Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier

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Contact between various epithelial cell lines and HIV chronically infected mononuclear cell lines results in a massive and rapid budding of HIV virions toward the epithelium followed by their internalization into epithelial endosome-like structures. Here it is shown that as early as 30 minutes after apical contact, primary virus isolates generated from primary peripheral blood leukocytes from HIV-infected patients can cross an epithelial cell line barrier using transcytosis, the characteristic epithelial transcellular vesicular pathway. As the next step in the spread of infection, transcytosed HIV particles can productively infect mononuclear cells located at the basolateral side of the epithelial barrier. These observations suggest an alternative, rapid and efficient mechanism for transmission of HIV across an intact epithelial barrier.

The gastrointestinal, anorectal and genitourinary tracts are considered to be the major routes of natural infection for HIV (ref. 1), but the very first cellular targets in the first hours of infection have not been characterized. These mucosae have in common that each is covered by a polarized epithelial cell layer. Vagina, exocervix and anus surface exhibit a stratified organization whereas rectum, endocervix, intestine and prepuce are covered by a simple epithelial monolayer[2]. Polarized simple epithelial cells exhibit a plasma membrane separated into two clearly distinct domains by tight junctions: the apical, which faces the tract lumen, and the basolateral, which faces the serosal side and the internal milieu[3]. To perform their vectorial functions, epithelial cells have evolved transcytosis, a characteristic pathway of membrane trafficking, which allows selective and rapid transcellular vesicular transport from the apical to the basolateral pole of an epithelium[3,4].

The various secretions that act as vectors of HIV transmission contain, besides cell-free HIV particles, numerous HIV-infected mononuclear cells[1]. Contact of the HIV chronically infected mononuclear cell lines with the apical epithelial cell line surface induces an efficient and rapid budding of HIV virions at the contact site. In contrast to cell-free HIV particles, these freshly released viruses are internalized into epithelial endosome-like structures from which, after several days, eventual epithelial cell infection may proceed[5]. However, *in vivo*, epithelial cells have not been found to be consistently infected[6]. Indeed in the macaque, the first targets found infected 1 day after simian immunodeficiency virus (SIV) intravaginal inoculation[7] are dendritic cells in stratified squamous mucosa or mononucleated cells located under the simple endocervix epithelia. In this study as well, the early events in infection, that is, from virus contact with the mucosal luminal face to infection of these target cells, have not been identified.

In order to investigate these early steps of transmucosal penetration across a tight epithelial monolayer that mimics simple epithelium, epithelial cell lines and HIV-seropositive patients' peripheral blood leukocytes (PBLs), which produce HIV primary isolates, were used. An alternative mechanism for virus spread involving transcytosis of endosome-internalized HIV particles generated by the contact of HIV-infected cells with the apical surface of an epithelial cell line is described. Transcytosed viruses rapidly access the serosal side of the epithelial barrier within 1 hour without infecting the epithelium itself. In turn, transcytosed HIV could infect host submucosal mononucleated target cells and spread the infection.

HIV transcytosis across a tight epithelial monolayer

To investigate whether HIV would cross mucosal barriers by transcytosis, an *in vitro* system was established (Fig. 1*a*) that was based on previously described systems[4,8]. The model epithelium (intestinal I407, HT-29 or Caco-2 or endometrial HEC1 cell lines) was grown as a tight and polarized monolayer as it would be *in situ*, under conditions optimized for polarity development and that offered experimental independent access to both apical and basolateral media[4,9]. The apical pole of the epithelial I407 cell line monolayer was exposed to mononuclear (U1-HIV-1-LAI) or $CD4^+$ T-lymphoid (CEM-HIV-1-NDK or CEM-HIV-2-ROD) cell lines chronically infected with different HIV-1 or -2 isolates[10,11]. If HIV used the transcytotic pathway to gain access to the basolateral (serosal) side of the monolayer, then virus should be detected in the basolateral medium (Fig. 1*a*, step 2). After apical cell–cell contact, HIV-1 particles generated from either lymphocytic CEM-NDK or monocytic U1-LAI cell lines were rapidly detected in the basolateral medium (Fig. 1*b*), as were HIV-2 particles generated from CEM-ROD cell lines (see below Fig. 1*c*).

