Productive Infection of a Cervical Epithelial Cell Line with Human Immunodeficiency Virus: Implications for Sexual Transmission

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The human cervix-derived epithelial cell line (ME180) used in this study displays a characteristic epithelial morphology, including numerous desmosomes, tonofilaments, and epidermal filaments. When T-cell lines infected with human immunodeficiency virus (HIV) are added to epithelial cultures, they rapidly adhere to the epithelial monolayer. Within a few minutes, the T cells shed numerous virions into narrow spaces formed between the epithelial cell and the adherent T cells. Virions subsequently enter the ME180 cells via large vesicles. A few days after infection, cytopathic effects and syncytium formation were observed. Infected clones of ME180 cells have remained infected for 8 months. p24 enzyme-linked immunosorbent assay and infectivity assays show that one subclone of the cell line produces virus titers equivalent to those of high-secreting HIV-infected T-cell lines. Electron microscopy reveals numerous virions budding from both the basal and apical surfaces of the epithelium. These observations suggest that cervical epithelium has the potential to serve as a site of HIV infection.

Although the principal route of transmission of human immunodeficiency virus (HIV) is through heterosexual intercourse (6), there is no consensus regarding the mechanism(s) of HIV infection. It is clear that HIV can enter through lesions (18), but it has been suggested that in some cases HIV may be transmitted by infecting intact epithelia at the portal of entry, as do many other viruses. Productively infected epithelial cells would subsequently produce new virus which might infect CD4+ lymphocytes and macrophages in the connective tissue below the epithelium. Although there is no direct evidence for this hypothesis, there are several reports which present autoradiographic evidence that gut epithelia from AIDS patients are infected (3, 7, 12, 14, 15). These data are not altogether convincing, as the structures under the silver grains cannot be visualized in the micrographs. It is possible that the label could be over intraepithelial lymphocytes which lie between epithelial cells. It has also been demonstrated that epithelial cells derived from the human intestine can be infected in vitro (5, 19, 20, 25). These data give some support to the theory that sexual transmission of HIV can occur via infection of intact epithelia. Even if epithelia can be sites of infection, it is not clear which epithelial cell type(s) is involved. In women, the cervical epithelium, vaginal epithelium, and urethra are possibilities. In addition, Langerhans cells within the vaginal epithelium have been suggested as possible targets (10, 13). In men, the penile urethra is a likely site.

It is not clear whether infection during sexual contact occurs primarily via free virions (4) or is mediated by HIV-infected lymphocytes or monocytes/macrophages. The observations that semen contains considerable numbers of mononuclear cells (24) and that mononuclear cells isolated from semen of HIV-positive men (2, 11, 28, 29) or genital secretions of infected women (22, 23) can infect lymphocytes in vitro suggest that sexual transmission of HIV can be cell mediated. We report here on an in vitro model that we have developed to explore the possibility that the cervix may be a site of HIV infection. Our findings show that a cervix-derived epithelial cell line can be infected with HIV and that infection of this epithelium is likely to be cell mediated. We believe that these observations could be relevant to HIV transmission in vivo.

MATERIALS AND METHODS

Electron microscopy. For electron microscopy, we used techniques which we have described recently (20).

Cell culture. H9/HIV-1MN, ACH2/LAV, MT-2, HT-6C (CD4-transfected HeLa), and HeLa cells were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (ARRRP). The human cervical epithelial cell line, ME180, was purchased from the American Type Culture Collection (Rockville, Md.). All cells were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum. Medium for HT-6C cells was supplemented with 10 µg of G418 (GIBCO, Grand Island, N.Y.) per ml.

Production of chronically infected ME180 cells. ME180 cells were seeded in six-well plates at a density of 2 × 10⁵ cells per well and cultured for 24 h at 37°C in 5% CO₂. After 24 h, 5 × 10⁵ H9/HIV-1MN cells, which had been pretreated with 200 µg of mitomycin C (Sigma, St. Louis, Mo.) per ml for 60 min, were added to each well and cocultured for 18 h. At 24 and 48 h, the cultures were washed six times. T cells were not observed after the medium was changed at 48 h. Cultures of HIV-infected ME180 (ME180/HIV) cells were subcultured once a week. Before subculture, the medium in which cells were grown for 24 h was collected and assayed for HIV p24 antigen.

