5-1-1997

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JOURNAL OF VIROLOGY, May 1997, p. 3498–3506 Vol. 71, No. 5
0022-538X/97/$04.00+0

Human Immunodeficiency Virus Type 1 Infection of Cells and Tissues from the Upper and Lower Human Female Reproductive Tract

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Received 4 November 1996/Accepted 7 January 1997

Viable tissue sections and isolated cell cultures from the human fallopian tube, uterus, cervix, and vaginal mucosa were examined for susceptibility to infection with human immunodeficiency virus type 1 (HIV-1). We examined infectivity by using the monocytotropic strain HIV-1_{JR-FL} and several primary isolates of HIV-1 obtained from infected neonates. HIV-1 infection was measured by p24 production in short-term culture and by immunofluorescence detection of HIV-1 Nef and p24 proteins by laser scanning confocal microscopy. Three-color immunofluorescence was used to phenotype HIV-infected cells within tissue sections from each site. Our findings indicate that epithelial, stromal, and dendritic cells and cells with $CD14^+$ $CD4^+$, $CD14^-$ $CD4^-$, and $CD4^+$ $CD14^-$ phenotypes from the female reproductive tract are infectable with HIV-1. Of importance is the finding that tissues from the upper reproductive tract are susceptible to infection with HIV-1. Moreover, tissue samples from women in all stages of the menstrual cycle, including postmenopausal women (inactive), could be infected with HIV-1. Female reproductive tract cells required a minimum of 60 min of exposure to HIV-1 in order for infection to occur, in contrast to peripheral blood lymphocytes, which became infected after being exposed to HIV-1 for only 1 min. These findings demonstrate that HIV-1 can infect cells and tissues from different sites within the female reproductive tract and suggest that multiple cell types, including epithelial cells, may be targets for the initial infection by HIV-1.

The rate of heterosexual transmission of human immunodeficiency virus type 1 (HIV-1) continues to increase worldwide. In the United States, 40,000 to 80,000 new cases of HIV infection occur each year (22), and it is estimated that 70% of these occur via heterosexual transmission. Although female-to-male transmission of HIV can occur, the vast majority of cases involve transmission of virus or virus-infected cells from male to female. HIV-1 can be transmitted either as free virions or within infected cells (1, 3, 14, 19, 42, 48), although it is more likely that infection is transmitted by virus-infected cells, as these are present in higher numbers than free virus in seminal fluid (20). Moreover, cell-to-cell contact within the female reproductive tract is increased via factors such as prostaglandins that are in high concentrations in semen (31).

Epidemiological studies established an association between the presence of lesions or abrasions in the vagina or cervix and an increased susceptibility to infection by HIV. This finding suggested that HIV required direct contact with the circulation for infection to occur. Moreover, this assumption helped to explain the high level of heterosexual spread of HIV-1 in Africa, where cervical ectopy and genital ulcers from venereal diseases are associated with increased HIV seroprevalence (17, 25, 32), and in the United States, where herpes simplex and syphilis infections correlated with an increased risk of HIV transmission (15). In fact, HIV antigens were demonstrated in monocyte-derived macrophages and endothelial cells within the submucosa of cervical biopsy specimens from HIV-seropositive women who also were shown by immunohistochemistry to have cervicitis (33). In animal studies, transmission of HIV has been reported in a chimpanzee inoculated with high levels of cell culture-derived HIV directly into the vaginal canal (10). Evidence obtained from cell culture studies suggests that HIV is found within macrophages present in the cervix and that these cells may function to transmit the virus to other sites (26, 33). Infection of a cervical epithelial cell line by infected lymphocytes and macrophages (39) and by free virions (11) has also been demonstrated.

Despite these findings, little is known about the events surrounding HIV-1 transmission and infection of the human female reproductive tract. It is well documented that virus can be isolated from mucosal secretions from HIV-infected women (33). Moreover, virus recovery is increased in pregnancy, during oral contraceptive use, and in cervical ectopy (5, 13), suggesting that hormone levels may regulate virus production and/or infectivity. Mucosal epithelial cells may play an active role in the infectivity of HIV-1, as studies of polarized epithelial cells derived from a canine kidney cell line demonstrated that the secretion of HIV-1 envelope glycoprotein is restricted to the basolateral (serosal) surfaces (27). This finding suggests that HIV-1 matures at the basolateral surface membrane and provides a mechanism whereby HIV-1 can traverse epithelial cells to gain entry into tissues and the systemic circulation by a route other than by physical breakdown of the epithelium-blood barrier.

We performed a systematic study of HIV-1 infectivity of normal female reproductive tract tissues obtained from women undergoing hysterectomy for various clinical conditions. The overall goal of this study was to identify the types of cells within the female reproductive tract that were susceptible to infection with HIV-1 and the conditions under which infectivity could be established. Our hypothesis is that epithelial cells lining the

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TABLE 1. Patient characteristics and relevant medical histories[a]

Patient no.	Age (yr)	Hormone therapy	Diagnosis	Menstrual cycle stage
346	46	Lupron	Fibroids	Proliferative
349	36	None	Adenomyosis	Proliferative
353	45	None	Adenomyosis	Mild hyperplasia
357	44	None	Fibroids, adenomyosis	Proliferative
359	50	None	Uterine cancer	Proliferative
364	41	None	Premalignant ovarian tumor	Inactive
372	31	Estrogen + progesterone	Adenomyosis	Secretory
387	37	None	Fibroids	Secretory
388	78	None	Uterine cancer	ND[b]
396	34	Unknown	Premalignant ovarian tumor	Proliferative
413	35	None	Pelvic relaxation	Secretory
421	37	None	Fibroids	Proliferative
422	52	None	Endometriosis, fibroids	ND
440	30	None	Menorrhagia[c]	Proliferative
449	46	Unknown	Uterine cancer (stromal cell)	ND
454	36	None	Adenomyosis	Secretory
468	42	None	Fibroids, adenomyosis, endometriosis	Proliferative
473	34	None	Benign ovarian cyst	Secretory
488	48	None	Fibroids, endometriosis, fallopian tube inflammation	Secretory
491	65	Unknown	Benign ovarian cyst	ND
502	53	None	Endometriosis (bicornate uterus)	Inactive
506	44	Unknown	Adenomyosis	Proliferative
513	39	None	Fibroids	Proliferative
518	40	None	Fibroids	Secretory
524	39	None	Dysmenorrhea[c]	Secretory
527	51	None	Ovarian cancer	Inactive