The appearance of HIV in the basolateral medium is consistent with the characteristics of transcytosis[4,12]: it is rapid and is inhibited at 4 °C and by irreversible depolymerization of epithelial microtubules with colchicine (Fig. 1, *b* and *c*). Note that epithelial cells were only pretreated with colchicine (see the Methods section), the drug was absent during the transcytosis assay to protect HIV-infected cell lines from microtubule disassembly and to preserve their full migration properties. Two additional criteria confirmed the trans- rather than para-cellular nature of HIV transport toward the basolateral medium. First, CEM-NDK cells were loaded with the fluorescent dye calcein-AM (ref. 13) before the onset of the assay. Up to 3 hours after apical contact, no fluorescent cells could be detected in the basolateral chamber (Fig.

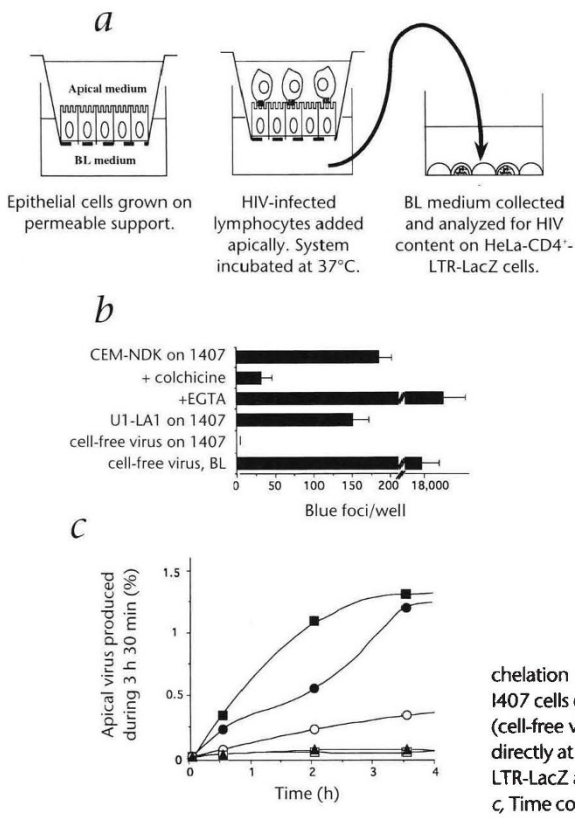

Fig. 1 HIV transcytosis across human epithelium induced by contact of HIV-infected mononuclear cells with apical surface. *a*, Scheme of the steps used to monitor HIV transcytosis coupled to cell–cell transmission. *b*, Histochemical detection of the virus in basolateral (BL) medium after 3 h of apical contact between the I407 epithelial cell line and HIV chronically infected CEM cell lines (CEM-NDK on I407). Transport is impaired by preincubation of I407 epithelium with colchicine (10 μM) (+ colchicine) but greatly increased following Ca^{2+} chelation with EGTA (5 mM) (+ EGTA). HIV-infected monocytic cell lines can induce transport through I407 cells (U1-LAI on I407).Transport does not occur when assayed with cell-free virus on human I407 cells (cell-free virus on I407). The infectious capacity of cell-free viruses is monitored by adding cell-free viruses directly at step 3 (Fig. 1*a*) (cell-free virus, BL). Values shown are numbers of blue foci formed by HeLa-CD4⁺-LTR-LacZ and are means ± s.e.m. of at least three independent experiments. Matched controls were used. *c*, Time course of HIV transcytosis across the human epithelial cell line barrier obtained in the conditions described above but detected with a more sensitive assay using *o*-nitrophenyl-β-D-galactopyranoside as β-galactosidase substrate. HIV transcytosis (■) plateaued at 2 h 30 min, was significantly impaired with colchicine (10 μM) (○), at 4 °C (□) or when monitored with cell-free virus (▲). HIV-2 released by contact between HIV-2-infected CEM (CEM-ROD) and epithelium can also be transcytosed (●). Results are expressed as percentage of apical virus produced per filter monolayer during 3-h and 30-min coculture and simultaneously quantified in triplicate together with the basolateral media on HeLa-CD4⁺-LTR-LacZ cells. All the experiments reported here were carried out in a 5% CO_2 atmosphere incubator at 37 °C. *d*, The potential translocation of the HIV-infected cell lines across the epithelial monolayer into the basolateral chamber was investigated. HIV⁺ cells were fluorescently labeled before the onset of transcytosis assay and the basolateral chamber (BL row) was observed by epifluorescence after 3 h of cell–cell contact. During a standard assay (standard column), the tight junctions remain intact as shown by the regular pattern of ZO1, the tight junction-associated protein (ZO1 row), and no HIV⁺ fluorescent cells are detected in the basolateral medium. In contrast, opening the tight junctions with EGTA (+ EGTA column) allows the paracellular translocation of the HIV-infected cells.

