HIV binding, penetration, and primary infection in human cervicovaginal tissue

Diane Maher*, Xiaoyun Wu†, Timothy Schacker‡, Julie Horbul*, and Peter Southern*§

Departments of *Microbiology and ‡Medicine, University of Minnesota Medical School, MMC 196, 420 Delaware Street Southeast, Minneapolis, MN 55455; and ¹Department of Medicine, University of Alabama at Birmingham, 1530 Third Avenue South, Birmingham, AL 35294

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We have developed human cervicovaginal organ culture systems to examine the initiating events in HIV transmission after exposure to various sources of HIV infectivity, including semen. Newly infected cells were detected in the cervical submucosa 3–4 days after exposure to a primary HIV isolate. At earlier times, extensive and stable binding occurred when cervical surfaces were exposed to virions or seminal cells. Cervical mucus provided some protection for the endocervical surface, by physically trapping virions and seminal cells. Confocal microscopy combined with 3D surface reconstruction revealed that virions could both bind to the external surface of the cervical epithelium and actually penetrate beneath the epithelial surface. In quantitative assays, pretreatment with a blocking antibody directed against β1 integrin reduced HIV virion binding. Collectively, these results highlight a continuum of complex interactions that occurs when natural sources of HIV infectivity are deposited onto mucosal surfaces in the female reproductive tract.

confocal microscopy | female reproductive tissue | HIV transmission | organ culture | tissue surface reconstruction

Today women account for more than half of the newly HIV-infected adults worldwide, and most women acquire HIV through heterosexual exposure (1–6). There is, however, only limited information available concerning the specific cellular and molecular events that lead to HIV transmission through sexual activity. Semen contains either cell-free virus or cell-associated virus (7–12), and each source of HIV infectivity may require different mechanisms to establish infection. Clear understanding of the molecular interactions between sources of infectivity and mucosal surfaces will be crucial in developing topical agents to prevent sexual HIV transmission.

The current view of sexual HIV transmission has largely been developed from simian immunodeficiency virus (SIV) infections of female nonhuman primates (reviewed in ref. 4). When macaques were exposed to cell-free SIV intravaginally, infected resident cells were consistently detected in reproductive tract tissues before being detected in the draining lymph nodes (13). Taken together, the data from macaque infections indicate that SIV (100% infectious dose of cell-free virus) readily traverses an epithelial surface and establishes focal infections within susceptible resident cells in female reproductive tissues and then the infection spreads systemically. For heterosexual HIV transmission, the properties of the infectious inoculum are highly variable as cell-free HIV virions (12) or HIV-infected cells may be shed in semen (9). Furthermore, both soluble and cellular constituents of semen may induce transient changes in exposed epithelial surfaces, resulting in increased susceptibility to primary HIV infection (14, 15).

The complex nature of mucosal surfaces presents a major challenge for comprehensive cellular and molecular studies of microbial infections (16). The development of organ cultures with human mucosal tissues has provided some insight into HIV infection at these sites. Although methods for organ culture have varied, previous studies with human cervical tissue have shown that cells in the submucosa are susceptible to HIV infection (17–19). Additionally, ex vivo organ cultures have been used to evaluate inhibitors of HIV infection and replication (19–21). We have previously described binding of HIV virions and seminal cells to the mucosal surface of palateine tonsil in a reconstruction of HIV transmission after oral exposure (22). Here, we set out to study sexual transmission of HIV at mucosal surfaces of the cervix. The selection of human cervical tissue allowed the study of both the stratified epithelium of the ectocervix, which closely resembles the vaginal epithelial surface, and the columnar epithelium of the endocervix. Each epithelial surface may interact differently with sources of HIV infectivity. Our organ culture methods have the advantage of using human materials for the target mucosal tissue and biologically relevant sources of infectivity: semen from HIV-seropositive donors and a cell-free stock of HIV virions obtained by limited passage in vitro of a primary patient isolate.