Indirect immunofluorescence assay of CD4 expression. ME180, HT-6C, and HeLa cells, used as positive and negative controls, were grown on chamber slides (Nunc, Inc., Naperville, Ill.) for 48 h. Slides were fixed with 2% paraformaldehyde for 20 min at 0 to 4°C. Sheep antisem to human CD4 (ARRRP) was diluted 1:300 in a volume of 100 µL of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). After fixation, slides were washed and incubated with anti-CD4 for 1 h at room temperature. The
slides were washed with PBS and incubated with fluorescein isothiocyanate-conjugated anti-sheep immunoglobulin G (Pierce, Rockford, III.) for 30 min at 4°C.

**Immunoblotting for CD4.** Chronically infected ME180 cells were lysed with 0.25% Nonnal P-40. Protein extracts were separated by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis. Gels were electrophoretically transferred to nitrocellulose membranes. The membrane was blocked with 3% BSA overnight at 4°C and incubated with sheep anti-CD4 polyclonal antibody (ARRRP) at a dilution of 1:500. Immunoreactive proteins were visualized by a 3,3'-diaminobenzidine peroxidase substrate kit (Vector, Burlingame, Calif.), using an immunopure protein A/G-peroxidase conjugate (Pierce) as the second antibody.

**Polymerase chain reaction (PCR) and Southern blot detection of proviral sequences.** After a 3-month culture period, ME180 cells were trypsinized and washed with PBS. A total of 10⁶ cells were lysed with 1% Triton X-100 in 10 mM Tris buffer (pH 8.0). The DNA was extracted by using an Oncor nonionic DNA extraction kit (Oncor, Gaithersburg, Md.). The primers for the amplification reaction, SK38 and SK39 (Perkin Elmer Cetus, Norwalk, Conn.), were derived from a highly conserved region of the 136-bp fragment within the gag gene. One hundred microliters of reaction mixture contained 10 µl of chromosomal DNA (approximately 0.1 µg of DNA), 1 µM each primer, 200 µM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, and 2.5 U of Taq polymerase. After incubation at 95°C for 5 min, 40 cycles of denaturation, primer annealing, and chain elongation were performed. After amplification, sample DNA was electrophoresed in a 3% agarose gel and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Filters were incubated for 1 h at 65°C in a prehybridization solution and hybridized to a 32P-labeled, nick-translated HIV full-length probe (Oncor) by incubation at 65°C overnight. Filters were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS solution for 15 min at 55°C and then once with 0.5× SSC-0.1% SDS for 15 min at 65°C and exposed overnight to Kodak X-Omat AR film at -70°C.

**p24 ELISA and infectivity.** The amount of HIV antigen production was quantitatively estimated by enzyme-linked immunosorbent assay (ELISA), using a commercial kit for detection of HIV-1 p24 antigen (Coulter, Hialeah, Fla.) as instructed by the manufacturer. Infectivity of supernatants from infected cultures was determined by the method of Johnson and Byington (9).

**RESULTS**

**Characteristics of the ME180 cell line.** ME180 cells were originally isolated from a human omental metastasis of a rapidly spreading cervical carcinoma (21). Morphologically, the ME180 cells are remarkable in that they have retained many of the features of differentiated stratified squamous epithelia. When confluent monolayers are viewed with phase microscopy, small dense structures are seen between cells. In the scanning electron microscope, these features appear as numerous thin processes which extend from cell to cell (Fig. 1a). In transmission electron micrographs, the processes are seen to be derived from the opposing cells and to be connected by prominent desmosomes with emanating tonofilaments (Fig. 1b and c). The ME180 cells are characterized by prominent epidermal filaments which occur throughout the cytoplasm (Fig. 1b and c). The numerous desmosomes and epidermal filaments indicate that these cells display the typical ultrastruc-
tural characteristics of the cervical epithelial cells from which they were derived. This type of epithelium is also characteristic of the urethra, vagina, and skin. Thus, on the basis of morphology, the ME180 cell line is an appropriate model for a genital tract epithelium which may be a site of HIV infection.

