Parameters of Human Immunodeficiency Virus Infection of Human Cervical Tissue and Inhibition by Vaginal Viricides

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Heterosexual transmission of human immunodeficiency virus (HIV) is the most frequent mode of infection worldwide. However, the immediate events between exposure to infectious virus and establishment of infection are still poorly understood. This study investigates parameters of HIV infection of human female genital tissue in vitro using an explant culture model. In particular, we investigated the role of the epithelium and virucidal agents in protection against HIV infection. We have demonstrated that the major target cells of infection reside below the genital epithelium, and thus HIV must cross this barrier to establish infection. Immune activation enhanced HIV infection of such subepithelial cells. Furthermore, our data suggest that genital epithelial cells were not susceptible to HIV infection, appear to play no part in the transfer of infectious virus across the epithelium, and thus may provide a barrier to infection. In addition, experiments using a panel of virucidal agents demonstrated differential efficiency to block HIV infection of subepithelial cells from partial to complete inhibition. This is the first demonstration that virucidal agents designed for topical vaginal use block HIV infection of genital tissue. Such agents have major implications for world health, as they will provide women with a mechanism of personal and covert protection from HIV infection.

Heterosexual transmission of human immunodeficiency virus (HIV) infection occurs through mucosal surfaces and is the major route of infection worldwide. This mode of transmission is increasing in prevalence more rapidly than any other in the West (31, 33, 42). Male-female transmission rates (per contact infectivity estimated to be 0.0009 in North American women) are reported to be approximately eight times more efficient than female-male rates, with history of concomitant sexually transmitted diseases (STDs) being the most strongly associated risk factor (22). Conflicting data have been reported on the selective pressure of mucosal transmission on the phenotypic and genotypic characteristics of transmitted viral isolates from a heterosexual inoculum. Predominant isolation from peripheral blood of virus able to infect both macrophages and CD4+ T lymphocytes (non-syncytium inducing [NSI], M-tropic) over those that preferentially infect T lymphocytes (syncytium inducing [SI], T-tropic), during or near to acute HIV infection have lead to the suggestion that NSI viruses may be more readily transmitted via mucosal routes (40). The mechanisms for such selective transmission of HIV isolates may be one of either selective penetration or selective amplification within the infected host (33). Analyses of genital biopsies from HIV-infected women and preliminary studies from this laboratory, using cervical organ culture as a model of primary infection, have demonstrated that HIV-infected cells reside within subepithelial mucosa, with no evidence of HIV infection of epithelial cells (20, 21, 23, 27, 35). Furthermore, it has been demonstrated that the primary targets of simian immunodeficiency virus (SIV) infection, following intravaginal infection of macaques, are cervical and vaginal subepithelial cells (35). Such data indicate that establishment of HIV infection requires transepithelial penetration. Whether intact genital epithelium presents a barrier to, or is an active participant in, HIV transmission has not been tested in primary human mucosal tissue.

Epithelium along the female genital tract differs in structural cellular organization: the vagina and ectocervix, the site most exposed to a natural inoculum, are composed of stratified non-keratinizing epithelium, whereas the endocervix is composed of a single epithelial monolayer. Multiple mechanisms for HIV transmission across genital epithelia have been proposed: direct HIV infection of epithelial cells, transcytosis of HIV through epithelial cells, epithelial transmigration of HIV-infected donor cells, uptake of HIV by intraepithelial Langerhans cells, or circumvention of epithelium via breaches in epithelial integrity (14, 33). Evidence for HIV infection of, or transcytosis through, epithelial cells is derived from in vitro studies using epithelial cell lines, which may bear little relation to primary intact genital epithelium (1, 38). Furthermore, infection or transcytosis in such models is dependent on cell-associated virus, an observation at odds with efficient mucosal cell-free SIV or feline immunodeficiency virus infection (3, 35). Strong epidemiological association of inflammatory ulcerative venereal disease with HIV transmission and observation that mucosal SIV transmission may be enhanced following thinning of vaginal epithelium by progesterone implants suggests a barrier role for genital epithelium (18, 31). Furthermore, recent studies have demonstrated that STDs increase both the number of CD4 cells in genital mucosa and the expression of chemokine receptors known to function as HIV coreceptors, thereby increasing the number of target cells (16, 24, 30). While HIV may achieve transepithelial penetration by more than one mechanism, the relatively low incidence of per-contact infectivity suggests that this is unlikely to reflect a constitutive mechanism. However, male-female transmission is also influenced by factors relating to the male partner, including seminal viral load and incidence of STDs (31), all of which have an impact on contact infectivity.

