Samples from different donors were then pooled and centrifuged at $700 \times g$ for 10 min. The supernatant containing only seminal plasma was carefully removed and immediately frozen. Samples were stored at -70°C until thawed for use. For the inoculations, 0.25 ml of huSP was infused into the vagina of each animal just prior to infusion of the viral inoculum.

Intravaginal inoculation of female rhesus macaques. The inoculation procedure involved placing either cell-free SIV alone or cell-free SIV and huSP into the vaginal vault of each animal. In animals exposed to seminal plasma, huSP was infused through a 2.5-mm-outer-diameter (8 French) soft-plastic pediatric nasogastric-feeding tube (American Pharmaseal, Valencia, Calif.) into the vaginal canal. Within 5 min of the huSP infusion, 1 ml of the SIV inoculum was infused through a pediatric nasogastric feeding tube into the vaginal vault of each immobilized female rhesus macaque. After each inoculation, the animal remained immobile for at least 20 min.

Virus isolation from peripheral blood and lymph nodes. Virus was isolated from heparinized whole blood and lymph nodes obtained from genitally inoculated macaques. Periph-

eral blood mononuclear cells (PBMC) were isolated by Ficoll gradient separation (Lymphocyte Separation Medium; Organon Teknika, West Chester, Pa.) and cocultured with CEMX174 cells (10) (provided by James A. Hoxie, University of Pennsylvania, Philadelphia) as previously described (13). To detect virus in peripheral lymph nodes, axillary and inguinal lymph nodes were obtained by transcutaneous biopsy and aseptically teased into single-cell suspensions of mononuclear cells (LNMC) in RPMI 1640. Except where otherwise noted, 5 to 20 million PBMC or 20 to 80 million LNMC were cocultivated with 2 to 3 million CEMX174 cells; cell numbers were determined with a Neubauer hemocytometer. Aliquots of the culture media were assayed regularly for the presence of the SIV major core protein (p27) by antigen capture enzyme-linked immunosorbent assay (ELISA) (13). Cultures were considered positive if they were antigen positive at two consecutive time points. A detailed description of the technique and criteria used to determine if an aliquot was antigen positive has already been published (16). All cultures were maintained for 8 weeks and tested for SIV p27 by ELISA before being scored as virus negative. Blood samples and lymph node biopsies were collected at the times indicated in Tables 1 and 2. For animals which died during the study, a final blood sample was obtained at necropsy.

Preparation of tissue for PCR amplification. PBMC and LNMC were pelleted, resuspended at 10^7 /ml in PCR lysis buffer (10 mM Tris-HCl [pH 8.3], 0.1% Nonidet P-40, 0.1% Triton X-100, 100 μg of proteinase K [Boehringer Mannheim] per ml). These lysates were incubated for 2 h at 55°C to digest cellular protein, and proteinase K was inactivated by incubation at 95°C for 10 min. Cell lysates were stored at -70°C until used for PCR amplification. Ten to 20 μl of each lysate, equivalent to the DNA from 1×10^5 to 2×10^5 mononuclear cells, was used for PCR amplification.

PCR amplification. Nested PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus, Emeryville, Calif.). Two rounds of 30 cycles of amplification were performed with aliquots of plasmid DNA containing the complete genome of SIVmac1A11 (15) (positive control) or aliquots of crude cell lysates by using SIVmac-specific *gag* primers and conditions described elsewhere (35). DNA from uninfected CEMX174 cells was amplified as a negative control in all assays to monitor potential reagent contamination. This nested PCR amplification procedure allows visual detection of a single copy of the SIV *gag* sequences in as many as 200,000 PBMC (35). PCR amplification (30 cycles) of the β -globin DNA sequences from all PBMC lysates with primers PC03 and KM38 (32) was also performed to quantify total cellular DNA and to detect potential inhibitors of *Taq* polymerase in cell lysates.

Serum antibody responses in inoculated animals. Titers of SIV-specific antibodies in sera, diluted serially fourfold beginning at 1:100, were measured by a whole-virus ELISA as previously described (14). All dilutions of each sample serum and a positive control serum from an SIV-infected animal were assayed in duplicate, and mean optical density values were calculated. Data are presented as the inverse of the highest sample dilution that was greater than three times the mean optical density of serum from each animal sampled prior to inoculation. Immunoblots were also performed to detect specific SIV proteins as previously described (34).