[a] All patients described in this study are Caucasian.
[b] ND, not determined; in the two patients with ovarian cancer, menstrual cycle stage could not be determined due to the type of pathology.
[c] No pathological diagnosis was obtained.

upper reproductive tract are susceptible to infection by HIV-1 and that once infected, they can transmit infection to T cells, monocytes, and macrophages within the female reproductive tract. Transmission of virus from infected epithelial cells to T cells and monocytes/macrophages could explain how infection is transmitted systemically, as T cells and myeloid cells traffic between the reproductive tract and the periphery. In this study, we examined infectivity of purified cultures of epithelial and stromal cells as well as viable sections of tissues from the fallopian tube, uterus, ectocervix, endocervix, and vagina and demonstrated HIV infection of both epithelial and stromal cells at multiple sites within the female reproductive tract, including the upper tract. We also determined that reproductive tract epithelial cells require a longer time period of exposure to HIV-1 than do peripheral blood lymphocytes for infection to occur. This prolonged time suggests that receptors other than CD4 are involved in the initial stages of HIV infection in these cells. These receptors may not be as efficient as CD4 at binding and mediating internalization of virus. These findings are the first to report infectivity of normal female reproductive tract tissues and demonstrate that HIV can infect cells in the upper reproductive tract. Moreover, these findings have important implications for the pathophysiology of HIV infection resulting from heterosexual transmission of virus.

MATERIALS AND METHODS

Source of tissues. Reproductive tract tissues were obtained immediately following surgery from 26 women who had undergone a hysterectomy for various clinical conditions (Table 1). All tissues obtained and used in this study were distal to the sites of pathology and were determined to be unaffected with disease upon inspection by a trained medical technologist. Tissues obtained for study were fallopian tube, uterus, ectocervix, endocervix, and vaginal mucosa, although not all types of tissues were obtained from each patient.

Antibodies. Antibodies were obtained commercially and used for phenotypic characterization of cells. Murine monoclonal antibodies were either unconjugated (rabbit anti-p24) or conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), cyanin 3 (Cy3), or cyanin 5 (Cy5). Antibodies to leukocytes included anti-CD4 (monocytes and lymphocytes), anti-CD14 (monocytes), anti-HLA class II (Caltag, South San Francisco, Calif.), and anti-CD45 (pan-leukocyte). Ber-EP4 (Dako, Carpinteria, Calif.) binds to an extracellular epithelial cell protein (18), and anticytokeratin (pan-cytokeratin proteins 8 and 18, FITC conjugated; Sigma, St. Louis, Mo.) binds to an intracellular filament protein in epithelial cells. Antivimentin (Cy3 conjugated; Sigma) binds to intracellular filaments in stromal cells, fibroblasts, and leukocytes. Anti-HIV antibodies included anti-Nef (FITC conjugated; Intracell, Cambridge, Mass.) and anti-p24 (Abbott Laboratories, Abbott Park, Ill.). Anti-Nef has been used to detect infected cells in frozen sections of lymph nodes from HIV-1 infected individuals (29).

Anti-CD4 (OKT4) was conjugated to Cy3 and anti-CD14 (AML-2-23) and anti-HLA class II (IVA12) were conjugated to Cy5 in our laboratory. Antibodies were purified from hybridoma cell culture supernatants (hybridoma cell lines obtained from the American Type Culture Collection, Rockville, Md.) by using HiTrap protein G-Superose columns (Pharmacia Biotech, Piscataway, N.J.) and conjugated, where indicated, with Cy3 or Cy5 Fluorolink protein labeling kits (Amersham, Arlington Heights, Ill.) according to the manufacturer's recommendations.

Cell and tissue preparation. Reproductive tract tissues obtained from hysterectomy patients were placed immediately in sterile ice-cold phosphate-buffered saline (PBS). Tissue specimens were dispersed by enzymatic digestion by using a PHC enzyme mixture that contained final concentrations of 3.4 mg of pancreatin (Gibco BRL, Grand Island, N.Y.), 0.1 mg of hyaluronidase (Worthington Biochemical Corporation, Freehold, N.J.), 1.6 mg of collagenase (Worthington), and 2 mg of D-glucose per ml in 1× Hanks' balanced salt solution (Gibco) containing penicillin (50 U/ml) and streptomycin (50 mg/ml). Enzymes were chosen to maximize digestion of the extracellular matrix while minimizing digestion of cell surface antigens (43a). After mincing of tissues and incubation in PHC-Hanks' balanced salt solution for 2 h at 37°C, cells were dispersed through a 250-μm-pore-size mesh screen, washed, resuspended in complete medium (Leibowitz L-15 medium supplemented with 20 mM HEPES, 20 μg of gentamicin per ml, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum [HyClone, Logan, Utah]) and analyzed for cell number and viability. In some experiments, these cells were plated directly into multiwell plates and allowed to adhere for 48 h, and all nonadherent cells (including leukocytes) were removed by washing. This cell preparation was termed mixed cells because it contained a mixture of epithelial and stromal cells.

Epithelial cells were separated from stromal cells by serial filtration through 40- and 20-μm-pore-size nylon mesh filters (Small Parts Inc., Miami Lakes, Fla.).

TABLE 2. Summary of results of HIV-1 infection of female reproductive tract cells[a]

Patient no.	Organ type	Cell type	Virus(es)	HIV infectivity results
346	CX, ECX, VM	Stromal	JR-FL	Not infected
	FT	Vibratome	JR-FL, primary[b]	Infected
349	UT	Stromal	JR-FL, primary	1 of 2 cultures infected
	UT	Epithelial	JR-FL, primary	Infected
353	UT	Stromal	JR-FL	Infected
357	UT	Mixed cell	Harwi	Infected
	FT	Stromal	Harwi	Poor culture viability
359	UT	Mixed cell	JR-FL	Infected
372	UT	Stromal	JR-FL, Rodro	Infected
387	UT	Stromal	JR-FL, Jamwi	1 of 2 infected with Jamwi; 1 of 4 infected with JR-FL
388	FT	Vibratome	JR-FL	Infected
413	UT	Stromal	Rodro, Jamwi	Not infected
	FT, ECX, CX	Mixed cell	JR-FL, Harwi	Only FT infected
421	UT	Mixed cell	JR-FL, Harwi	Infected
422	FT	Vibratome	JR-FL	Infected
454	UT	Stromal	JR-FL, Harwi	1 of 2 cultures infected
	UT	Epithelial	JR-FL, Harwi	3 of 4 cultures infected
468	UT, ECX, CX	Vibratome	Harwi	3 of 3 cultures infected
473	UT	Epithelial	Harwi	Infected
502	UT, ECX, CX, FT	Vibratome	Harwi	4 of 4 cultures infected
527	UT, ECX, CX	Epithelial	Harwi	3 of 3 cultures infected
	UT, ECX, CX	Stromal	Harwi	3 of 3 cultures infected