Methods

Tissue Samples. Cervical tissues from premenopausal women with conditions not involving the cervix were processed in the laboratory within 1–3 h of completion of surgery. The experimental protocols had full Institutional Review Board approval. Cervical tissue with external epithelial surfaces (10–25 mm²) was mounted in agarose medium such that the intact epithelial surface remained exposed and all cut surfaces were covered with agarose (22). The stratified epithelium of the ectocervix remained largely intact for up to 48 h, then squamous epithelial cells sloughed away but the basal epithelial cell layer remained intact for 7–10 days. An intact endocervical columnar epithelium was retained for 4–6 days and continued to secrete mucus.

Tissue pieces in agarose were infected and processed as described (22, 23). Alternatively, in some infections, cervical tissue was submerged in cell-free virus and then the tissue was cultured on collagen sponges at the gas-medium interface. In all experiments reported here, infections were performed with a dual trophic primary patient isolate (HIV 96–480; refs. 22 and 23), and binding studies were performed with a noninfectious X4 tropic virion preparation, HIV-GFP (ref. 22; see Supporting Methods, which is published as supporting information on the PNAS web site). Single-cell immunohistochemistry was performed as reported (24).

Blocking Assays, Image Collection, and Quantitative Analysis. Populations of primary human cervical epithelial cells or 3-mm biopsies punches of intact human ectocervical epithelium were incubated with antibodies directed against β1 integrin, galactose ceramide, or a mouse IgG control. Antibody treatment began 1–2 h before the addition of HIV-GFP virions (2.5 × 10⁷ virions) in seminal plasma (final concentration: 50% seminal plasma, 50% original

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§To whom correspondence should be addressed. E-mail: peter@mail.ahc.umn.edu.

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blocking solution). After 3 h, samples were washed extensively, fixed, and quantified for virion binding.

**3D Analysis.** Tissue surface representations were created with AMIRA (Mercury Computer Systems/TGS Unit, San Diego) at the Supercomputing Institute, University of Minnesota.

For further details, see Supporting Methods.

**Results**

**HIV Infection of Cervical Leukocytes.** When cell-free HIV (HIV 96-480; a low-passage patient isolate) was applied directly onto intact endocervical epithelium and continued secretion of mucus (arrow) after 4 days of culture (hematoxylin/eosin, same tissue as shown in A–C). (F) HIV infection of cervical tissue by submersion: numerous p24-positive intraepithelial leukocytes within the submucosa of endocervical tissue. (G) High magnification of the boxed area shown in D. (Scale bars: A, E, and F, 50 μm; B, C, and G, 20 μm; D, 100 μm.)

**Cells from Semen Bind and Penetrate Mucosal Surfaces but Also Remain Trapped in Cervical Mucus.** In monitoring the organ cultures during the 3- to 4-day incubation period required to detect newly infected cells, we recognized that significant changes were occurring at the exposed epithelial surfaces. Cell sloughing and degeneration of stratified epithelium during the extended incubation obscured the earliest physical interactions supporting the initiation of HIV infection in ectocervix organ cultures. Therefore, we developed procedures to characterize events occurring within the first 24 h after exposure to HIV. Tissue pieces were embedded in agarose and exposed to sources of HIV infectivity (seminal cells or cell-free virions) and then large areas of undisturbed epithelial surface were surveyed by confocal microscopy with a water immersion lens. Viable seminal cells

Fig. 1. Reconstruction of sexual HIV transmission: de novo infection of cervical leukocytes by cell-free HIV. (A–C, F, and G) Immunohistochemical detection of HIV p24 gag (brown signal) 4 days (A–C) or 3 days (F and G) postinfection. Images are representative of experimental infections with cervical tissue from six independent tissue donors. (A) Transfer of infectivity through an intact endocervical epithelium: a p24-positive intraepithelial leukocyte directly beneath the columnar epithelium. (B) High magnification of the boxed area shown in A. (C) High-power image showing an additional p24-positive cell in close proximity to the epithelium. (D and E) Retention of columnar epithelium in organ culture. Representative image showing the integrity of columnar epithelium and continued secretion of mucus (arrow) after 4 days of culture (hematoxylin/eosin, same tissue as shown in A–C). (F) HIV infection of cervical tissue by submersion: numerous p24-positive cells within the submucosa of endocervical tissue. (G) High magnification of the boxed area shown in D. (Scale bars: A, E, and F, 50 μm; B, C, and G, 20 μm; D, 100 μm.)