While condoms provide an effective barrier against trans-
mission of HIV and other STDs, they require the consent of the male partner, which cannot always be negotiated by women at risk for infection. Thus, there is an urgent need to develop prevention strategies that are under the personal control of women. The potential of effective topical vaginal viricides to prevent sexual transmission of HIV and other STDs is widely recognized (8). However, proper evaluation of the efficacy of such agents in blocking HIV infection of female genital tissue has been hampered by the lack of appropriate experimental models.

Thus, understanding the first critical events in genital mucosal transmission of HIV infection is important in developing strategies to block or limit such transmission. In this study, human mucosal tissue from premenopausal seronegative women was been used to define primary target cells for HIV infection within genital mucosa, differential susceptibility of such tissue to M-tropic and T-tropic HIV isolates, and the interaction of HIV with genital epithelium. Furthermore, this in vitro model has been used to determine the efficacy of potential vaginal viricides designed to protect women from HIV infection.

MATERIALS AND METHODS

Virus culture and infection. The following strains of HIV-1 were used in this study: BaL (grown in monocyte-derived macrophages), RF and HXB (grown in NC1 cells) (AIDS reagent project, National Institute for Biological Standards and Control, Potters Bar, United Kingdom), and SL-2, 2044, and 2076 (generously donated by Paul Clapham, Imperial Cancer Research Fund, United Kingdom), and SL-2, 2044, and 2076 (generously donated by Paul Clapham, Imperial Cancer Research Fund, United Kingdom). Cervical and vaginal explants, comprising of both epithelial and stromal tissue, were used for either 3- or 8-mm diameter biopsies (25). In some experiments, where indicated, explants were either processed for quantitative PCR or fixed in 3% glutaraldehyde and presence of cytokeratin markers (confirming cell origin) [K13, stratified squamous epithelial cells; K18, columnar endocervical cells] [data not shown]. The intestinal epithelial cell line I407 (American Type Culture Collection) was cultured in RPMI 10%. Minimal epithelial monolayers were exposed to cell-free virus (30 to 70 ng of HIV p24 per ml, pretreated with DNase) or chronically infected cells (pretreated with mitomycin C) for 2 or 24 h at 37°C in 5% CO2. Epithelial monolayers were washed five times immediately after exposure to cell-free HIV or chronically HIV-infected cells and washed again 48 h later. Culture medium was collected every 2 days for p24 ELISA. Eight days postinfection, monolayers were processed for PCR or cocultured with PM-1 T cells, seeded at a 1:1 ratio for 10 days for virus isolation. PM-1 T cells are susceptible to both M- and T-tropic HIV, expressing both CXCR4 and CCR5 coreceptors.

Virus transmission across polarized epithelial sheets. Cervical epithelial sheets (8 mm), isolated as described above, were analyzed by light microscopy (>200) for any visible perforation of the epithelial cell surface. Viability and functional analysis of epithelial sheets was carried out as described above. In addition, presence of functional intercellular junctions (which exclude inulin) were demonstrated by the detection of inulin transfer following EGTA treatment. Epithelial sheets were clamped in diffusion chambers (12) consisting of two acrylic plates, each of which had a central aperture (3.5-mm diameter) connected to vertical chambers providing independent access to apical and basolateral surfaces. A leakage seal was obtained by the means of concentrically positioned O-rings (6-mm diameter). Cell-free virus (30 to 70 ng of HIV p24 per ml, equivalent to 10^5 TCID50) or chronically infected cells in culture medium containing 0.5 mCi of [14C] inulin were added to the apical chamber (1 ml), while culture medium alone was added to the basolateral chamber, and polarized tissue was incubated for 2 h at 37°C in 5% CO2. Culture medium was subsequently withdrawn from apical and basolateral chambers, and the epithelial tissue was removed. Medium was tested for the presence of [14C]inulin, p24 antigen, and infectious virus by coculture with PM-1 T cells. Epithelial layers were subsequently either processed for quantitative PCR or fixed in 3% glutaraldehyde in PBS, and sent for processing for transmission electron microscopy (TEM) (Department of Anatomy, St. George’s Hospital Medical School).