[a] HIV-1 infection was confirmed either by p24 production or by immunostaining. Samples from patients 440, 449, 506, 513, and 518 were contaminated with fungus and could not be evaluated. Samples from patients 488, 491, and 524 could not be evaluated because of poor culture viability. Stromal cells from the fallopian tube (FT), ectocervix (ECX), cervix (CX), and (VM) vaginal mucosa from patient 364 and vibratome samples from the fallopian tube, uterus, ectocervix, and cervix from patient 396 were not infected with HIV-1.

[b] Primary indicates that all four primary isolates of HIV-1 (Jamwi, Rodro, Arilu, and Harwi) were used.

Epithelial cells were retained on the 40- and 20-μm-pore-size filters, while stromal cells passed through the filters and were recovered in the effluent. Epithelial cells were collected by inverting and washing the filters and were plated in complete medium (containing serum) at 10^5 cells/well in 96-well microtiter plates (Costar, Cambridge, Mass.). Stromal cells were washed, resuspended in complete medium, and plated in 96-well microtiter plates at a concentration of 10^5 cells/well. Leukocytes and other cells were removed from these cultures by washing out nonadherent cells after incubation for 24 h (stromal cells) or 48 h (epithelial cells). Monocytes/macrophages were also washed out, as these cells did not stick to plastic in the presence of serum-containing medium. The remaining adherent cells were allowed to proliferate in complete medium. It was not always possible to prepare stromal and/or epithelial cell cultures from each patient because in some cases an insufficient amount of tissue was obtained. We have summarized the tissue sites, types of cells studied, and results of HIV-1 infectivity studies for each patient in Table 2.

Purity of epithelial and stromal cells was evaluated by immunofluorescent staining with lineage-specific antibodies and was performed on replicate wells prior to each experiment. Determination of antibody binding was performed by scanning wells containing stained cells by confocal microscopy. Positive and negative cells were enumerated in areas representing approximately 200 cells. Epithelial cell cultures exhibited uniform positive staining for the epithelial antigens Ber-EP4 and cytokeratin and were negative for CD45 and vimentin. Stromal cell cultures demonstrated strong positive staining for vimentin and were negative for Ber-EP4 and CD45. Neither epithelial nor stromal cells express CD4.

Additionally, we prepared fresh, unfixed, viable tissue sections for infectivity studies. Blocks of freshly excised tissue were trimmed of excess fat and underlying musculature, and 30- to 70-μm sections were cut by using a vibratome (VT1000; Energy Beam Sciences, Agawam, Mass.). Sections were maintained in ice-cold PBS throughout processing, then transferred to wells of a 96-well plate containing complete medium, and cultured for up to 15 days. Because vibratome sectioning was performed on the bench, maintaining sterility was often difficult. Thus, in some cases (Table 2), vibratome sections became contaminated with fungus and could not be evaluated for infectivity. This problem was corrected midway through the study when a new vibratome that could be used in a laminar flow hood was obtained.

HIV-1 infection. Several strains of HIV-1 were used to determine susceptibility to infectivity. HIV-1_{JR-FL}, a monocytotropic laboratory strain that infects both T cells and monocytes, was grown and its titer was determined as previously described (7). Four pediatric isolates were isolated and characterized by John Sullivan (University of Massachusetts, Worcester) and obtained from the NIH AIDS Repository (Rockville, Md.). These strains, designated Rodro, Arilu, Harwi, and Jamwi, were isolated from HIV-infected newborns who were infected either in utero or at the time of birth. Pediatric isolates were propagated in phytohemagglutinin (PHA) blasts, and titers were determined by endpoint dilution as described elsewhere (35). These strains infect primary cultures of monocytes as well as T cells.

Infectivity of purified epithelial, stromal, or mixed cell cultures and of vibratome sections was performed by adding 100 to 300 50% tissue culture infective doses ($TCID_{50}$) of HIV-1 to microtiter wells containing the cells or tissue section. In most experiments, virus was allowed to incubate with cells for 5 days, followed by washing out the unbound virus by repetitive removal and replenishment of serum-free medium (RPMI 1640) into each well. The cells were refed with complete medium, and a sample of the medium removed for p24 determination (day 0). In experiments in which p24 levels were quantitated, PHA blasts (10^5 cells) prepared from an allogeneic seronegative donor were added to one-half of the replicate wells after the 5-day exposure to virus, and the medium was supplemented with 10 IU of interleukin-2 (Boehringer Mannheim). PHA blasts were prepared by culturing mononuclear cells for 72 h with 4 μg of PHA per ml and then washed three times to remove PHA before culture. After 0, 5, and 10 days following PHA blast addition, 100 μl of supernatant representing one-half of the volume from each well was collected and pooled from triplicate microwells, and infectious virus was inactivated with 0.5% Triton. Each well was refed with 100 μl of fresh medium following harvesting of supernatant.

To determine whether HIV-1 would stick nonspecifically to female reproductive tract cells and then infect PHA blasts that were subsequently added, semiconfluent monolayers of LN-CaP cells, a human epithelial carcinoma cell line derived from prostate (16), were incubated with HIV-1 in the same manner as the female reproductive tract cell cultures, washed, and cocultured with PHA blasts. Culture supernatant was collected on days 0, 5, and 10 after PHA blast addition and tested for p24.

Time course of HIV-1 infectivity. Replicate cultures of uterine epithelial cells were grown for 21 days to achieve 80% confluency and then infected for various times from 1 min to 12 h with 300 $TCID_{50}$ of HIV-1_{Harwi}. Virus was added to triplicate wells at specific time points, starting with the longest time period and ending with the shortest time period. At the end of the incubation, all wells were washed extensively to remove any unincorporated virus and incubated overnight in complete medium, and PHA blasts were added. p24 levels were evaluated in culture medium pooled from triplicate wells 10 days after addition of PHA blasts.