To confirm the results of the whole-virus assays, 16-week-postinoculation (p.i.) sera were tested with a gp120 ELISA. The details of the assay have already been described (25). Briefly, culture supernatant from SIVmac-infected H9 cells was treated with 1% Nonidet P-40 nonionic detergent to provide a source of SIV envelope glycoproteins. These anti-

TABLE 1. Virus isolation from rhesus macaques intravaginally inoculated with SIVmac251 propagated in huPBMC

Animal	Virus inoculum ^a	Result ^b at p.i. wk:								Death (B)		
		1 (B ^c)	2 (B)	4 (B)	8 (B)	10		12–14 (B)	30			
						B	L ^d		B		L	
19175	10 ³ /sp	–		–		+					+	(51) ^e
19716	10 ³ /sp	–	–	–		+					–	(55)
19168	10 ³	–	–	–							–	(92)
20424	10 ³	–	–	+							+	(50)
18998	10 ³	+	+	+	–						+	(40)
20563	10 ³	+	–	–								
19753	10 ³	+	+	+	–						–	(94)
8102	10 ² /sp	+	+	+	+	+			+		+	(37)
18462	10 ² /sp	+	+	+	+	+			–			
19065	10 ² /sp	+	+	+	+	+			+			
7938	10 ² /sp	+	+	+	+	+			–			
17562	10 ² /sp	+	+	+	+	+			–			
18407	10 ² /sp	+	+	+	+	+					+	(23)
24842	10 ² /sp	–	+	+		–	–			–		
24300	10 ²	–	+	–		–	–			–	–	
19147	10 ²	–	+	–		–	–			–	–	
25178	10 ²	–	+	–		–	–			+	–	
22995	10/sp	–	–	–		–	–			+		
24071	10/sp	–	–	+		–	–			+	–	
23676	10/sp	–	–	–		+	–			–	–	
23051	10/sp	–	+	–		–	–			–	–	
17240	10	–	–	–	–	–	–			–	–	
19761	10	–	–	–	–	–	–			–	–	– (34)
23205	10	+	–	+	+	+	–			–	–	
23545	1/sp	+	+	–		+	–			–	–	
23061	1/sp	–	–	–		–	–			–		
24074	1/sp	–	–	–		–	–					– (26)

^a The virus inoculum is the cell-free SIVmac251 inoculum. The virus dose used is expressed in TCID₅₀. If seminal plasma was used for the inoculation procedure, the abbreviation sp follows the TCID₅₀ value.

^b +, on the basis of the criteria described in Materials and Methods, the sample was SIV isolation positive. —, on the basis of the criteria described in Materials and Methods, the sample was SIV isolation negative.

^c B, PBMC.

^d L, LNMC.

^e The values in parentheses are weeks p.i.

gens were captured from a 1:3 dilution of supernatant onto a solid phase by absorbed sheep antibody D7369 (Aalto BioReagents, Dublin, Ireland), which recognizes a peptide sequence at the carboxy terminus of gp120. After removal of unbound envelope glycoproteins by washing, macaque sera (1:300 dilution) were added in TMTSS buffer (Tris-buffered saline [pH 7.5], 2% nonfat milk, 20% sheep serum, 0.5% Tween 20) and bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Accurate Chemicals Inc., Westbury, N.Y.) and AMPAK, an ELISA amplification system (Dako Corp., Carpinteria, Calif.). Each serum was tested in triplicate against D7369-coated wells containing or lacking SIV envelope glycoproteins, and the mean optical density at 492 nm from the control wells was subtracted from that of the test wells to correct for nonspecific antibody absorption. Corrected optical densities at 492 nm of >0.100 are considered significant.

Necropsy collection and preparation of tissue samples. A complete necropsy examination was performed on all animals that died during the course of the study. Tissues collected at necropsy were fixed in 10% buffered formalin, embedded in

paraffin, sectioned at 6 μ m, stained with hematoxylin and eosin, and examined by light microscopy. In addition, tissues were snap-frozen in liquid nitrogen.

RESULTS

Study 1. A total of 27 animals were intravaginally inoculated with the huPBMC SIVmac stock (Table 1). Each animal received a single inoculation of 1 ml of the viral inoculum either with or without huSP. Two of two animals intravaginally inoculated with 1 ml of the undiluted SIVmac stock (10³ TCID₅₀) with huSP became viremic. These two animals were inoculated at the beginning of the study, and only 1 $\times$ 10⁶ to 2 $\times$ 10⁶ PBMC were used for the virus isolation attempts. For both animals, the first PBMC sample from which SIV was isolated was collected at 10 weeks p.i. (Table 1). One animal died with clinical signs and pathologic changes consistent with AIDS at approximately 1 year p.i. (19175 at 51 weeks p.i.). SIV was isolated from the PBMC collected at necropsy, but the animal did not have a detectable serum antibody response to SIV (Table 3). The other animal was healthy at the time it was

TABLE 2. SIV PCR from rhesus macaques intravaginally inoculated with SIVmac251 propagated in huPBMC

Animal	Virus inoculum ^a	Result ^b at p.i. wk:	
		4	10
19175	10 ³ /sp		
19716	10 ³ /sp		
19168	10 ³		
20424	10 ³		
18998	10 ³		
20563	10 ³		
19753	10 ³		
8102	10 ² /sp	+	(B ^c)
18462	10 ² /sp	-	(B)
19065	10 ² /sp	-	(B)
7938	10 ² /sp	-	(B)
17562	10 ² /sp	-	(B)
18407	10 ² /sp	+	(B)
24842	10 ² /sp		
24300	10 ²		- (B)
19147	10 ²		- (B)
25178	10 ²		- (B)
22995	10/sp		- (L ^d)
24071	10/sp		- (L)
23676	10/sp		- (L)
23051	10/sp		- (L)
17240	10		- (B)
19761	10		
23205	10		- (L)
23545	1/sp		- (L)
23061	1/sp		- (L)
24074	1/sp		- (L)

^a See Table 1, footnote a.^b See Table 1, footnote b.^c B, PBMC.^d L, LNMC.

euthanized for experimental reasons unrelated to this study (19716 at 55 weeks p.i.). SIV was not isolated from PBMC collected at necropsy, and the animal did not have serum antibodies against SIV antigens (Table 3).