Measurement of tissue viability. Potential toxicity of virucidal agents was quantitated using the principle of MTT (Sigma) dye reduction by viable explant tissue into a methanol soluble formazan product. In brief, ecotropic explants (3-mm diameter) were incubated in virucidal agent (0.01 to 100 μg/ml) overnight and subsequently washed five times in PBS. Tissue was then either immediately tested for p24 antigen (by ELISA) or fixed for EMEM for virology testing for viability (10). To assess tissue viability, washed explants were incubated in medium containing MTT (250 μg/ml) for 3 h at 37°C. Tissue viability was determined by dividing the optical density of the formazan product at 570 nm by the dry weight of the explant. Toxicity was determined by comparison of viability between treated explants and untreated control tissue. Virucidal agents were considered to be nontoxic only at concentrations that demonstrated no reduction in tissue viability at either T1 or T2. A minimum of three independent experiments using tissue from separate donors were performed for duplicate for each condition.

Immunohistochemistry. Tissues were fixed overnight in neutral buffered formalin, and 4-μm sections of paraffin-embedded tissue were prepared. Endogenous peroxidase was inactivated in paraffinized sections with a 30-min treatment in methanol-0.3% (vol/vol) H2O2. Antigens in paraffin sections were unmasked with a 10-min treatment with pronase (Dako Ltd., High Wycombe, United Kingdom). Slides were washed between incubations with Tris-buffered saline. Slides were incubated with 20% (vol/vol) rabbit serum in Tris-buffered saline for 30 min followed by overnight incubation at 4°C with one of the following primary monoclonal antibodies diluted in 20% (vol/vol) rabbit serum: anti-p24 (Kal-1) CD2 (MTF910), CD68 (PGM1), CDla (NA34), CD45RO (CD71), CD45RA (CD18), and MHC class II (WR18 [Serotec, Oxford, United Kingdom]) and L243 (American Type Culture Collection). Biotinylated rabbit anti-mouse immunoglobulin G antibody (Dako) was applied to the sections, followed by avidin-biotin peroxidase complex (Dako) and diaminobenzidine (DAB). Sections were counterstained with Mayer’s hematoxylin. For dual immunohistochemistry with primary antibodies raised in the same species, the above procedure was applied in two
RESULTS

HIV infection of genital mucosa is potentiated by immune activation. Susceptibility of female genital tract mucosa to HIV infection was investigated in vitro using mucosal tissue explants, obtained from seronegative premenopausal women undergoing hysterec- tomy as previously described (23). Ectocervi- cal, endocervical, and vaginal explants (5 mm²) were cultured under resting (medium alone) or activating (in the presence of PHA and IL-2) conditions. Explant tissue was exposed, in a nonpolarized manner, to primary NSI and laboratory-adapted SI HIV-1 isolates with known coreceptor restriction: BaL (NSI, CCR5/CCR3 restricted), IIIB, and RF (SI, predominantly CXCR4 restricted). Ectocervical and endocervical explant cultures were demonstrated to be susceptible to infection with HIV-1str., as indicated by increased accumulation of HIV p24 release independent of culture conditions (Fig. 1). In con- trast, T-tropic strains of HIV (IIIB and RF) induced significant levels of productive HIV infection only in immune activated tissue. However, productive infection with either IIIB or RF could still be rescued if tissue stimulation (with PHA) was delayed by 8 days after exposure to HIV (Table 1). Persistence of HIV within explants for 8 days prior to stimulation was dependent on HIV infection, as these effects were inhibited in the presence of zidovudine. A similar pattern of HIV replication was also observed in vaginal explants (data not shown). To determine whether other primary isolates of HIV replicated similarly to HIV-1str., ectocervical explants were exposed to primary isolates SL-2 (NSI, predominantly CCR5 restricted), 2044 (SI, CXCR4 restricted), and 2076 (SI, dual tropic, able to use CCR5, CCR3, and CXCR4). Explants were inoculated with virus within 4 h of obtaining tissue from surgery to mini- mize any potential changes in coreceptor expression and culture in the presence or absence of activating conditions. Un- like HIV-1str., (at an equivalent dose [data not shown]), all three primary strains required immune activation to induce significant levels of viral replication (Fig. 2).