The CD4 antigen. Evidence from a number of laboratories suggests that it is possible to infect cells which appear to lack CD4, the principal HIV receptor (1, 5, 8, 16, 26, 27), prompting the suggestion that there may be alternate pathways for HIV entry. We carried out immunocytochemical studies with antibodies to CD4 to determine whether the cell surface CD4 molecule was present. HeLa cells were used as a negative control, and HT-6C cells (HeLa cells that have been transfected with CD4 and are known to express surface CD4) were used as a positive control. ME180 cells were found to be CD4 negative by this method (data not shown). We also carried out Western blot (immunoblot) analysis to determine whether ME180 cells contained CD4, using CD4-positive MOLT-4 cells as a positive control and HeLa cells as a negative control. We observed a faint band in the ME180 cells which migrated at the same position as the strong CD4 band in the MOLT-4 cells; however, we observed a similar band in the lane corresponding to HeLa cells, suggesting that this band was likely to be background. From these experiments, we concluded that ME180 cells do not express detectable amounts of CD4. However, as the sensitivity of both immunocytochemistry and Western blots is limited, we cannot rule out low-level expression.

Infection of epithelial cells by cell-cell contact. To examine the structural relationships between T cells and epithelia, we grow epithelia to confluence on insert filters, add the T cells, and fix the filters at 15 min to 3 h after the T cells are added. After the filters are embedded for transmission electron microscopy (TEM), we cut thin sections perpendicular to the filter. Using this method, we have previously described structural aspects of T-cell-mediated HIV infection of epithelia derived from the human gut and trophoblast (19, 20). As was the case with those epithelia, adhesion of T cells to the cervix-derived epithelium was rapidly followed by budding of virus into spaces between the T cell and the epithelium (Fig. 2a). Within an hour after addition of the HIV-infected T cells, we observed virions which were taken into the cytoplasm of the ME180 cells via smooth vesicles in a process resembling nonspecific phagocytosis (19) (Fig. 2b).

Cytopathic effect. When we infected ME180 cells with HIV-infected T cells, we observed some morphologic effects a few days after infection. One week after infection, the effect was characterized by a reduction in cell number. Many of the remaining cells contained large vacuoles, and large multinucleated syncytial cells were frequently observed (Fig. 3a and b). To confirm that the cytopathology which we saw in living cells was caused by a direct effect of HIV-1, we examined cultures by TEM 1 week after infection. We observed virions budding from the cell surface. In addition, the vesicles within the cytoplasm of the epithelial cells contained numerous virions (Fig. 3c and d). Infection of ME180 cells with ACH2/LAV cells resulted in more intense cytopathic effects than when H9/HIV-1$_{MN}$ cells were used. Addition of uninfected H9 cells or supernatant from HIV-1-infected cultures did not cause syncytium formation or affect the morphology of the cultures.

Infection by free virus. To determine whether we could infect with free virus, we added supernatants of H9/HIV-1$_{MN}$ We did not observe cytopathic effects and have been unable to detect infection by p24 ELISA. However, we detected infection of the epithelium by PCR analysis (data not shown).

Chronically infected cell lines. The initial infection was established by coincubation with H9/HIV-1$_{MN}$ cells. When we monitored the course of virus production by p24 ELISA, we found an increase in virus production 5 to 7 days after infection. Virus production subsequently declined, remained
low for a few weeks, and then rose to stable high levels (Fig. 4).