Fig. 2. Interactions between viable seminal cells and the intact mucosal surface of the cervix. seminal cells were prelabeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (green), and the epithelium was stained with either Cell Tracker orange (red, A, D, and E) or ethidium homodimer-II (red, B and C). Images are representative of binding experiments with four independent tissue donors. (A) Seminal cells bind to the stratified epithelium of the ectocervix in the presence of seminal plasma: 24-h exposure. Laser scanning confocal image showing a brightest point projection of 81 images collected at 1-μm steps in the z axis. (B) Seminal cells remain suspended in mucus secreted by the columnar epithelial cells of the endocervix: 18-h exposure. Brightest point projection of 15 images collected at 3-μm steps in the z axis. (C) Seminal cells penetrate the mucus and reach the columnar epithelial surface. The same field as shown in B, focusing on the endocervical tissue surface. Brightest point projection of 19 images collected at 3-μm steps in the z axis. (D and E) Penetration of the stratified epithelium by seminal cells. Each image shows an 8-μm cross section cut from the tissue shown in A (counterstained with DAPI, blue). (Scale bars: 20 μm.)

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HIV Virions Remain Trapped in Cervical Mucus. In a series of related binding experiments, we exposed endocervical epithelium to a reconstituted inoculum: seminal plasma plus fluorescent HIV virions (HIV-GFP; a noninfectious preparation of HIV that incorporates GFP into virions, see Supporting Methods). The majority of virions remained suspended in mucus but limited numbers of virions were detected in cross sections at or beneath the endocervical epithelial surface (Fig. 3 A and B). Because each cross section contains only information from a tiny area of epithelium, we recognized that a broader perspective could be gained by analyzing confocal information from intact, undisrupted tissue surfaces. Confocal images are frequently displayed as brightest point projections (Fig. 3C) but this format does not reveal spatial information contained within the data set. By using the 3D visualization and volume modeling software program AMIRA, we created images that display the 3D structure of an epithelial surface and the spatial locations of HIV virions (Fig. 3 D). When the 3D image was rotated (compare Fig. 3 E with F, also see Movie 1, which is published as supporting information on the PNAS web site), it became clear that most of the virions were suspended in mucus above the columnar epithelial cells. These findings provide evidence that the amount and composition of cervical mucus may influence sexual routes of HIV transmission (26, 27).

**HIV Virions Bind and Penetrate the Stratified Epithelium.** When the stratified epithelium of ectocervix was exposed to HIV-GFP virions in seminal plasma, confocal images revealed stable virion binding to the epithelial cell surface and penetration of some virions beneath the epithelial cell surface (Fig. 4). The precise location of virions, with respect to the ectocervical surface, was established from 3D representations combined with adjustments to the transparency of the surface cells (Fig. 4 C and D, AMIRA). Rapid penetration of HIV virions beneath the ectocervical
epithelial cell surface was confirmed by conventional cross-sectional analysis in independent experiments (Fig. 4E). The finding that HIV virions in seminal plasma readily penetrate into the epithelium of the ectocervix is particularly significant because a similar type of stratified epithelium lines the entire vaginal cavity. Additional experiments, with tissue from a different donor, focused on virion binding in the cervical junction region, corresponding to the transition from stratified epithelium of the ectocervix to columnar epithelium of the endocervix. In this location there was minimal mucus secretion, and both virion binding and penetration beneath the epithelial cell surface were readily observed in the presence of seminal plasma (Fig. 5).