Primary target cells of HIV infection within genital mucosa. Mucosal explants were processed for immunohistochemistry 7 days after in vitro exposure to HIV isolates to determine the primary target cells of HIV infection within genital mucosa. In a previous study, we have described the normal cellular distri- bution of immune cells in such cultures (23). Such studies demonstrated that CD1a Langerhans cells are exclusively found in cervical epithelium, CD3 cells are predominantly de- tected in the mucosa, closely associated with the epithelium, and CD14/68-positive macrophages are restricted to subepi- thelial mucosa. Analysis of tissue infected with M-tropic HIV-1str., in the absence or presence of stimulation, demonstrated numerous cells, within cervical subepithelial mucosa, positive for HIV p24 expression (Fig. 3A). The majority (>90%) of these cells were dual positive for p24 expression and the macro- phage marker CD68 (Fig. 3C). Few p24 cells were positive for CD3 expression, and there was no difference in their frequency between activated or resting explants. Some p24-positive cells were detected in immune activated cervical tissue exposed to T-tropic HIV isolates (IIIB and RF); however, their frequency was far lower than that seen with M-tropic strain of BaL, and it was not possible to determine their phenotype (Fig. 3B). HIV-infected epithelial cells or CD1a-positive Langer- hans cells were not detected by immunohistochemistry in any HIV-exposed explants; furthermore, in all experiments no p24-positive cells were ever detected in the epithelium regardless of their phenotype.

Lack of evidence for epithelial infection or transcytosis of HIV. Further investigations were carried out to exclude a low level of HIV infection of genital epithelial cells. In vitro cultures of primary ectocervical and endocervical monolayers were estab- lished based on previously described methods (37). Epithe- lial monolayers were exposed to either cell-free M-tropic HIV-1str., or T-tropic HIV-1RF, or to cell-associated HIV in the form of PM-1 cells (a T-cell line expressing both CCR5 and CXCR4 coreceptors) or human PBMC, chronically infected with either BaL or RF. The human intestinal epithelial cell line I407, previously demonstrated to be susceptible to HIV infec- tion and to transcytose HIV (1, 25) was used as a positive control. Primary cultures of ectocervical and endocervical epithe- lium were either very infrequent or not detectable by either light or electron microscopic analysis of primary epithelial cultures (Fig. 4B and C).

Polarized genital epithelium is impervious to cell-free or cell-associated HIV. To determine whether HIV can cross intact genital epithelium, epithelial sheets were polarized using a diffusion chamber allowing independent bathing of apical and basolateral epithelial surfaces as previously described (12). Such a system facilitated apical exposure of epithelial surfaces to HIV, as presented in vivo. Ectocervical stratified epithelial sheets were isolated following overnight digestion with dispase as previously described. Integrity of epithelial surfaces was assessed prior to experiments by direct light microscopic ex- amination of the mucosal surface and permeability assessed during experiments by [14C]inulin diffusion. Polarized cultures...
FIG. 1. Kinetics of HIV replication in cervical explant tissue. (A) Accumulation of HIV-1 DNA in ectocervical explants was determined 7 days after infection with HIV-1BaL, HIV-1AMR, or HIV-1HXB, as assayed for LTR DNA by quantitative real-time PCR as described in Materials and Methods. Explants either were prestimulated with PHA 2 days prior to HIV infection and subsequently cultured with IL-2 (filled bars) or were cultured in medium alone before and after infection (open bars). Data represents the mean and standard error of three independent experiments using paired explants from separate donors. p24 antigen release from ectocervical (B) and endocervical (C) explants was measured by ELISA. Explants were cultured either alone (open symbols, solid lines) or prestimulated with PHA (5 μg/ml) 2 days prior to viral exposure and restimulated 8 days post exposure. Stimulated explants were cultured in the presence of IL-2 (10 U/ml) (closed symbols, broken lines). Explants were exposed to HIV-1BaL (10^5 TCID_{50}) without (○) or with (●) PHA, HIV-1AMR (10^5 TCID_{50}) without (●) or with (■) PHA, or HIV-1HXB (10^5 TCID_{50}) without (□) or with (♦) PHA. Data represent the mean from a minimum of three independent experiments using paired explants from separate donors. (t test; *, P < 0.05; **, P < 0.01).
of ectocervical epithelial sheets were exposed to cell-free or cell-associated HIV as described above. Measurement of basolateral p24 release and infectious coculture assays demonstrated that stratified epithelium, which excluded [14C]inulin, was impervious to cell-free or cell-associated HIV (Table 3). Furthermore, TEM analysis of polarized ectocervical and endocervical tissue exposed to either cell-free or cell-associated HIV demonstrated no evidence of HIV transcytosis or paracellular penetration of either cell-free HIV or HIV-infected cells. In addition, no interaction between exogenous HIV-infected mononuclear cells and epithelium was observed. Lack of paracellular penetration of cervical epithelial sheets by HIV-infected mononuclear cells was confirmed by demonstration that epithelial sheets were negative, by PCR, for proviral DNA following exposure to such infected cells (data not shown).