Four of five animals inoculated intravaginally with 10³ TCID₅₀ of SIVmac without huSP became viremic (Table 1). Two of the infected animals were viremic at the time they died with SAIDS (20424 at 50 weeks p.i.; 18998 at 40 weeks p.i.), while one infected animal (20563) was alive and viremic at 77 weeks p.i. One of the infected animals (19753 at 84 weeks p.i.) was healthy at the time it was euthanized for experimental reasons unrelated to this study, and SIV was not isolated from the PBMC collected at necropsy. One of these four infected animals (20424) had detectable serum antibodies to SIV antigens (Table 3).

Seven of seven animals intravaginally inoculated with 10² TCID₅₀ of SIVmac with huSP became viremic. Six animals were persistently viremic (Table 1), and two of these animals had PCR-positive PBMC at week 4 while the PBMC of the other four were PCR negative at this time (Table 2). One animal (24842) was viremic at 2 and 4 weeks p.i., but its PBMC and LNMC were negative at 10 weeks p.i. (Table 1) and its PBMC were negative at 30 weeks p.i. Two of the persistently

TABLE 3. Serum anti-SIV antibody response in animals intravaginally inoculated with SIVmac251 propagated in huPBMC

Animal	Virus inoculum ^a	Result ^b at p.i. wk:			
		8 (ELISA)	16		
			ELISA	Immunoblot	gp120 ELISA
19175	10 ³ /sp	<1:100	<1:100	—	—
19716	10 ³ /sp	<1:100	<1:100	—	—
19168	10 ³	<1:100	<1:100	—	—
20424	10 ³	1:1,600	1:25,600	+	+
18998	10 ³	<1:100	<1:100	—	—
20563	10 ³	<1:100	<1:100	—	—
19753	10 ³	<1:100	<1:100	—	—
8102	10 ² /sp	1:1,600	>1:6,400	+	+
18462	10 ² /sp	<1:100	<1:100	—	—
19065	10 ² /sp	<1:100	<1:100	—	—
7938	10 ² /sp	<1:100	<1:100	—	—
17562	10 ² /sp	<1:100	<1:100	—	—
18407	10 ² /sp	<1:100	<1:100	—	+
24842	10 ² /sp	<1:100	<1:100	—	—
24300	10 ²	<1:100	<1:100	—	—
19147	10 ²	<1:100	<1:100	—	—
25178	10 ²	<1:100	<1:100	—	—
22995	10/sp	<1:100	<1:100	—	—
24071	10/sp	<1:100	<1:100	—	—
23676	10/sp	<1:100	<1:100	—	—
23051	10/sp	<1:100	<1:100	—	—
17240	10	<1:100	<1:100	—	—
19761	10	<1:100	<1:100	—	—
23205	10	<1:100	<1:100	—	—
23545	1/sp	<1:100	<1:100	—	—
23061	1/sp	<1:100	<1:100	—	—
24074	1/sp	<1:100	<1:100	—	—

^a See Table 1, footnote a.^b See Table 1, footnote b.

viremic animals died of SAIDS (8102 at 37 weeks p.i.; 18407 at 23 weeks p.i.), and SIV was isolated from the terminal PBMC samples of both animals. One of the animals that died of SAIDS (8102) is the only animal in this group that seroconverted to SIV antigens as determined by the whole-virus ELISA and an immunoblot assay, but 18407 had a weak anti-SIV envelope antibody response that was detected only with the gp120 ELISA (Table 3).

Three of three animals intravaginally inoculated with 10² TCID₅₀ of SIVmac without huSP became transiently viremic. SIV was isolated from PBMC of all animals at 2 weeks p.i., but the virus could not be isolated from the PBMC of the animals on later attempts (Table 1). SIV could not be detected by PCR in PBMC at 10 weeks p.i. (Table 2). LNMC from all three animals were also negative at two later time points (Table 1). Anti-SIV antibodies were not detected in the sera of these animals (Table 3).

Transient viremia was detected in all four animals intravaginally inoculated with 10 TCID₅₀ of SIVmac with huSP (Table 1). In these animals, the timing of SIV isolation was variable. SIV was not detected in the PBMC of one animal until 30 weeks p.i. SIV could not be isolated from the LNMC of these animals, although the PBMC of two animals were SIV positive at the time of the lymph node biopsy (23676 at 10 weeks p.i.

and 24071 at 30 weeks p.i.). PCR analysis of the LNMC obtained at 10 weeks p.i. from these animals failed to detect SIV (Table 2). None of these transiently infected animals had detectable SIV-specific serum antibodies.