A similar study was performed to determine the minimum time of exposure to HIV-1 necessary to infect peripheral blood T cells. For this study, PHA blasts were prepared as described above, incubated with HIV-1_{Harwi} for time periods ranging from 1 to 60 min, washed extensively to remove any unincorporated virus, and cultured in triplicate in wells of a 96-well microtiter plate. Cultures were refed on day 5 postinfection, and supernatant collected on day 10 was assayed for p24 by enzyme-linked immunosorbent assay (ELISA).

Determination of HIV-1 infectivity and immunophenotyping of cell cultures and tissue sections. HIV-1 infection of cells and tissue sections was determined by p24 antigen quantitation in supernatant by ELISA (HIVag kit; Abbott Laboratories) and by immunofluorescence detection of intracellular viral antigens.

For immunofluorescence staining, cells or tissue sections were first washed extensively in PBS containing 1% bovine serum albumin and 0.5% azide (PBA) to remove extracellular viral particles and resuspended in PBA containing human immunoglobulin (Ig; 6 mg/ml) to block nonspecific binding of antibodies to cells. Then 1 μg of primary or isotype control fluorochrome-conjugated antibodies to extracellular antigens was added, and the mixture was incubated for 30 min (confluent cell cultures) or overnight (vibratome sections) at 4°C in the dark with continuous gentle agitation. Extracellular antigens included Ber-EP4, CD14, HLA class II, and CD4. Combinations of Cy3-, Cy5-, and FITC-conjugated antibodies to these antigens were used to obtain the most relevant phenotypic information for each cell culture well or tissue fragment. Unbound antibody was removed by aspiration followed by four 20-min washes in PBA. Washed sections were then fixed overnight in PBA containing 1% paraformaldehyde. Intracellular staining for expression of the intracellular antigen Nef, p24, vimentin, or cytokeratin was then carried out. After washing, cell and vibratome sections were permeabilized by incubation for 2 h at 23°C in 200 μl of Fix and Perm (Caltag) in the presence of human Ig block and either primary antibody or a matched FITC-labeled isotype control. Following this incubation, the cells and vibratome sections were washed once in PBA containing 0.5% saponin, followed by two additional washes in PBA. Cells stained with the unconjugated anti-p24 antibody were then incubated with PE-conjugated goat anti-rabbit IgG antibody (Caltag) for 30 min at 23°C, followed by one wash with PBA-saponin and two washes with PBA as described above. The negative control for rabbit anti-p24 was normal rabbit serum at a 1:500 dilution. All cells and tissue fragments were then fixed in 1% paraformaldehyde and stored for up to 7 days before analysis.

Analysis by laser scanning confocal microscopy. Antibody-stained vibratome sections were removed from the microwell and wet mounted in antifade (Molecular Probes, Eugene, Oreg.) on a standard glass microscope slide, sealed with nail varnish, and analyzed for fluorescence on a Bio-Rad MRC 1024 laser scanning confocal microscope equipped with a krypton-argon laser. Sections exposed to HIV-1 were compared to identically treated sections that had not been exposed to HIV-1. Vibratome sections stained with isotype-matched control antibodies were optically sectioned, and photomultiplier tube gain and enhancement factors were then determined for the FITC, Cy3, and Cy5 channels, using these single fluorochrome-stained sections to ensure effective cross-channel compensation. The threshold of the FITC channel was set such that no fluorescence was seen either in the isotype control-stained sections or in uninfected sections that had been stained with FITC-Nef. Each section was scanned at a Z step setting of 2 μm until a representative image was obtained. The resulting image files were then used to create three-color images of the infected cells within the sections.

Confluent cultures of epithelial and stromal cells were analyzed for FITC or PE fluorescence on a Meridian Ultima UV/VIS argon laser system with an inverted microscope and stage scanning mechanism. Isotype control antibody-stained wells were analyzed first to verify that there was no nonspecific staining and that background levels of fluorescence were within acceptable levels. Antibody-stained wells were then analyzed, and dual images representing FITC and PE were obtained. PMT settings were adjusted to minimize crossover between fluorochromes. Images obtained by laser scanning confocal microscopy were analyzed by using the Bio-Rad Laser Sharp Analysis software.

RESULTS

HIV infectivity of female reproductive tract cells and tissues. Vibratome sections, purified cultures of epithelial and stromal cells, and/or mixed cell cultures from different sites of the female reproductive tract from 26 patients were evaluated for the ability to become infected with HIV-1 in vitro. Samples from 16 of the 26 patients studied showed evidence of in vitro infection with HIV-1 as demonstrated by p24 production or by immunofluorescence staining of HIV-1 viral proteins Nef and/or p24. None of the samples from the 26 patients demonstrated evidence of HIV-1 antigens prior to the addition of HIV-1 in the laboratory. Of the 10 patients in whom HIV infection could not be determined, cell cultures from 5 patients could not be evaluated because of contamination with fungus during the culture period, and cultures from 3 patients had low cell yield or poor culture viability. Stromal cell cultures from the fallopian tube, cervix, ectocervix, and vaginal mucosa from patient 364 and vibratome sections from the fallopian tube, cervix, ectocervix, and uterus from patient 396 did not demonstrate evidence of infection although the culture was healthy and viable throughout the 3 weeks of culture.

A summary of the 26 patients studied is shown in Table 1. Patients ranged in age from 31 to 78 years and represented proliferative, secretory, and inactive (postmenopausal) stages of the menstrual cycle. One patient was on sex steroids, and one was on Lupron therapy. The pathologic diagnoses were identified to benign conditions in 20 of the patients, including 4 patients with endometriosis. Benign conditions also included fibroids, adenomyosis, and benign ovarian cysts. Adenocarcinoma of the uterus was diagnosed in two patients, stromal cell carcinoma was diagnosed in one patient, and ovarian cancer was found in one patient. Two patients had premalignant conditions.