We established several clones from these cultures by using limiting dilution. Although all of the clones were positive, the amount of p24 varied among clones, and the highest-secreting clone produced an amount of virus equivalent to that produced by high-secreting T-cell lines on a per-cell basis. The infected ME180 cells produced about the same amount of infectious HIV-1 as did H9/HIV-1MN cells (2 × 10^4 50% tissue culture infective doses per ml). PCR-Southern blot analysis was carried out to estimate the number of copies of HIV provirus contained in the chronically infected epithelium. The data indicated that the ME180/HIV cell line contained several copies per cell (Fig. 5).

**Apical versus basolateral secretion.** Experiments with CD4-transfected monkey epithelial cells, as well as human epithelial cells, have indicated that HIV secretion is primarily from the basolateral surface, as measured by virus release into the basal versus the apical compartment of bicameral culture chambers (5, 17). However, more recent evidence suggests that secretion is primarily apical (25). Our high-secreting, chronically HIV-infected cell line provided an opportunity to examine this question in a system relevant to heterosexual transmission and in which the virus production was sufficient to view HIV budding directly in the electron microscope. Although we observed virus associated with many cells, we found that some cells appeared to be producing very few virions while others were producing large amounts of virus. We observed two mechanisms of virus production. The first mechanism was budding; in cells secreting large amounts of virus, profuse budding was seen from both apical and basal surfaces (Fig. 6a). In the second mechanism, cells bud virions into vesicles within the basal cytoplasm. Viruses were subsequently released from the base of the cell when these vesicles fused with the basal plasma membrane (Fig. 6b and c).

**Production of lytic virus.** We carried out experiments to determine whether the chronically infected ME180 cells were producing infectious HIV. The medium from infected ME180/HIV cells was capable of infecting CEM and MT-2 cells; syncytia were observed about a week after addition of the supernatant. Interestingly, we found that we could induce
thelial cells from which they were derived. Adhesion between HIV-infected T cells and the ME180 cells appears to induce directional secretion of virus onto the surface of the epithelium. Large numbers of secreted virions are apparently readily taken up by the epithelium via a process which resembles nonspecific phagocytosis. Although several workers have demonstrated that epithelial cells can be productively infected with HIV, to our knowledge this is the first demonstration of a lytic infection of an epithelium and the cloning of a high-secreting epithelial cell line.

The high-secreting ME180/HIV cells have provided a useful model for the sexual transmission of HIV. Direct observation of the epithelium by TEM has shown that virus buds profusely from both basal and apical surfaces. In addition to direct budding, ME180 cells produce virus by a novel system involving budding of virus into vesicles in the basal cytoplasm which subsequently fuse with the basal plasmalemma. Theoretically, if virus is shed basally, virions would be in a position to infect underlying epithelial cells in a stratified epithelium; in the case of the basal epithelial cells, virus would be released below the epithelium where lymphocytes and macrophages are located.

PCR-Southern blot analysis suggests that ME180/HIV cells contain multiple copies of HIV proviral DNA. The virus secreted by the epithelium is infectious, since T cells which are added to the epithelium form syncytia and became vacuolated within a few days. The observation that infection of T cells occurs very rapidly when these cells are added directly to the epithelium suggests that epithelia may be capable of infecting T cells by a direct cell-cell mechanism, just as T cells readily infect epithelia by a cell-cell mechanism.

We caution that the experiments described here have been conducted with transformed cell lines. Studies carried out with normal T cells or macrophages as well as normal cervical epithelial cells are needed to determine the relevance of these results to sexual transmission of HIV.

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FIG. 6.  (a) TEM of a chronically infected ME180 cell in the process of viral budding from apical and basal surfaces. Magnification, × 7,000. (b) Infected cell containing numerous basally located vesicles. These structures contain HIV-1 virions, and viral budding into the vesicles is often observed. Magnification, × 8,000.

syncytia in just 24 to 48 h if we added T cells directly to the ME180/HIV epithelium (data not shown).

DISCUSSION

We have studied an epithelial cell line which has retained many of the differentiated characteristics of the cervical epi-