One of three animals intravaginally inoculated with 10^5 TCID₅₀ of SIVmac without huSP became viremic (Table 1). In this animal (23205), SIV isolation was fairly consistent soon after inoculation but viremia was not detected at 30 weeks p.i. SIV could not be isolated from the LNMC from this animal, although the PBMC of the animal were SIV positive when one lymph node biopsy was obtained (10 weeks p.i.). PCR failed to detect SIV in the LNMC obtained at 10 weeks p.i. (Table 2). This animal did not seroconvert to SIV antigens (Table 3).

Intermittent viremia was detected in the one of three animals intravaginally inoculated with 1 TCID₅₀ of SIVmac with huSP (Table 1). In this animal (23545), SIV was isolated immediately after inoculation and at 10 weeks p.i. but viremia was not detected at 3 and 30 weeks p.i. SIV could not be isolated from the LNMC collected at 10 and 30 weeks p.i. (Table 1) or detected by PCR at 10 weeks p.i. (Table 2), although the PBMC were SIV positive at 10 weeks p.i. This animal did not develop detectable SIV-specific serum antibodies (Table 3).

Study 2. A total of 26 animals were intravaginally inoculated with the rhPBMC SIVmac stock (Table 4). Each animal

received a single inoculation of 1 ml of the viral inoculum either with or without huSP. Two of two animals intravaginally inoculated with 1 ml of the undiluted SIVmac stock (10^5 TCID₅₀) with huSP became persistently viremic. Virus isolation from PBMC was consistently positive in these two animals (Table 4). One animal (20584) died with clinical signs and pathologic changes consistent with AIDS at approximately 12 weeks p.i., and SIV was isolated from the PBMC obtained at necropsy. SIV was isolated from LNMC collected from the other animal (19242 at 14 weeks p.i.) at a similar time point p.i. The results of the PCR analyses of PBMC were generally consistent with the virus isolation results (Table 5). Both animals had moderate anti-SIV serum antibody responses (Table 6).

Eight of eight animals intravaginally inoculated with 10^5 TCID₅₀ of SIVmac without huSP became viremic (Table 4). Five animals were persistently viremic, and SIV was consistently isolated from the PBMC of these animals. SIV was isolated from the LNMC of three of four of these animals tested at 2 weeks p.i., five of five animals tested at 8 weeks p.i., and two of two animals tested at 12 weeks p.i. (Table 4). These results were consistent with PCR analysis of PBMC and LNMC samples (Table 5). All five persistently viremic animals had serum antibody responses to SIV antigens (Table 6). One animal (19103) was transiently viremic. SIV was isolated from

TABLE 4. Virus isolation from rhesus macaques intravaginally inoculated with SIVmac251 propagated in rhPBMC

Animal	Virus inoculum ^a	Result ^b at p.i. wk:												Death (B)	
		1 (B ^c)	2		4 (B)	6 (B)	8		12-16		20		40		
			B	L ^d			B	L	B	L	B	L	B		L
19242	10 ⁵ /sp	—	+		+	+	+		+	+					+ (12) ^e
20584	10 ⁵ /sp	+	+		+	+	+								
16954	10 ⁵	+	+	+	+	+	+	+	+						
17970	10 ⁵	—	—	—	—	—	—	—	+						
19103	10 ⁵	+	+	—	+	—	—	—			—	—			
21982	10 ⁵	—	+	+	—	—	—	—			—	—			
22429	10 ⁵	—	+		+	+	+		+	+					
23204	10 ⁵	—	+		+	+	+		+	+					
24877	10 ⁵	+	+	+	+	+	+	+	+						
24925	10 ⁵	+	+	+	+	+	+	+	+						
20624	10 ⁴ /sp	+	+	+	+	+	+		+	+					+ (42)
23374	10 ⁴ /sp	—	—		—	+	—		—	—			—	—	
17720	10 ⁴	+	+	+	+	+	+	+	+						+ (21)
18540	10 ⁴	+	+	+	—	—	—	—			—	—			
18997	10 ⁴	+	+	+	+	+	+	+	+						
19825	10 ⁴	+	+	—	—	+	—	—	—		—	—			
23008	10 ⁴	+	+	+	+	+	+	+	+		+	+			
23363	10 ⁴	+	+	+	—	—	—	—	—		—	—			
25117	10 ⁴	—	—	—	—	+	—	—	—						
25566	10 ⁴	+	+	+	+	+	+	+	+						
18421	10 ³ /sp	—	—		—	+	+		—	—			—	—	
22201	10 ³ /sp	—	—		—	+	—		—	—			—	—	
20570	10 ³	—	—	—	—	+	—	—	+			—			
24739	10 ³	—	—	—	—	—	—	—	—			+			
24936	10 ³	—	—	—	—	+	—	—	+			+			
25343	10 ³	—	—	—	—	+	—	—	—			—			

^a See Table 1, footnote a.

^b See Table 1, footnote b.

^c B, PBMC.

^d L, LNMC.

^e The values in parentheses are weeks p.i.