Inhibition Assays Indicate a Role for β1 Integrin in Virion Binding to Cervical Epithelium. As a step toward defining the specific interactions supporting virion binding at epithelial surfaces, we developed quantitative assays for HIV virion binding to epithelial cells, in the presence of seminal plasma. Because CD4 is generally not expressed on epithelial cells, the fundamental interaction between cell surface CD4 and HIV gp120 cannot account for virion binding to epithelial cells (28). However, in considering the natural process of HIV virion shedding in semen, we recognized that high levels of fibronectin in semen would coat virions by binding to gp120 (29, 30). Fibronectin-coated virions could then bind to β1 integrin expressed on epithelial surfaces. For initial binding experiments, we used primary human epithelial cells, grown from cervical tissue explants, that expressed cytokeratins and consistently formed tight junctions (data not shown for cervix; see refs. 22 and 23 for a description of primary tonsil epithelial cells). HIV-GFP virions readily bound to primary cervical epithelial cells in the presence of seminal plasma (Fig. 6A, 3-h exposure). Pretreatment of primary cervical cells with an antibody that blocked the interaction of β1 integrin with fibronectin reduced the number of bound virions relative to paired samples exposed to a control antibody (Fig. 6D). We also tested the inhibitory effects of an antibody to galactose ceramide, an alternative HIV receptor that is present on some epithelial cells (31), and virion binding was reduced in one of three primary cervical epithelial cell populations (Fig. 6D). The variability in blocking may be explained by differences in the expression levels for galactose ceramide at the cell surface in independent populations of primary cervical epithelial cells.

Binding inhibition assays were also performed with intact tissue pieces, using 3-mm punch biopsies collected in adjacent sequence from a large contiguous area of ectocervix (Fig. 6B). The anti-β1 integrin and anti-galactose ceramide antibodies used in these experiments bound to the external cells of the stratified ectocervical epithelium (Fig. 6C and data not shown). Substantial inhibition of HIV-GFP virion binding was observed with two of five tissue donors tested with the anti-β1 integrin antibody and two of four donors tested with anti-galactose ceramide antibody (Fig. 6E). Immunocytochemical detection of β1 integrin expression on stratified squamous epithelium from 10 randomly selected cervix tissue samples (tissues fixed immediately on receipt in the laboratory) revealed variable levels of expression between adjacent regions of the same tissue and large differences between tissue donors (data not shown). The variation of β1 integrin expression may explain the broad range of inhibitory effects observed in the quantitative binding assays but, in situations where extensive inhibition of HIV-GFP virion binding did occur, our results strongly suggest that a major component of HIV binding to epithelial surfaces involves recognition of β1 integrin.

Discussion
We have developed organ culture systems to reconstruct key elements of HIV sexual transmission, by combining viable human cervicovaginal tissue, HIV virions, and the cellular and soluble constituents of human semen. Other studies that used human organ culture techniques to document physical transfer of HIV virions across epithelial surfaces drew criticism because of uncertainties regarding virion leakage around the tissue pieces (18, 32, 33). With these concerns in mind, we focused exclusively on the detection of individual HIV-infected cells and direct visualization of bound virions and cells at and beneath the exposed mucosal surfaces. A primary patient isolate of HIV established infection in resident endocervical leukocytes, at low frequency, when the virus was applied to an intact epithelial surface. This mode of HIV
In cervicovaginal organ cultures that have been designed to simulate sexual HIV transmission, we have observed rapid and extensive binding of HIV virions and penetration beneath the most external epithelial cell layers. For HIV transmission to occur in a community exposure, progression to the next event may require a chance encounter with a susceptible cell [CD4-positive T cell or macrophage or uptake by a dendritic cell (38, 39)] or the exposure may not result in primary infection. Virion binding without transmission may occur frequently, as epidemiological studies suggests that 1 in 200–1,000 exposures leads to HIV infection (1, 40). Immunohistochemical staining of 10 randomly selected cervical tissues (10 different tissue donors; tissue fixed immediately on receipt in the laboratory) with antibodies directed against CD3, CD4, and CD45RO [clone UCHL-1 (41)] revealed a broad distribution of activated T cells, ranging from occasional positive cells to focal accumulations of activated T cells. Overall, the submucosa of the endocervix consistently contained more CD4-positive cells than the ectocervix (data not shown). The differences observed in T cell numbers and distribution between different tissue donors may relate to the variability of HIV transmission rates in community settings.

Our results with human tissue and HIV are consistent with findings from vaginal SIV infections in macaques because HIV exposure in the organ culture systems also leads to initial infection of only small numbers of resident leukocytes in cervicovaginal tissues. However, as a consequence of extensive virus amplification and rapid systemic dissemination, observed in both macaques and patients with acute retroviral syndrome, any realistic strategy to curtail HIV transmission must include protection of resident tissue leukocytes that could become infected after HIV exposure (4). Given the extent of virion and cell binding to mucosal epithelial surfaces, any mechanism to reduce binding could diminish HIV transmission.