**Vaginal virucides block HIV infection of genital mucosa.** Further investigations were carried out to determine the efficacy of potential virucides to block HIV infection of cervical explants. Compounds included nonoxynol-9 (N-9; a nonionic surfactant known to be active against HIV and other sexually transmitted agents [36]), gramicidin (GD; a peptide antibiotic with virucidal activity [2]), and PRO 2000 (a naphthalene sulfonate polymer that appears to disrupt the initial binding and fusion events of HIV infection [32]). Agents were tested (range, 100 to 0.01 μg/ml) for potential tissue toxicity following overnight culture with ectocervical explants. Viability of cervical explants, assessed by MTT assay, was reduced following exposure to N-9 or GD at concentrations of >1 μg/ml, while PRO 2000 demonstrated no toxicity at 100 μg/ml (data not shown). As potential virucidal agents should demonstrate no tissue toxicity, which could reduce barrier effects of genital epithelium, for all subsequent investigations we used N-9 and GD at 1 μg/ml and PRO 2000 at 100 μg/ml. To determine the efficacy of these agents to block HIV infection of cervical tissue, explants were preincubated with virucides for 1 h prior to overnight exposure to virus in the continued presence of virucidal agent. Explants were exposed to virus in a nonpolarized manner, allowing direct access to both subepithelial and epithelial cells, analogous to conditions of compromised genital epithelium in vivo. Following overnight exposure to virus, explants were washed to remove any exogenous virus and virucidal agent and subsequently cultured for 10 days in the presence or absence of activating conditions prior to measurement of p24 production and proviral accumulation. Presence of N9, GD, and PRO 2000 during exposure of ectocervical explants to HIV-1 (10^5 TCID50/ml) resulted in a 30%, 71%, and 97% inhibition of p24 production, respectively. PRO 2000 was the only agent to completely block proviral formation, as determined by PCR, and this agent was also able to efficiently block HIV infection with three other primary HIV strains, SL-2, 2044, and 2076, under activating conditions (PHA and IL-2) (Fig. 5).

**DISCUSSION**

**HIV infection of genital mucosa is enhanced by immune activation.** The observation that immune activation (PHA and...
IL-2) enhanced HIV infection, using a range of viral isolates (RF, IIIB, 2044, 2076, and SL-2), suggests that transmission and productive infection of genital mucosa are likely to be enhanced by concomitant immune activation in vivo. Thus, infection with STDs resulting in local inflammation, and activation of local inflammatory cells potentially renders an individual more susceptible to HIV infection. Such observations fit with reported epidemiological evidence of a correlation between STDs and HIV infection (10, 19), although this was not observed by others (41). Furthermore, our in vitro studies are supported by recent studies demonstrating an association between STDs and increased CD4 levels and coreceptor expression in genital mucosa (16, 24, 30). Demonstration that these isolates can establish productive HIV infection of cervical explants even if immune activation is delayed up to 8 days after in vitro exposure suggests that immune stimulation caused by venereal infection in vivo potentially facilitates and amplifies localized HIV infection subsequent to the transmission event. It is beyond the scope of this experimental model to determine how long such a reservoir of localized HIV infection may persist; however, the potential ability of venereal infection to

<table>
<thead>
<tr>
<th>Origin of epithelial cells</th>
<th>Virus Type</th>
<th>Strain</th>
<th>p24 antigen</th>
<th>PCR</th>
<th>Virus isolation by coculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectocervix</td>
<td>Cell free</td>
<td>IIIB</td>
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<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RF</td>
<td>0/3</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Cell associated</td>
<td>RF</td>
<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BaL</td>
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<td>0/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Endocervix</td>
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<td>IIIB</td>
<td>0/3</td>
<td>0/6</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>RF</td>
<td>0/5</td>
<td>0/4</td>
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<tr>
<td></td>
<td>Cell associated</td>
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<td></td>
<td></td>
<td>BaL</td>
<td>0/6</td>
<td>0/6</td>
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* Monolayers of primary ectocervical, endocervical, and intestinal (I407) epithelial cells were established as described in the text, and duplicate wells were exposed to either cell-free or cell-associated HIV-1<sub>INT</sub>, HIV-1<sub>INT</sub>, or HIV-1<sub>INT</sub> for 2 h. Monolayers were washed at least five times with PBS and refed every 2 days. p24 antigen levels were monitored over 10 days by ELISA. After 10 days, either ectocervical and endocervical primary epithelial cells were trypsinized and the cell pellets tested for viral DNA, or they were cocultured with PM-1 cells for a further 10 days for viral isolation. p24 levels remained below detection in the period following exposure of primary cervical epithelial monolayers to virus, and after 10 days virus could not be detected by coculture or by PCR. Similar results were obtained when primary epithelial layers were exposed to cell-free or cell-associated virus for 24 h. Cell-associated virus in the form of chronically infected PM-1 cells or PBMC produced identical results. The intestinal cell line I407 became productively infected with both cell-free and cell-associated HIV-1<sub>INT</sub> but not HIV-1<sub>INT</sub>. ND, not determined.