TABLE 5. SIV PCR from rhesus macaques intravaginally inoculated with SIVmac251 propagated in rhPBMC

Animal	Virus inoculum ^a	Result ^b at p.i. wk:							
		1 (B)	2		4 (B)	6 (B)	8		12-16
			B	L			B	L	
19242	10 ⁵ /sp	—	+		+	+	+		+
20584	10 ⁵ /sp	—	+		+	+	+		+
16954	10 ⁵	+	+	+	+	+	+	+	+
17970	10 ⁵	—	—	—	—	—	—	—	—
19103	10 ⁵	—	—	—	—	—	—	—	—
21982	10 ⁵	—	—	—	—	—	—	—	—
22429	10 ⁵	—	+		+	+	+		+
23204	10 ⁵	—			+	+	+		+
24877	10 ⁵	+	+	+	+	+	+	+	+
24925	10 ⁵	+	+	+	+	+	+	+	+
20624	10 ⁴ /sp	+	+		+	+	+		+
23374	10 ⁴ /sp	—	—		—	+	—		—
17720	10 ⁴	—	+	+	+	+	+	+	+
18540	10 ⁴	—	—	—	—	—	—	—	—
18997	10 ⁴	—	+	+	+	+	+	+	+
19825	10 ⁴	—	—	—	—	—	—	—	—
23008	10 ⁴	+	+	+	+	+	+	+	+
23363	10 ⁴	—	—	—	—	—	—	—	—
25117	10 ⁴	—	—	—	+	—	—	—	—
25566	10 ⁴	—	+	+	+	+	+	+	+
18421	10 ³ /sp	—	—		—	—	—		—
22201	10 ³ /sp	—	—		—	—	—		—
20570	10 ³	—	—	—	—	—	—	—	—
24739	10 ³	—	—	—	—	—	—	—	—
24936	10 ³	—	—	—	—	—	—	—	—
25343	10 ³	—	—	—	—	—	+	—	—

^a See Table 1, footnote a.^b See Table 1, footnote b.

PBMC early after inoculation (1, 2, and 4 weeks p.i.) and not isolated subsequently (12, 14, and 20 weeks p.i.) (Table 4). SIV was not isolated from LNMC of monkey 19103 at 2 or 8 weeks p.i., despite virus isolation from PBMC at 2 weeks p.i. SIV was not detected by PCR analysis at any time point tested (Table 5). In one transiently viremic animal (21982), SIV was isolated both from PBMC at 2 and 12 weeks p.i. and from LNMC at 2 weeks p.i. However, at numerous later time points, the virus was not isolated from the PBMC or LNMC of this animal (Table 4). PCR results obtained with PBMC and LNMC from this animal were uniformly negative (Table 5). SIV was isolated from the PBMC of one animal (17970) only at 12 weeks p.i. (Table 4), and virus was never detected by PCR analysis (Table 5). All three of the transiently viremic animals remained seronegative for SIV antigens (Table 6).

Two of two animals intravaginally inoculated with 10⁴ TCID₅₀ of SIVmac with huSP became viremic (Table 4). One animal (20624) was persistently viremic, and SIV was consistently isolated from its PBMC. SIV was isolated from both PBMC and LNMC of this animal at 2 and 16 weeks p.i. This animal had a weak serum antibody response to SIV (Table 6) and died at 42 weeks p.i. with clinical signs and pathologic lesions consistent with AIDS. The other animal (23374) was transiently viremic (Table 4). SIV was isolated from PBMC only at 6 weeks p.i., and LNMC obtained at 16 and 40 weeks p.i. were virus negative. PCR results for these two animals were

consistent with the virus isolation results. Anti-SIV antibodies were not detected in the serum of this animal (Table 6).

Eight of eight animals intravaginally inoculated with 10⁴ TCID₅₀ of SIVmac without huSP became viremic (Table 4). Of these, four animals were persistently viremic and SIV was consistently isolated from their PBMC. SIV was isolated from LNMC of these four animals at 2 and 8 weeks p.i. Results of PCR analysis of PBMC and LNMC were generally consistent with the virus isolation data. However, at 1 week p.i., PCR detected SIV in only one of four virus isolation-positive PBMC samples (Table 5). This is likely a reflection of the low virus load in these animals at the time the samples were obtained. Three of these monkeys had moderate-to-strong serum antibody responses to SIV antigens (Table 6). One persistently infected animal (23008) did not have a serum antibody response to SIV (Table 6) and died of AIDS at 21 weeks p.i. SIV was isolated from the PBMC and LNMC obtained at necropsy. The other four animals (18540, 19825, 23363, and 25117) were intermittently or transiently viremic. SIV was isolated from PBMC of monkeys 18540 and 23363 at 1 and 2 weeks p.i. only, and LNMC obtained from these two animals were virus positive at 2 weeks p.i. but negative at 8 and 20 weeks p.i. (Table 4). One animal (19825) was viremic at 1, 2, and 6 weeks p.i., but SIV was not detected in its PBMC at 4, 12, 16, and 20 weeks p.i. The LNMC of this animal were virus negative at 2, 8, and 20 weeks p.i., despite SIV-positive PBMC at 2 weeks p.i. (Table 4). PCR analysis of PBMC and LNMC failed to detect SIV infection in three of these four transiently viremic monkeys (Table 5). The PBMC from one animal (25117) were virus positive only at 6 weeks p.i. LNMC from this animal were negative at 2 and 8 weeks p.i. (Table 4). PCR results obtained with PBMC and LNMC from this monkey were negative, with the exception of one PCR-positive, virus isolation-negative PBMC sample obtained at 4 weeks p.i. (Table 5). SIV-specific antibodies were not detected in the sera of any of the transiently viremic animals (Table 6).