1*d*, left column). Similarly, if calcein labeling was performed on the basolateral medium at the end of the assay — to rule out any alteration of infected cells' dynamic properties — translocation of apically added infected cells in the basolateral chamber could not be demonstrated. In contrast, opening of the tight junctions with the chelating agent EGTA resulted in the appearance of HIV⁺ fluorescent cells in the basolateral medium (Fig. 1*d*, right column), presumably because of paracellular transport. In addition, the possibility was ruled out that HIV-infected cells might have passed tight junctions but remained in the epithelium above the filter where they could have produced HIV as follows: At the end of the transcytosis assay, the apical side of the epithelial monolayer was extensively washed to remove fluorescent HIV-infected cells located in the apical chamber. The monolayer was then carefully observed by confocal microscopy in a series of 30 optical sections, 0.4 μm apart and using an (*x*, *z*) resolution of 0.6 μm. No fluorescent cells were detected in the monolayer. Taken together, this set of experiments indicated that during the transcytosis assay, HIV-infected cells remained on the apical side of the monolayer. Second, epithelial monolayer tightness, as assessed by two well-established criteria, transepithelial resistance and measurement of [^{14}C]inulin transport[4,14,15], remained stable throughout the experiment unless EGTA was added to chelate calcium and to open tight junctions (Table 1).

Table 1 [^{14}C]Inulin transport across the epithelium and transepithelial resistance

	Time (min)	CTRL	+ CEM	+ CEM-NDK	+ CEM-NDK 4 °C	+ EGTA
[^{14}C]Inulin	0	0	0	0	0	0
(BL % apical)	30	0.075	0.05	0.075	0	96
	60	0.1	0.075	0.125	0	99
	120	0.15	0.15	0.165	0	100
	180	0.21	0.23	0.28	0	100
TER	0	130	130	130	130	130
(ohms × cm²)	30	131	128	132	129	0
	60	133	126	135	127	0
	120	125	129	129	127	0
	180	129	134	127	125	0

Tightness of the epithelial monolayer during the transcytosis assay was measured using two independent criteria: transepithelial resistance (TER) and [^{14}C]inulin transport from the apical to the basolateral pole of the monolayer. CTRL, control cells without lymphocytes. The following cells (1 million/filter) were added apically at time zero: + CEM, uninfected CEM lymphocytes; + CEM-NDK, HIV-1-NDK-infected CEM lymphocytes; + CEM-NDK 4 °C, same as + CEM-NDK but the assay was performed at 4 °C to inhibit transcytosis; + EGTA, EGTA (5 mM) was added to the monolayer to open the tight junctions and to allow paracellular transport. Values are means of at least five independent experiments, in which s.e.m. was less than 7%. Matched controls were used.

Table 2 HIV transcytosis across intestinal or endometrial epithelial cell line barriers

Epithelial cell line	Standard % apical (s.e.m.)	Transcytosis at 4 °C % apical (s.e.m.)	Cell-free HIV % apical (s.e.m.)
I407 (intestinal)	1.31 (0.06)	0 (0.01)	0 (0.01)
Caco-2 (intestinal)	1.65 (0.08)	0 (0.01)	0 (0.01)
HT-29 (intestinal)	1.43 (0.09)	0 (0.01)	0 (0.01)
HEC1 (endometrial)	1.89 (0.11)	0 (0.01)	0 (0.01)

Transcytosis of HIV generated from CEM-NDK cells as described in Fig. 1*a*. and monitored as in Fig. 1*c* . Selected human cell lines were used to form the tight epithelial monolayer. Transcytosis values are calculated as in Fig. 1*c* and given for the time point 3 h 30 min of CEM-NDK cell-to-epithelium contact. Transcytosis occurred across the various epithelial cell lines tested (standard), is inhibited at 4 °C (transcytosis at 4 °C), and is not detected for cell-free HIV (cell-free HIV) as in Fig. 1*c*. Values are means ± s.e.m. of at least four independent experiments.