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Fig. 6. Interactions between HIV virions and cervical epithelial cells: quantitative binding and blocking assays. (A and B) Representative bright-point projections used for quantitative image analysis showing HIV-GFP virions bound to primary cervical epithelial cells (A) and intact ectocervical epithelium (B). (C) Representative image showing the blocking antibody to β1 integrin (brown) binding to the external cells of the ectocervical epithelium. (D and E) Quantitative image analysis of HIV virion binding in the presence of seminal plasma to primary epithelial cells (D, four independent experiments with different primary cell populations from four different cervical tissue samples, Cx1–Cx4); or intact ectocervical epithelium (E, five independent experiments with tissue from five different cervical tissue samples, Cx5–Cx9). Data shown have been normalized to the isotype control antibody (mIgG) and represent particle counts from 10 fields for each experimental condition. Error bars indicate the SEM.

Percent Bound

- mlgG, 50 μg/ml
- B1, 50 μg/ml
- B1, 25 μg/ml
- B1, 2.5 μg/ml
- Gal Cer, 16 μg/ml

Cx1 Cx2 Cx3 Cx4

Percent Bound

- mlgG, 50 μg/ml
- Cx5 Cx6 Cx7
- Cx8 Cx9

We presume experiments to block virion binding to intact cervical epithelium produced more variable results, presumably reflecting variability in β1 integrin surface expression levels across a tissue piece and additional variability between tissue donors. One element of donor-to-donor variability may be attributed to hormonal effects on the epithelial surfaces, according to the stage of the menstrual cycle (37). The apparent discrepancies between virion binding to primary cells and tissue pieces emphasize the importance of maintaining the closest possible link between the assay and the biological process under consideration. As virion binding to either primary cells or intact cervical mucosa was not inhibited completely by the blocking antibodies tested, other cell surface molecules may be involved in virion–epithelial cell binding. Additionally, in related experiments we observed that inert latex spheres (0.1 μm diameter) can bind or become trapped on mucosal surfaces (data not shown). If nonspecific mechanisms can also cause retention of virions, then nonspecific events may account for part of the binding not blocked by treatment with specific antibodies. In developing a comprehensive understanding of primary HIV transmission, it is important to recognize that a spectrum of both specific and nonspecific interactions may contribute to retention of HIV infectivity at mucosal surfaces until the first cycle of productive infection occurs.

infection deliberately excluded external activation of target cervical tissues to make the experimental systems conform as closely as possible to the biological reality of HIV transmission. However, preexisting inflammation in the female reproductive tract can increase susceptibility to HIV infection (5, 27, 34). The tissue distribution of susceptible CD4-positive T cells, together with morphological abnormalities in different mucosal surfaces, may contribute to the variability associated with community HIV transmission.

Studies using immortalized epithelial cells or primary populations of epithelial cells have suggested that epithelial cells are able to sequester HIV virions and that the heparan sulfate moieties of cell surface proteoglycans are involved in virion binding without transmission. We therefore speculated that heparan sulfate in the submucosal surfaces, may contribute to the variability associated with apparent discrepancies between virion binding to primary cells and tissue pieces. Therefore, the most external epithelial cell layers. For HIV transmission to occur in a community exposure, progression to the next event may require a chance encounter with a susceptible cell [CD4-positive T cell or macrophage or uptake by a dendritic cell (38, 39)] or the exposure may not result in primary infection. Virion binding without transmission may occur frequently, as epidemiological studies suggests that 1 in 200–1,000 exposures leads to HIV infection (1, 40). Immunohistochemical staining of 10 randomly selected cervical tissues (10 different tissue donors; tissue fixed immediately on receipt in the laboratory) with antibodies directed against CD3, CD4, and CD45RO [clone UCHL-1 (41)] revealed a broad distribution of activated T cells, ranging from occasional positive cells to focal accumulations of activated T cells. Overall, the submucosa of the endocervix consistently contained more CD4-positive cells than the ectocervix (data not shown). The differences observed in T cell numbers and distribution between different tissue donors may relate to the variability of HIV transmission rates in community settings.

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