A range of tissue types demonstrated evidence of HIV infection, including uterus (from 11 patients), fallopian tube (5 patients), cervix (3 patients), and ectocervix (3 patients). The only site in which infectivity was not demonstrated was vaginal mucosa, where samples from five patients were either contaminated (three cases) or did not become infected (two cases). In some patients, there was variability in infectivity among different cell types or cultures. For example, in patient 346 (Table 2), vibratome sections from the fallopian tube were infected with HIV-1_{JR-FL}, yet stromal cell cultures established from the cervix, ectocervix, and vaginal mucosa were not infected. In patient 387, two of six stromal cell cultures were infected with virus, one with HIV-1_{Jamwi} and one with HIV-1_{JR-FL}. Only one of the two stromal cell cultures from patient 349 demonstrated infection with HIV-1.

Representative laser scanning confocal microscopic images following immunofluorescence detection of HIV antigens in cells and tissues from all five reproductive tract sites are shown in Fig. 1. The sources of the cell and tissue samples are identified in the legend to Fig. 1. No binding of anti-Nef antibody was seen in confluent cell cultures that were not infected with HIV-1 (insets in Fig. 1e, f, h, and i) or in uninfected vibratome sections (data not shown). Uterine and fallopian tube epithelial cell cultures were dually stained with the epithelial cell-specific antibody Ber-EP4, and stromal cell cultures were stained with PE-antivimentin to verify cell lineage and homogeneity of these cultures. Stromal cell and epithelial cell cultures exhibited uniform staining on 100% of the cells with their respective lineage-specific antibodies (data not shown). Vibratome sections stained with lineage-specific antibodies and FITC–anti-Nef show that cells of multiple lineages are infected with HIV. For example, in Fig. 1a, a cell expressing CD4 and HLA class II displayed high reactivity with anti-Nef antibody. Based on phenotyping and morphology, this cell is most likely a macrophage. Figure 1b shows a cluster of cells that are $CD14^+$ $CD4^+$ but Nef negative surrounding a smaller cluster of $CD14^-$ $CD4^-$ Nef-positive cells. These nef-positive cells could be either stromal, epithelial, or dendritic cells. In panel c, a cell expressing CD4 and Nef and negative for CD14 most likely represents a T cell. In Fig. 1d, a cell with dendritic morphology negative for CD14 and CD4 was highly positive for Nef.

p24 production by purified epithelial and stromal cells. Initial studies involved infecting cells with HIV-1 for 10 days followed by analysis of p24 in culture supernatant. These studies demonstrated that p24 levels were often below the limit of detection by ELISA, and so subsequent studies involved the addition of PHA blasts to the cells on day 5 postinfection to amplify virus production. For these studies, purified cultures of stromal or epithelial cells were incubated with HIV-1 for 5 days and then extensively washed to remove all extracellular virus particles. PHA blasts were then added to one-half of the replicate wells, and p24 values were determined immediately (day 0) and at 5 and 10 days after PHA blast addition (Table 3). p24 levels were all below the level of detection (<10 pg/ml) immediately following the addition of blasts but increased markedly 5 and 10 days after incubation with PHA blasts.