**FIG. 3.** (A and B) Immunohistochemical staining of p24 expression (blue/purple) in an ectocervical explant 7 days after infection with HIV-1<sub>INT</sub> (A) and HIV-1<sub>INT</sub> (B). (C) Dual staining for p24 expression (blue/purple) and macrophage marker CD68 (brown) in an ectocervical explant 7 days after infection with HIV-1<sub>INT</sub>. Original magnification for all panels, ×400.
activate localized HIV infection in this manner has serious implications for transmission of HIV. In contrast to the isolates mentioned above, HIV-1 BaL (able to use both CCR5 and CCR3 coreceptors) infected and replicated in both immunologically silent and activated cervical tissue with equal efficiency. The ability of HIV-1BaL to replicate efficiently in cervical tissue, without immune activation, is unlikely to be coreceptor dependent, determined by V3 hypervariable regions of the viral envelope, since both SL-2 and 2076 can efficiently utilize CCR5, and 2076 can utilize CCR3 (34). Furthermore, such preferential replication is unlikely to be dependent on macrophage tropism, as SL-2, 2044, and 2075 can all infect macrophages in vitro (34). It is possible that the observed preferential replication of HIV-1BaL may reflect expression of sequences within V1 and V2 hypervariable regions that appear to modulate efficiency of viral spread in macrophages (39). Indeed, recent observations that a concentration-dependent direct or indirect interaction between CCR5 and CD4 governs HIV infection of macrophages (26) suggests that in the absence of increased CD4 and CCR5 levels stimulated by concomitant immune activation (16, 24, 30), such regions outside V3 may provide a selective advantage for infection of genital tissue. The contribution of such regions to HIV infection of cervical tissue is the subject of ongoing investigations.

TABLE 3. Polarized ectocervical epithelium is impervious to HIV

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Virus</th>
<th>No. of patients</th>
<th>Mean % apical (SEM)</th>
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<tbody>
<tr>
<td>Intact</td>
<td>Cell free</td>
<td>6</td>
<td>0.01 (0.00)</td>
</tr>
<tr>
<td></td>
<td>Cell associated</td>
<td>6</td>
<td>0.03 (0.02)</td>
</tr>
<tr>
<td>Perforated</td>
<td>Cell free</td>
<td>4</td>
<td>3.20 (1.11)</td>
</tr>
<tr>
<td></td>
<td>Cell associated</td>
<td>3</td>
<td>4.98 (2.95)</td>
</tr>
</tbody>
</table>

* Ectocervical epithelial layers were placed in diffusion chambers, and the apical side was exposed to either cell-free or cell-associated HIV-1RIH in culture medium containing [14C]inulin for 2 h. Medium was withdrawn from apical and basolateral chambers, and the epithelium was removed. Medium was tested for the presence of [14C]inulin and p24 antigen (data shown as basolateral percentage of apical concentration) and cocultured with PM-1 cells for 10 days for viral isolation. Epithelial layers were processed for TEM. When epithelial layers were intact, no [14C]inulin or p24 antigen was detected in the basolateral chambers and no virus was detected by coculture. However, if epithelial integrity was disrupted, [14C]inulin and p24 antigen could be detected in basolateral chambers and virus could be detected by coculture in all samples. Similar results were obtained using cell-free (n = 5) or cell-associated (n = 3) HIV-1RF, both chronically infected PM-1 cells and PBMC produced identical results.