Two of two animals intravaginally inoculated with 10³ TCID₅₀ of SIVmac with huSP became transiently viremic (Table 4). SIV was isolated from PBMC of the animals only at 6 weeks p.i. (22201) or at 6 and 8 weeks p.i. (18421). SIV was not isolated from PBMC or LNMC obtained from either animal at 16 and 40 weeks p.i. PCR failed to detect SIV in all of the samples tested (Table 5). Neither of these monkeys developed a detectable anti-SIV serum antibody response (Table 6).

All four animals intravaginally inoculated with 10³ TCID₅₀ of SIVmac without huSP became transiently viremic (Table 4). SIV was isolated from PBMC of three animals (20570, 24936, and 25343) at 6 weeks p.i. and again from PBMC of two animals (25070 and 24936) at 12 weeks p.i. One of the animals (24936) was also viremic at 20 weeks p.i. One animal (24739) was virus positive at only one time point, 20 weeks p.i. SIV was not isolated from two LNMC samples of these transiently viremic animals. The PCR results were generally consistent with the virus isolation results; however, PCR did not detect SIV in the three PBMC samples that were virus isolation positive and one virus isolation-negative PBMC sample was PCR positive (Table 5). The four transiently viremic monkeys remained seronegative for SIV antigens (Table 6).

DISCUSSION

The titration studies described here utilized two stocks of SIVmac and demonstrated that a single intravaginal inoculation of cell-free SIV can reliably produce infection in rhesus macaques. With the cell-free SIV stock grown in huPBMC,

TABLE 6. Serum anti-SIV antibody response of animals intravaginally inoculated with SIVmac251 propagated in rhPBMC

Animal	Virus inoculum ^a	Result ^b at p.i. wk:				
		8		16		
		ELISA	Immunoblot	ELISA	Immunoblot	gp120 ELISA
19242	10 ⁵ /sp	1:1,600	+	1:6,400	+	+
20584	10 ⁵ /sp	1:100	+	1:6,400	+	+
16954	10 ⁵	1:1,600	+	1:6,400	+	+
17970	10 ⁵	<1:100	—	<1:100	—	—
19103	10 ⁵	<1:100	—	<1:100	—	—
21982	10 ⁵	<1:100	—	<1:100	—	—
22429	10 ⁵	1:6,400	+	>1:100,000	+	+
23204	10 ⁵	1:1,600	+	1:25,600	+	+
24877	10 ⁵	1:6,400	+	1:25,600	+	+
24925	10 ⁵	1:6,400	+	1:25,600	+	+
20624	10 ⁴ /sp	1:100	+	1:100	+	+
23374	10 ⁴ /sp	<1:100	—	<1:100	—	—
17720	10 ⁴	1:1,600	+	1:6,400	+	+
18540	10 ⁴	<1:100	—	<1:100	—	—
18997	10 ⁴	1:25,600	+	>1:100,000	+	+
19825	10 ⁴	<1:100	—	<1:100	—	—
23008	10 ⁴	<1:100	—	<1:100	—	—
23363	10 ⁴	<1:100	—	<1:100	—	—
25117	10 ⁴	<1:100	—	<1:100	—	—
25566	10 ⁴	1:6,400	+	1:25,600	+	+
18421	10 ³ /sp	<1:100	—	<1:100	—	—
22201	10 ³ /sp	<1:100	—	<1:100	—	—
20570	10 ³	<1:100	—	<1:100	—	—
24739	10 ³	<1:100	—	<1:100	—	—
24936	10 ³	<1:100	—	<1:100	—	—
25343	10 ³	<1:100	—	<1:100	—	—

^a See Table 1, footnote a.^b See Table 1, footnote b.

intravaginal inoculation with as little as 10² TCID₅₀ in the presence of huSP produced infection in seven of seven animals. Intravaginal inoculation with 10⁵ to 10⁴ TCID₅₀ of the cell-free stock grown in rhPBMC infected 20 of 20 animals, and the presence of huSP did not have a clear effect on the efficiency of transmission. This will be useful for developing challenge studies to test the ability of vaccines to prevent vaginal transmission of SIV. Unexpectedly, this study also demonstrated that some animals intravaginally inoculated with cell-free SIVmac became transiently viremic. The transient viremia was characterized by the ability to isolate virus from PBMC obtained at only a few time points and an undetectable antibody response to SIV antigens. In the transiently viremic animals, the initial SIV-positive PBMC samples were often collected in the first few weeks p.i. but were also collected as late as 30 weeks p.i. We have also shown that intravaginal inoculation of rhesus macaques with as little as 1 TCID₅₀ of SIVmac251 can produce viremia (Table 1).