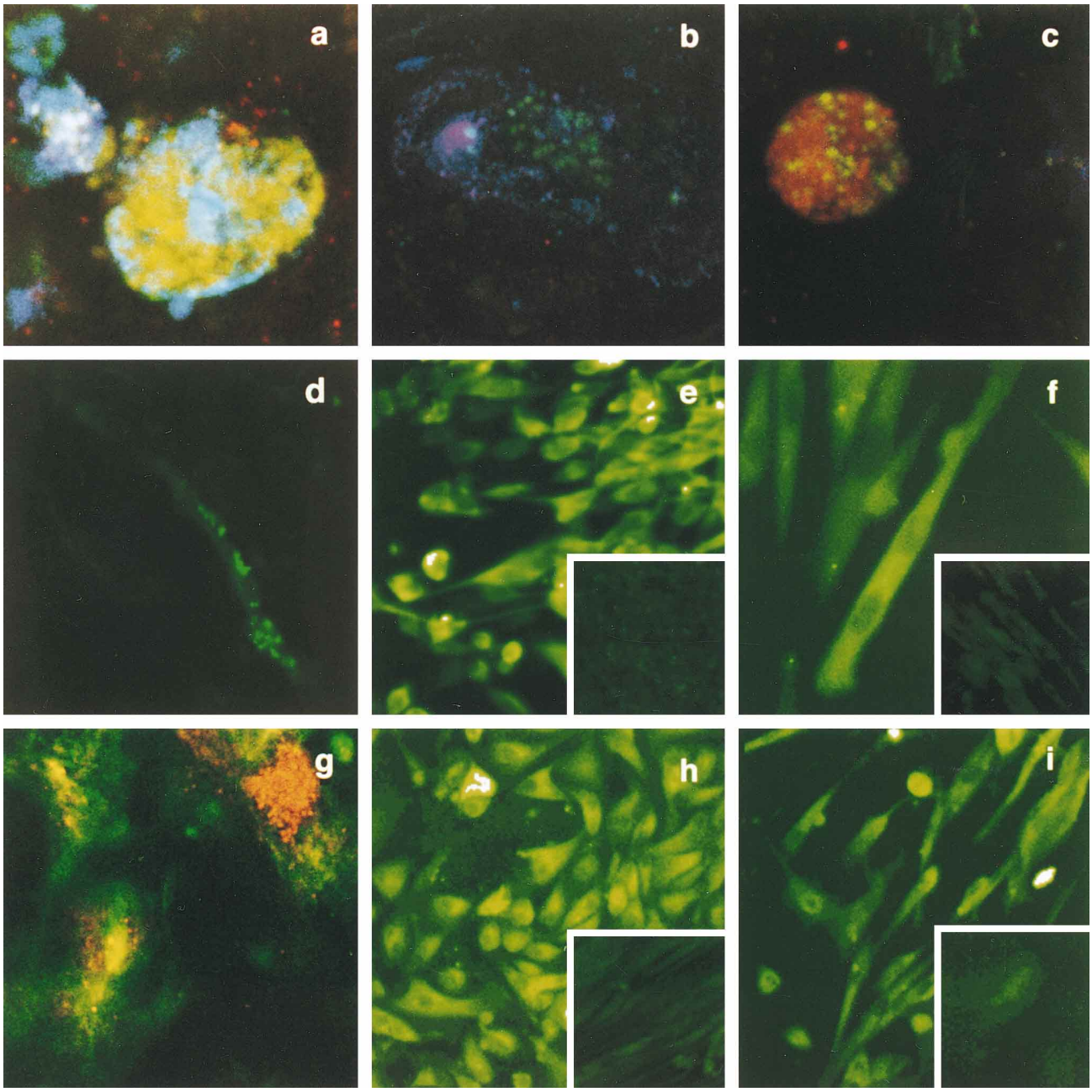

FIG. 1. HIV infection of vibratome sections and purified cell cultures form the female reproductive tract. Vibratome sections or purified epithelial or stromal cell cultures were infected with HIV-1 for 5 days, washed extensively, and then examined for expression of Nef and lineage-specific cell surface markers by immunofluorescence and laser scanning confocal microscopy. Vibratome sections from fallopian tube (a) were stained with Cy5 (blue)–anti-HLA class II, Cy3 (red)–anti-CD4, and FITC (green)–anti-Nef, and those from uterus (b), ectocervix (c), cervix (d), and vaginal mucosa (g) were stained with Cy5-CD14, Cy3-CD4, and FITC–anti-Nef. The Nef-positive cell in panel a is most likely a monocyte/macrophage, as it is positive for both class II and CD4. Panel b shows a cluster of cells that are $CD14^+$ $CD4^+$ but Nef negative surrounding a smaller cluster of $CD14^-$ $CD4^-$ Nef-positive cells. The cell in panel c is negative for CD14 and positive for CD4 and Nef and is most likely a T cell. The Nef-positive cell in panel d is a dendritic cell, based on morphology and negative staining for both CD4 and CD14. Purified cultures of epithelial cells from the fallopian tube (e) and uterus (h) were uniformly positive for Nef staining, and uninfected cells from these sites were Nef negative (the inserts in panels f and h show no fluorescence staining with anti-Nef antibody). Similarly, purified cultures of stromal cells from fallopian tube (f) and uterus (i) were also uniformly positive for Nef staining. Uninfected stromal cells from these sites were Nef negative (inserts in panels f and i).

To verify that p24 production reflected PHA blast infection by uterine stromal or epithelial cells and was not due to nonspecific sticking of virus to the extracellular membrane of the female reproductive tract cells or to the plastic wells, we performed an identical study using purified cultures of epithelial cells from a prostate carcinoma cell line, LN-CaP (16). As shown in Table 3, LN-CaP cells infected identically to epithelial and stromal cell cultures did not demonstrate any detectable levels of p24 10 days after PHA blast addition.

Vibratome sections from the fallopian tube were infected with $HIV\text{-}1_{JR\text{-}FL}$ in a manner similar to that used with epithelial and stromal cell cultures (Table 4). Due to the three-

TABLE 3. P24 production from purified cultures of stromal and epithelial cells from the uterus infected with HIV-1[a]

Patient no.	Cell type	Virus strain	p24 (ng/ml)		
			Day 0	Day 5	Day 10
349	Stromal	JR-FL	0.005	97.8	ND[b]
353			<0.01[c]	0.2	2.5
372			<0.01	<0.01	12.3
387			<0.01	15.3	50
387		Jamwi	<0.01	6.18	49
372		Rodro	<0.01	63.3	97.2
372			<0.01	54.2	84.4
413			<0.01	ND	7.9
349	Epithelial	Harwi	<0.01	17.6	ND
473			<0.01	16.8	ND
	LNCaP	JR-FL	<0.01	<0.01	<0.01
			<0.01	<0.01	<0.01

[a] Cells (uterine stromal, uterine epithelial, or LN-CaP) were plated in wells of 96-well plates and allowed to reach 75% confluency before being infected with HIV-1. HIV-1 was added at 300 $TCID_{50}$ per well and incubated with cells for 1 to 5 days. Each well was extensively washed to remove unincorporated virus, and blasts were added to triplicate wells. p24 levels were sampled immediately after blast cell addition (day 0 p24) and on days 5 and 10. p24 was not detected in replicate wells that did not receive PHA blasts (data not shown).
[b] ND, not determined.
[c] Limit of detection of the p24 ELISA is 0.01 ng/ml.

dimensional nature of the tissue section, it was not possible to completely eliminate all extracellular virus by washing. Thus, day 0 p24 values are above the lower limit of detection, although they are lower than values on days 5 and 10 after blast addition. The values in the wells that did not receive PHA blasts also increased over time, albeit at a much lower rate, demonstrating that virus was being produced by these tissues even in the absence of PHA blast amplification.

Time course of HIV infectivity. To further evaluate the kinetics of HIV infectivity, we performed a detailed time course using uterine epithelial cells. As shown in Fig. 2a, virus production from uterine epithelial cells amplified by PHA blasts was first detected in cultures that were exposed to HIV-1 for 60 min. No virus production was detected in cultures that were exposed to HIV-1 for less than 60 min (Fig. 2A). In contrast, when we performed the same experiment with PHA blasts alone rather than with uterine epithelial cells, virus production on day 10 was observed in cells incubated with virus for only 1 min (Fig. 2B). In this study, PHA blasts were incubated with HIV-1 for the times noted, washed extensively to remove unincorporated virus, and cultured for 10 days. p24 levels were quantified in wells on day 10 by ELISA.

TABLE 4. p24 production from vibratome sections[a] from the fallopian tube infected with HIV-1

Virus strain	p24 concentration (ng/ml)			
	PHA blasts	Day 0	Day 5	Day 10
JR-FL	+	2.6	26.1	190
	−	2.6	6.2	27
Arilu	+	1.8	28.0	46.0
	−	2.7	3.0	6.0
Harwi	+	2.7	30.0	137.0
	−	2.7	3.0	7.0
Rodro	+	2.8	ND[b]	125.0
	−	2.7	0.86	ND

[a] Vibratome sections from fallopian tube from patient 346 were infected with HIV-1 and washed extensively on day 5. PHA blasts (10^5 cells) were added to one-half of the replicate wells, and p24 was collected immediately after blast addition (day 0) and on days 5 and 10 after blast addition.
[b] ND, not determined.