HIV INFECTION OF HUMAN CERVICAL TISSUE

FIG. 4. (A) TEM showing lymphocyte adherence to I407 epithelial monolayer (original magnification, ×7,800). Experiments representative of a minimum of five explants for each condition. (B) TEM showing ultrastructure of stratified cervical ectocervical epithelium following exposure to PM-1 T cells infected with HIV-1BaL (original magnification, ×260). The epithelial sheet has been removed from underlying stroma following overnight treatment with dispase. Epithelial tissue was exposed to cell-associated virus for 2 h in the described diffusion chamber and gently washed before processing for TEM. There is no evidence of adherence or penetration of PM-1 T cells into the epithelium (experiment representative of three). (C) TEM showing single endocervical epithelial monolayer following exposure to PBMC infected with HIV-1BaL (original magnification, ×1,200). Endocervical epithelial cells contain multiple mucus-containing vesicles and express multiple microvilli on their apical surface. Endogenous mononuclear cells can be seen in the underlying stromal tissue. The epithelial tissue was exposed to PBMC infected with HIV-1BaL for 2 h and processed as described above. There is no evidence of adherence of paracellular migration of donor PBMC (experiment representative of five).
would be in keeping with a selective amplification model of HIV transmission (33).

In contrast, isolates lacking such a potential selective advantage for replication in mucosal tissue might remain localized in genital mucosa at undetectable levels until amplified by immune activation following coincidental or subsequent infection with other STDs. Such findings correlate with the observation that recently infected women display a genotypic diversity in HIV populations isolated from genital secretions not reflected in peripheral blood (28).

Prime target cells for HIV infection of the female genital tract. The described study has confirmed that subepithelial cells are the prime target cells for HIV infection in female genital tract mucosal tissue in organ culture. Furthermore dual immunohistochemistry identified the majority of HIV-infected cells as subepithelial macrophages. Thus, these cells may represent the main target cells for HIV infection in the female genital tract. There was no evidence of HIV infection of cells within the cervical epithelium. This is in keeping with numerous previous studies demonstrating that following vaginal transmission of either HIV or SIV, infection is exclusively restricted to the subepithelial cells (20, 21, 23, 27, 35). The lack of HIV infection within the epithelium is highly likely to reflect recent observations that expression of CCR5, CCR3, and CXCR4 in cervical tissue is predominantly restricted to subepithelial cells (24, 44). However, it cannot be excluded that cell populations within the epithelium can harbor viral infection below limits of detection by immunohistochemistry.

Resistance of human endocervical, ectocervical, and vaginal epithelial cells to HIV infection, on exposure to cell-free or cell-associated HIV, was confirmed by PCR (able to detect 10 copies per 10⁵ cells), infectious coculture, and p24 release using primary epithelial cell lines. While an extremely low level of infection cannot be completely excluded, such observations contrast to previous reports of productive infection utilizing transformed epithelial cell lines (38). Chenine et al. recently reported that intestinal epithelial cells could be productively infected with laboratory (HIV-1NDK) but not primary HIV isolates (4). Such data suggest that strain differences may determine differing tropism for epithelial cells. However, our observation that HIV-1RF and HIV-1NDK (data not shown), able to infect intestinal cells, did not infect cervical epithelial cells suggests that the adaptation required for infection of intestinal cells is not transferable to those of the cervix. Such differences are more likely to reflect the lack of CXCR4 expression within the cervical epithelium (24, 44), previously demonstrated to be required for infection of intestinal epithelium (6). These results are in agreement with the consistent inability to detect HIV infection of genital epithelial cells in vivo (20, 21, 23, 27, 35) but contrast to reported detection of infected intestinal cells in vivo (17). One study has previously suggested that genital epithelial cells may be susceptible to HIV infection ex vivo (13), as demonstrated by immunohistochemical localization. Differences between this previous report and ours demonstrating a lack of infection by immunohistochemistry, proviral PCR, p24 production, and infectious coculture assay may reflect differences in the purity of epithelial cultures and/or effectiveness in the elimination of nonspecific
sticking of virus to the membrane of epithelial cells in the absence of productive infection. While transcytosis of HIV has been demonstrated in intestinal epithelial cell lines (1), extrapolation of these mechanisms to heteroepithelial transmission via intact genital mucosa would be premature. Indeed, data presented in this study demonstrate no evidence for transcytosis of HIV across primary human endocervical, ectocervical, and vaginal epithelial cell layers, strongly suggesting that transcytosis is not a major mechanism of transepithelial penetration across the female genital mucosa. Such findings are perhaps unsurprising since a principal strategic function of genital epithelium is protection from infection. Indeed, intestinal epithelial cells bear little resemblance to genital epithelial cells, being derived from endoderm, expressing no keratin markers, lacking stratification, and unlike genital epithelium, having a highly active endocytic phenotype. Thus, it is unlikely that genital epithelium would take on the transport function of specialized intestinal cells such as enterochromaffin cells reported to be infected in the rectal mucosa of HIV-positive subjects (17).