The small amount of [^{14}C]inulin detected in the basolateral chamber reflected bona fide fluid-phase transcytosis[4,14] and was not modified by apical addition of lymphoid cell lines. Furthermore, when the assay was performed at 4 °C, [^{14}C]inulin was not detected in the basolateral chamber, ruling out a breakdown of the cell line monolayer.

Optimization of the detection assay (see Fig. 1 and Methods) enabled the detection of transcytosed HIV as early as 30 minutes after contact of HIV-1- or HIV-2-infected cells with the apical side of the epithelial cells (Fig. 1*c*), a kinetics in agreement with the $t_{1/2}$ of fluid-phase transcytosis[4]. Transcytosis across the I407 cell line reached a maximum at 2 hours 30 minutes, and then seemed to plateau, as does HIV budding induced by cell–cell contact[8]. Other human epithelial cell lines: intestinal HT-29 and Caco-2 (ref. 16) and endometrial HEC1 (ref. 15) grown as tight monolayers were similarly permissive to transcytosis (Table 2).

In contrast to freshly released HIV particles induced by cell–cell contact, high doses of cell-free infectious viruses are not internalized by I407 cells[5,8]. Accordingly, infectious cell-free viruses were unable to cross the epithelial cell barrier up to 3 hours after apical addition (Fig. 1*b*, cell-free virus on I407, and Table 2). HIV is an envelope virus that incorporates in its membrane host cell components in addition to the viral envelope glycoprotein[17]. This may suggest that HIV particles budded after cell–cell contact have recruited in their membranes some specific host lympho- or monocytic cell components favoring endo- and transcytosis through the epithelial barrier.

Transcytosed HIV is still infectious

Next, the infectious capacity of transcytosed HIV toward potential target cells such as human macrophages or monocytic or CD4$^+$ T-lymphocytic cell lines was addressed, as depicted in Fig. 2*a*. Each of the mononuclear cell types, CEM, H9, Sup T1 (not shown) and U937 and macrophages, when placed at the basolateral side of the epithelium, was permissive to transcytosed HIV (Fig. 2*b* and not shown). This observation suggests that mononuclear cells from the submucosa may be downstream targets of transcytosed HIV *in vivo* and could in turn convey the virus toward lymph nodes and/or allow its propagation.

HIV transcytosis involves HIV envelope and galactosyl ceramide

By what molecular mechanism does transcytosis occur? Monoclonal antibody against gp120 or recombinant HIV-1 gp120 added to the apical medium during the assay inhibited HIV transcytosis (Table 3), indicating that the HIV envelope does participate in the transport process, most probably at a binding step. Then the role of HIV gp120 cell-surface receptors was examined. The major one, CD4, is not involved: I407 cells are CD4 negative (data not shown and ref. 8), and neither anti-CD4 monoclonal antibodies against the gp120 binding site (sim4) nor against another region (sim2) interfered with HIV transcytosis (Table 3). In contrast, the alternative gp120 galactosyl-ceramide receptor (GalCer)[18,19] detected on I407 cells by immunofluorescence (data not shown) may be involved. Indeed, monoclonal antibody anti-GalCer added apically significantly inhibited HIV transcytosis by I407 cells (Table 3). The same monoclonal antibody studies conducted on HT-29, Caco-2 and HEC1 cells gave similar results (not shown).