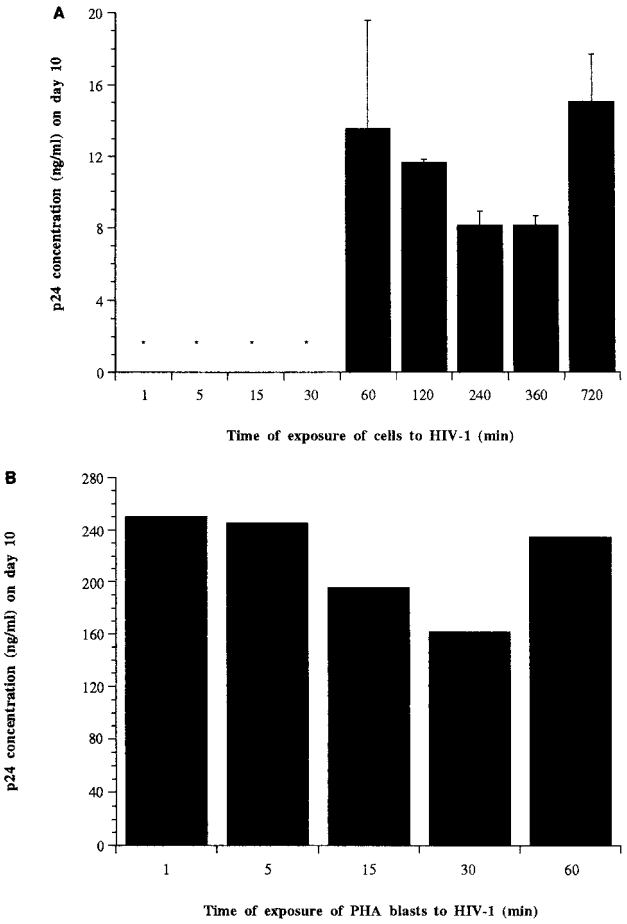

FIG. 2. Time course of HIV infectivity of uterine epithelial cells and peripheral blood T cells (PHA blasts). Purified uterine epithelial cells from patients 473 and 357 were infected with $HIV\text{-}1_{Harwi}$ for various times from 1 min to 12 h, washed extensively, and cultured overnight prior to the addition of PHA blasts. p24 levels were determined on day 10 of culture (A). In panel B, PHA blasts alone were incubated with $HIV\text{-}1_{Harwi}$ for various times from 1 min to 60 min, washed extensively to remove unincorporated virus, and cultured in triplicate wells. p24 values were measured on day 10 of culture. The values in panel A (mean ± standard deviation) represent the average of two experiments using cells from two different donors. Asterisks refer to p24 values of ≤10 pg/ml, which is the lower limit of detection of the p24 ELISA. The values in panel B represent a single experiment.

DISCUSSION

We performed a systematic study of HIV-1 infectivity of normal cells and tissues from the female reproductive tract. Purified epithelial cells, stromal cells, and tissue sections from five sites within the female reproductive tract representing both the upper and lower reproductive tract with the exception of ovary were infected with either a laboratory strain or one of four primary isolates of HIV-1 obtained from infected neonates. The viable tissue sections allowed analysis of HIV-1 infectivity in situ, where tissue architecture is conserved and physical relationships between different cell types is preserved. HIV-1 infectivity was determined by using three-color immunofluorescence and confocal microscopy and by measuring p24

production from cell cultures or tissue fragments. For analysis of p24 production, PHA blasts were added to cells and tissue fragments to amplify virus production, as the level of virus produced by infected female reproductive tract cells is often too low to measure by ELISA. This approach has been used to amplify virus production in CD4-negative cells (40). In all cases, infectivity was verified by immunofluorescence detection of HIV-1 viral proteins.

Our findings demonstrate that purified cultures of both epithelial and stromal cells from the fallopian tube and uterus can be infected directly with HIV-1. Moreover, a laboratory strain of HIV-1, HIV_{JR-FL}, as well as several primary pediatric isolates were able to infect these cells. As neither stromal nor epithelial cells from the female reproductive tract express CD4, this finding suggests that these cells express other receptors that mediate the binding and internalization of HIV-1 or that virus is taken up nonspecifically, possibly by fluid-phase pinocytosis, phagocytosis, or direct fusion. Others have shown that HIV can infect CD4-negative cells of epithelial cell origin from the gut and other tissues (9, 24, 49, 52). Moreover, galactosyl ceramide has been suggested to be a putative receptor for HIV on CD4-negative human colonic epithelial cells (50) and may well be the receptor for virus entry within female reproductive tract epithelial cells. Preliminary analysis of uterine epithelial cell monolayers for neutral glycolipids has demonstrated, however, that these cells express di- and triosylceramides but no significant levels of galactosyl ceramide (51). These findings suggest that epithelial and/or stromal cells may serve as initial sites of HIV-1 infectivity and may potentially mediate the transfer of virus throughout the female reproductive tract, perhaps via T cells and monocytes/macrophages that then traffic out of the reproductive tract into the periphery.

Infectivity of epithelial cells from the fallopian tube, uterus, and cervix indicates that the entire reproductive tract is potentially a target for HIV uptake and systemic spread. Others have suggested that blood contact from the vaginal trauma of sexual intercourse, or lesions within the vaginal canal or cervix resulting from sexually transmitted diseases, are portals of entry for HIV (12, 17, 32, 41). Our findings suggest that if HIV travels the length of the reproductive tract, then free virus and/or cell-associated virus present in semen may infect at any site along the tract independently of lesions or sites of trauma. That virus could reach the upper reproductive tract under normal physiological conditions is suggested by the finding that a small number of poorly motile sperm can be found in the fallopian tubes within minutes of vaginal deposition (37). It is possible that virus or virus-infected cells could establish an initial infection in cells within the fallopian tubes. Further studies are needed to demonstrate that virus and/or virus-infected cells present within semen reach the upper reproductive tract to infect cells beyond the cervix and vagina.

In other studies, we attempted to determine whether tissues are susceptible to HIV-1 infection under conditions in which the architecture of cells in the reproductive tract is maintained. These studies were performed with viable tissue sections from the female reproductive tract prepared in the absence of enzymatic treatment. Infectivity studies of vibratome sections revealed the presence of HIV-1 in $CD4^+$ $CD14^+$ cells that had that morphological characteristics of macrophages, in $CD4^+$ $CD14^-$ cells that were most likely lymphocytes, and in $CD4^-$ $CD14^-$ cells with dendritic cell morphology. Other $CD4^-$ $CD14^-$ cells in vibratome sections that were infected with HIV-1 included epithelial and stromal cells. Thus, multiple cell types of varied morphology and phenotype within the female reproductive tract, in addition to epithelial and stromal cells, are susceptible to HIV infection. These findings raise the possibility that the initial infection with HIV can occur in any number of different cell types.