Other studies, using epithelial cell lines or animal models, have suggested that donor HIV-infected cells may themselves invade genital mucosa (14, 43). In this study, we observed that while PBMC transiently adhered to the I407 intestinal epithelial cell line, adherence to primary endocervical or ectocervical epithelial cultures was not detected. Furthermore, there was no evidence of HIV-infected donor cell migration into either ectocervical, endocervical, or vaginal tissue, as assessed by light and electron microscopy and by PCR for proviral DNA. Thus, an active role for either epithelia or migration of donor cells in transmucosal penetration of HIV appears unlikely in our in vitro model. In contrast, the weight of evidence presented in this study suggests that intact normal cervical and vaginal epithelial cells, in the absence of inflammatory stimuli, provide a barrier to both cell-free and cell-associated HIV. Thus, transmission of HIV infection at such tissue sites is likely enhanced by any physical breach in epithelial integrity, such as might be caused by physical abrasion, ulceration, or inflammation. This conclusion is supported by observations that factors which have the potential to decrease epithelial integrity, in particular, (i) epithelial ulceration following venereal infection and (ii) cervical ectopy which may leave tissue more friable, are associated with increased rates of HIV transmission (31, 33). Furthermore, while this study provides no evidence of HIV infection of epithelial Langerhans cells, their potential role in passive transfer of HIV across genital epithelium cannot be excluded (29).

The demonstration in this study that the target cells for HIV infection in genital mucosal tissue are exclusively found directly below genital epithelium, and that genital epithelial cells are resistant to HIV infection, strongly suggest that intact genital epithelium provides a barrier to HIV transmission. Strategies designed to protect genital epithelium are highly likely to have a major impact on heterosexual transmission rates. In this respect, aggressive syndromic management of STDs has been demonstrated in a Tanzanian trial to reduce HIV transmission rates by 42% (10). Entry of HIV through epithelial breaches would be unlikely to provide selective genotypic or phenotypic pressure on the heterogeneity of transmitted virus. Indeed, data reported here suggest that such pressure is likely to come from localized levels of immune activation, influencing coreceptor expression, and/or selective amplification of strains able to efficiently replicate in genital tissue in the absence of immune stimulation. Thus, potential virucides designed to block HIV infection should demonstrate no tissue toxicity, which could reduce barrier effects of genital epithelium, should not induce inflammation, and should be active even when epithelial integrity is compromised and/or under inflammatory conditions. Recent studies to evaluate safety and tolerability of intravaginal N-9 have suggested that its use may cause adverse effects including inflammation and reduction in numbers of lactobacilli (36). Thus, in this study, all virucidal agents were used at concentrations demonstrated not to be toxic to cervical tissue. N-9 and GD, when used at non-toxic concentrations, did not provide complete protection from HIV infection in vitro. In contrast, the virucidal agent PRO 2000 at a concentration of 100 μg/ml efficiently blocked HIV infection of cervical tissue under conditions for which neither compromised epithelial integrity and inflammatory conditions. Previous studies have detected up to 10^7 HIV-1 RNA copies/ml of semen (11); however, this is unlikely to reflect the level of infectious virions. Indeed, quantitative microculture methods have demonstrated levels of only up to 10^5 infectious units per ejaculate (7). Thus, the demonstration that PRO 2000, at a concentration of 100 μg/ml, provided complete protection against a viral inoculum with a TCID_50 of 10^5/ml suggests that this agent is highly likely to provide protection against any natural inoculum. Furthermore, such studies were carried out using a concentration of PRO 2000 that is 400 times less than that currently proposed for intravaginal use.

These studies demonstrate that the described cervical explant model of HIV infection represents a suitable model for evaluation of potential virucidal agents. Furthermore, this is the first demonstration that virucidal agents can effectively block HIV infection of genital tissue. In the absence of the imminent approval of an effective mucosal vaccine, use of such virucidal agents, designed to provide women with unobtrusive protection, is likely to have a major impact on global heterosexual transmission rates and may also prove useful in prevention of vertical transmission.

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