In this study, the PCR data were generally consistent with the virus isolation results. No SIV culture-negative animals were positive by PCR. In fact, with most of the PBMC and LNMC samples, virus isolation was a more sensitive indicator of SIV infection than PCR. This probably reflects the fact that substantially more cells were analyzed in the virus isolation assay than in the PCR assay. Thus, under the conditions used in this study, the ability to detect SIV in samples with PCR is indicative of a relatively high virus load (>1 infected cell per 10⁵ PBMC).

The timing of seroconversion in individuals infected with HIV through sexual contact is not clear. Some investigators have reported that there is a prolonged period between infection and seroconversion in this population (11, 29), while others have found little evidence of prolonged seronegative HIV infection (36). The serum antibody response of the persistently viremic animals inoculated intravaginally with the huPBMC SIVmac251 stock was variable. In fact, only three animals inoculated intravaginally with this stock developed detectable serum anti-SIV antibodies and one of these animals was seropositive only in the SIV gp120 ELISA. The latter animal had a very weak anti-gp120 immune response; the ELISA signal was 10³ to 10⁴ times lower than those of strongly positive sera. The large number of seronegative, persistently viremic animals in study 1 was surprising, and the reason for this finding is not clear. It is possible that the assays used for these studies are not sufficiently sensitive to detect low levels of anti-SIV antibodies. However, it is significant that the results obtained with the whole-SIV ELISA and immunoblot assays were confirmed with a sensitive SIV gp120 ELISA. It is also important to note that two rhesus macaques inoculated intravenously with 100 TCID₅₀ became persistently viremic and developed strong anti-SIV serum antibody responses (18a). Thus, the virus stock is capable of generating immune responses in systemically infected animals. The transiently viremic animals in study 1 did not develop a SIV-specific serum antibody response. In study 2, persistently viremic animals inoculated with the rhPBMC SIVmac251 stock developed

serum antibodies against SIV antigens but the strength of the response varied. One persistently viremic animal failed to develop a serum antibody response and died of SAIDS in a relatively short time. This association among persistent viremia, a weak antibody response, and a short disease course in SIV-infected rhesus macaques has been described previously (6, 12, 20). As with the animals in study 1, transiently viremic animals did not develop a SIV-specific serum antibody response. It seems likely that the transiently viremic, seronegative monkeys in the studies reported here had low levels of plasma SIV antigen due to limited viral replication and therefore failed to mount a detectable serum antibody response. This hypothesis is consistent with the results of limited attempts to isolate virus from the plasma of the intravaginally inoculated animals in studies 1 and 2. At 2 weeks p.i., SIV was isolated from the plasma of 9 of 11 of the seropositive, persistently infected animals but SIV was not isolated from the plasma of 8 transiently viremic, seronegative animals (data not shown). This hypothesis is also consistent with a recent report that rhesus macaques inoculated intravenously with SIVmac251 became viremic and antigenemic and developed antibody responses by 14 days p.i. (30). Alternatively, viral replication may have occurred at a site that did not result in sufficient antigen reaching the systemic immune system. It will be of interest to determine if these animals have a detectable mucosal immune response to SIV antigens. In addition, we did not assess other markers of immune recognition of SIV antigens, including cellular immune responses to SIV.

These results clearly demonstrate the need to rigorously assess the postchallenge viral status of animals in vaccine studies utilizing mucosal challenge routes. In addition, the results also suggest that one measure of an effective immune response to vaginal challenge would be for a vaccinated animal to develop transient viremia following a vaginal challenge with a dose of SIV that uniformly produces persistent viremia in unvaccinated animals.

The effect of seminal plasma on vaginal SIV transmission was not clear and may have been different depending on the virus stock used. The seven animals inoculated with 10^2 TCID₅₀ of the huPBMC-grown stock in the presence of huSP were uniformly viremic, and six of the seven were persistently viremic, while three of three animals inoculated with the same virus dose without huSP were only transiently viremic. At lower doses of SIV, the animals inoculated with the huSP-SIV mixture were more often infected than the animals exposed to the virus alone. Thus, four of four animals inoculated with 10 TCID₅₀ of SIVmac251 and huSP became transiently viremic while only one of three animals inoculated with 10 TCID₅₀ of SIVmac251 without huSP became transiently viremic. The effects of huSP on vaginal transmission of the rhPBMC-grown stock are even less clear, and this is due in part to the small number of animals in the groups that were exposed to the huSP-SIV mixed inoculum. If the number of animals in these groups had been increased, it might have been possible to detect a difference in the efficiency of transmission in the presence of huSP. Seminal plasma is a complex biologic fluid, and any effect on SIV transmission that was observed may be due to properties other than its buffer effect. Seminal plasma contains high levels of prostaglandin E_{2a}, which can have a variety of local and systemic effects, including inhibition of phagocyte function (1). We intend to use strong buffer solutions to neutralize the vaginal pH prior to virus inoculation and thus directly test the effect of the vaginal pH on SIV transmission.