To define the minimum time interval of exposure to HIV-1 necessary for infection to occur, we incubated HIV-1 with purified uterine epithelial cells for time intervals from 1 min to 12 h. Extensive washing followed by PHA blast addition allowed determination of infectivity of cells incubated with virus at each time point. HIV-1 infection was not detected in any cultures in which virus remained in contact with the cells for less than 60 min prior to washing. This finding suggests that infectivity of uterine epithelial cells does not occur rapidly but requires a somewhat prolonged contact between virus and cells. This is in contrast to the kinetics of HIV infection of peripheral blood T cells (PHA blasts), in which infection was observed by day 10 in cells that had been exposed to virus for only 1 min. The prolonged contact required for infection of uterine epithelial cells may be due to virus binding to receptors other than CD4 or to the internalization of virus by mechanisms that are other than receptor mediated, mechanisms that may not be as efficient as the binding of gp120 to CD4.

That some individuals become HIV positive following a single exposure to virus, whereas others remain resistant to repeated challenge, indicates that factors beyond simple viral contact with a particular mucosal cell are involved in infection (4, 28, 32). For example, sex hormone and cytokine levels in the microenvironment of the virus and target cell may influence viral infectivity. Although our studies indicate that samples of cells and tissues representing each stage of the menstrual cycle could be infected with HIV, specific sex hormones or cytokines may still affect the ability of HIV to infect female reproductive tract cells. In studies using both human and rodent models, we and others have found that sex hormone changes during the reproductive cycle regulate both the composition and the function of immune cells throughout the female reproductive tract. For example, in the human, polymeric Ig receptor levels in uterine secretions are low during the proliferative phase of the menstrual cycle and are elevated during the secretory phase, when serum progesterone and estradiol levels are elevated (38). In contrast, IgA in cervical secretions is lowest at midcycle when ovulation occurs, relative to early proliferative and late secretory phases (36). Using a rat model, we demonstrated that antigen presentation by cells in the reproductive tract varies with the stage of the estrous cycle and the site analyzed (34, 46, 47). For example, antigen presentation in the vagina by dendritic cells and macrophages is suppressed by estradiol at midcycle (proestrus) at a time when antigen presentation by epithelial cells in the uterus is enhanced (47). Endocrine balance influences both the composition and the capacity of uterine and vaginal cells to present antigen (45). These events are closely regulated by sex hormones to ensure perpetuation of the species and effective immune protection against pathogens. It is against this endocrine-immune system interface that HIV has evolved to survive and infect women upon exposure to virus in the female reproductive tract.

As documented with several viral diseases, $CD8^+$ cytolytic T cells (CTL) are thought to play a critical role in the containment of HIV infection (2, 30, 44). With the demonstration that activated $CD8^+$ T cells derived from peripheral blood secrete one or more suppressive factors (21), it is highly likely that $CD8^+$ T cells in the reproductive tract contribute to the control of HIV infection in vivo. We have recently found that whereas $CD3^+$ $CD8^+$ cells are present in the fallopian tube, uterus, cervix, and vagina throughout the menstrual cycle, these cells in the uterus are noncytolytic during the secretory phase of the menstrual cycle when progesterone levels are highest and when

fertilization and implantation are most likely to occur (43). In contrast, CTL activity is higher in the uterus in postmenopausal women than in premenopausal women during the menstrual cycle. Should reproductive tract $CD8^+$ T cells produce RANTES, MIP-1α, or MIP-1β, which have been identified as major suppressive factors produced by peripheral blood $CD8^+$ T cells (6), then we would postulate that HIV infection would be more easily established at the uterine level in women during the secretory phase of the menstrual cycle. Whether uterine $CD8^+$ T cells produce suppressive factors remains to be determined. What is clear is that the endocrine regulation of the mucosal immune system in the female reproductive tract influences immune cell function that has the potential for either enhancing or suppressing the heterosexual transmission of HIV. Indeed, a recent report by Marx and coworkers (23) showed that primates that were given progesterone were more likely to contract simian immunodeficiency virus infection after vaginal challenge compared to placebo-treated animals, which suggested that women on progestin-containing birth control may be at higher risk for HIV infection (8, 23). This study concluded that the increased susceptibility to SIV infection was most likely due to the thinning of the vaginal wall by progesterone, which allowed for virus to penetrate the vaginal mucosae more easily. However, our studies demonstrating lower CTL activity in the uterus during the secretory stage of the menstrual cycle, in which progesterone levels are highest (43), suggest that the negative regulatory effect of progesterone on immune cell function in the reproductive tract is more likely to have been responsible for a higher incidence of infection after viral challenge.

While much remains to be understood about the events surrounding the heterosexual transmission of HIV, our finding that multiple cells and tissues in the female reproductive tract are susceptible to HIV infection provides a foundation to more fully define the mechanisms involved in virus transmission as well as approaches for inducing protection against viral spread.

ACKNOWLEDGMENTS

This study was supported in part by grant AI 34478 from the NIH and a grant from the Veterans Affairs. Image cytometry was done at Dartmouth Medical School in the Herbert C. Englert Cell Analysis Laboratory, which was established by a grant from the Fannie E. Rippel Foundation and is supported in part by an NIH core grant for the Norris Cotton Cancer Center (CA 23108).

We are grateful for the technical assistance of Kenneth Orndorff in the Herbert C. Englert Cell Analysis facility, to Esther Colby for processing the tissues from the female reproductive tract, and to Alice Given for editorial and technical assistance. We express our grateful appreciation to the following individuals for their assistance in this study: John Currie, Stephen Andrews, Jackson Beecham, Joan Barthold, John Ketterer, Eileen Kirk, Benjamin Mahlab, Paul Manganiello, Eric Sailer, Barry Smith, and William Young (surgeons); Jeannette Sawyer, Tracy Stokes, Fran Reinfrank, and Jaclyn Logren; (operating room nurses); pathology assistants Peter Seery, Maryalice Achbach, Judy Rook, and chief pathologist Vince Memoli; Joanne Lavin, Kris Ramsey, and Nancy Leonard (operating room scheduling); and Tamara Krivit and Laura Wolfe (clinical support).

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