Transcytosis of primary HIV isolates

It is well established that HIV passaged in immortalized cell lines acquires biological properties that differ from those of primary virus isolated from activated PBLs. Studies using only cell line-passaged viruses are in the process of being reevaluated. Consequently and to more closely approximate the *in vivo* situation, the capability of PBLs from HIV-seropositive patients for inducing transcytosis in this assay was also investigated. Primary PBLs from randomly selected HIV-1-infected patients, cultivated up to 2 weeks after collection, exhibited transcytotic behavior identical to that of HIV$^+$ cell lines in the assay (Fig. 3). Only HIV generated by cell–cell contact was able to transcytose across I407 cells over a 2-hour and 30-minute period. Transcytosis was markedly

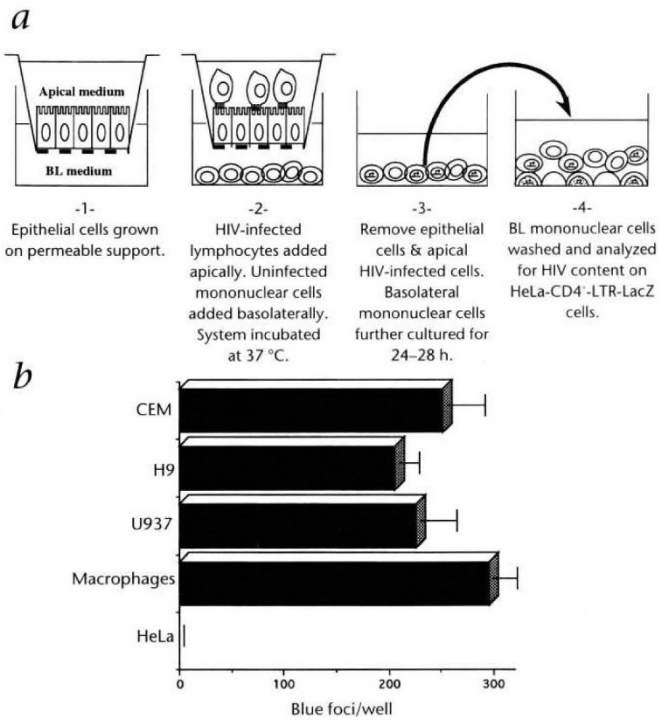

Fig. 2 Transcytosed HIV is still infectious. *a*, To monitor infectivity of transcytosed HIV, the experimental setup was modified by placing uninfected mononuclear cells in the basolateral chamber (step 2) at the onset of the assay. Their HIV content was further analyzed as depicted in steps 3 and 4. *b*, Transcytosed HIV generated from CEM-NDK cells is capable of productively infecting CD4$^+$ T-lymphocytic cell lines (H9 and CEM cells), monocytic cell lines (U937 cells) or macrophages from normal human PBLs (macrophages) after transcellular transport. Nonpermissive HeLa cell lines are shown as a negative control for infection. Values are means ± s.e.m. of at least four independent experiments. Matched controls were used.

inhibited by anti-GalCer antibodies and did not occur for primary cell-free viruses isolated from overnight culture of these same HIV-infected PBLs. These results indicated that HIV transcytosis is not a property restricted to cell lines chronically infected by HIV laboratory isolates but could also occur from PBLs infected by HIV primary isolates.

Table 3 Molecular characterization of HIV transcytosis

	mAb isotype	Percent of standard (s.e.m.)
Standard	–	100
+ mAb anti-gp120	IgG1	13 (4)
+ rec gp120	–	15 (3)
+ mAb anti-CD4 (sim4)	IgG1	96 (6)
+ mAb anti-CD4 (sim2)	IgG2b	98 (7.5)
+ mAb anti-GalCer	IgG3	12 (6)
+ irrelevant mAb	IgG1	99 (4)
+ irrelevant mAb	IgG3	101 (3)

Transcytosis of HIV generated from CEM-NDK cells and monitored as in Fig. 1*a* (standard) was inhibited by mAbs against gp120 (+ mAb anti-gp120) or by recombinant gp120 (+ rec gp120) added apically but unaffected by various mAbs against CD4 [+ mAb anti-CD4 (sim2) and (sim4)], the major HIV receptor. However, mAbs against galactosyl-ceramide (+ mAb anti-GalCer), an alternative receptor for gp120, significantly impaired HIV transcytosis. Matched irrelevant isotypes (+ irrelevant mAb, IgG1, and IgG3) were used as controls. Values are given as percent of transcytosis observed after CEM-NDK cell-to-epithelium contact for 3 h 30 min, the standard conditions. Values are means ± s.e.m. of at least four independent experiments.