There are two possible explanations for transient viremia in the intravaginally inoculated animals. It is possible that the SIV

infection was eliminated in the transiently viremic animals and the animals were transiently infected with SIV. Alternatively, the animals may remain infected with SIV but the infection is not detectable with standard virus detection techniques or serologic assays. The SIV infection in transiently viremic animals could be limited to a compartment of the lymphoid system that cannot be adequately assessed by monitoring SIV infection in PBMC and peripheral LNMC.

If the intravaginally inoculated rhesus macaques have cleared the transient SIV infection, it may be because of limited dissemination of the virus during the period in which antiviral immune responses are generated. If only a small number of target cells are infected following intravaginal inoculation, the infection may be slow to expand and disseminate. Thus, by the time effective anti-SIV immunity is present, only a relatively small number of infected cells and virions are present in the infected animal and the infection can be eliminated without damage to the immune system. However, the humoral immune response in the transiently viremic animals was weak or undetectable. Thus, if anti-SIV immune responses are responsible for eliminating or limiting the infection, it is unlikely that SIV-specific antibody is the critical element of effective anti-SIV immunity. It may be that cell-mediated immune responses, especially anti-SIV cytotoxic T lymphocytes, are the critical component in the effective anti-SIV immune response of transiently infected macaques.

It is also possible that the transient viremia is due to an abortive infection with an attenuated virus. Transient viremia has not been seen in animals intravenously inoculated with uncloned SIVmac251. However transient viremia is characteristic of rhesus macaques intravenously inoculated with an attenuated infectious clone derived from SIVmac251, i.e., SIVmac1A11 (16). The mechanism that accounts for the transient viremia in animals inoculated intravenously with SIVmac1A11 has not been identified. It is clear, however, that SIVmac1A11 does not replicate in tissues as well as uncloned SIVmac251. The animals infected with SIVmac1A11 do mount a weak immune response to SIV that wanes over time but the response is much less pronounced than that seen in macaques infected with uncloned SIVmac.

The population of viral variants in the SIV stocks used for these studies and the viral titer to which an animal was exposed may have contributed to the outcome of the animal inoculations. Most of the transiently viremic animals were inoculated with low doses of virus. Thus, transient viremia may be due to both the absolute virus dose and the relative frequency of attenuated viral phenotypes in the inoculum. Studies using recombinant viruses constructed from genetically similar clones of SIVmac (SIVmac239 and SIVmac1A11) demonstrated that following intravenous inoculation with the recombinant viruses, the duration of viremia is determined by the relative amounts of the genome derived from pathogenic (SIV239) and nonpathogenic (SIV1A11) parental clones. Of the animals inoculated with the recombinant viruses, those infected with the clone containing the largest amount of the SIV239 genome were most persistently viremic (16). These findings support the idea that the viral genotype determines the length of viremia in rhesus macaques and may indicate that an attenuated strain of SIV was transmitted to the transiently viremic animals in this study.

Alternatively, it is possible that the SIV infection persists undetected in intravaginally inoculated, transiently viremic animals. If this is the case, the virus is likely to be sequestered in an anatomic or functional compartment of the immune system that cannot be adequately assessed by attempting to isolate SIV from blood or peripheral lymph nodes. LNMC

from the persistently viremic animals were consistently SIV positive, while LNMC from the transiently infected animals were often SIV negative, even when paired PBMC samples were virus positive. Thus, in contrast to the findings on HIV-infected humans (7, 9, 27, 28), HIV-1 infected chimpanzees (33), and intravenously SIV-infected macaques (16, 31, 37), peripheral lymph nodes were not reservoirs of SIV infection in intravaginally inoculated, transiently viremic monkeys. These discordant results of virus isolation from paired PBMC and LNMC samples support the ideas that SIV is sequestered in a remote compartment of the lymphoid system and that SIV-infected PBMC detected in blood did not recirculate into peripheral lymph nodes. It was expected that if 5×10^6 to 20×10^6 circulating PBMC were SIV positive, then 2×10^7 to 8×10^7 LNMC would also be positive, as observed in intravenously inoculated animals. However, this was often not the case. On many occasions, PBMC were positive while LNMC were negative, suggesting that the infected cells in the blood did not spread the infection to peripheral lymph nodes. This hypothesis is possible if the trafficking of infected cells in the blood is limited to recirculation between the tissues of the mucosal immune system.

We have initiated studies to characterize the cellular immune responses in the transiently viremic animals. Recent reports have described individuals who have subtle immune recognition of HIV antigens despite being virus negative (3; reviewed in reference 4). This phenomenon may occur because these individuals were exposed to HIV but not infected or have an infection limited to the lymphoid tissues of the mucosal immune system. The observations described in this report may provide insight into the natural history of HIV infection in humans heterosexually exposed to HIV. The sexual transmission and subsequent dissemination of SIV and HIV are intimately associated with the cellular traffic of the genital mucosal immune system. We hypothesize that following vaginal inoculation, SIV is found primarily in a subpopulation of immune cells that recirculate between the genital mucosa and genital lymph nodes. Following genital transmission, dissemination of SIV to systemic lymphoid tissues may be a relatively late event following a period of amplification in the mucosal compartment of the immune system.

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