Discussion

Transcytosis of HIV generated by cell–cell contact. This work strongly suggests that transcytosis is a rapid (ca. 1 hour) and efficient mechanism that may occur at the very first steps of HIV primary infection. Transcytosis applies to both HIV-1 and -2 penetration across a tight, simple epithelial cell line mucosal barrier and does not require actual epithelial cell infection, in agreement with *in vivo* observations[1,7,20]. Transcytosis requires low amounts of HIV induced from HIV-infected cells, similar to that occurring in infectious secretions like sperm or milk from seropositive patients. This contrasts with other studies conducted with a much higher viral load[7]. The transcytosis-inhibitory activity of recombinant gp120, anti-HIV gp120 and anti-galactosyl ceramide suggests that HIV transcytoses by a receptor-mediated mechanism between gp120 and its alternate receptor galactosyl ceramide. Galactosyl ceramide is enriched at the apical pole of various epithelial cells as well as in human colon[21], where it may form microdomains through hydrogen bonding. One may suggest that the tyrosine serine and threonine residues on gp120 may transiently form hydrogen bonding with epithelial galactosyl ceramide, thereby inducing HIV receptor-mediated endocytosis.

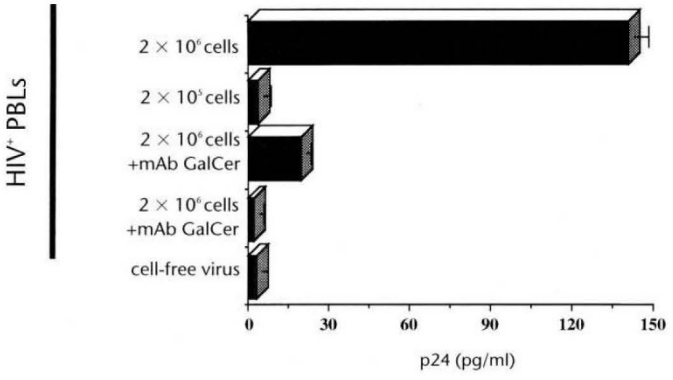

Fig. 3 Transcytosis of HIV viruses generated by contact of PBLs from randomly selected HIV-seropositive patients with the apical surface. Detection of the virus in the basolateral medium after 3 h of apical contact between I407 epithelial cells and 2×10^6 or 2×10^5 HIV-infected PBL cells. At both HIV-infected PBL quantities, transport is impaired by preincubation of the I407 epithelial cell line barrier with mAb anti-GalCer as in Table 1 (+ mAb GalCer). Transport does not occur when assayed with cell-free virus originating from the same HIV-infected PBLs (cell-free virus). Values shown are basolateral medium p24 content estimated by ELISA and are means ± s.e.m. of at least three independent experiments performed with different primary PBL samples. Matched controls were used.

Cell-free virus transcytosis is inefficient. As cell-free viruses produced in liquid culture without cell–cell contact are not internalized[8] and consequently not transcytosed (as shown here), it is surprising that virus particles budded at the site of HIV-infected lymphocyte/epithelial cell contact are immediately endocytosed and transcytosed. This tantalizing contrast between the two differently produced viruses, that is, with or without induction by cell–cell contact, is under investigation. Cell–cell contact does not modify the actin or microtubule network underlying the apical surface (not shown), even if the cytoskeleton of HIV-infected mononucleated cells is itself affected[5,22]. This suggests that there is no disappearance of the apical brush border that would facilitate retroviral endocytosis. Preliminary results (M.B., manuscript in preparation) suggest that epithelial cell–lymphoid cell contact induces the budding of viruses in which, in addition to the HIV envelope glycoprotein, some adhesion molecules from the lympho- or monocytic host cell have been actively recruited into the viral membrane. Accordingly, HIV membrane composition can be modulated by cell–cell contact[17,23]. This may explain the differences observed in endo-transcytosis.

HIV may cross mucosae by one of several mechanisms including transcytosis. Besides the transcytotic pathway that characterizes epithelial cells, HIV may penetrate mucosae (tight or loose) by other and not mutually exclusive mechanisms as previously reported: (1) by transcytosis through mucosal (M) cells as suggested from cell-free HIV-1 studies on HIV-nonpermissive rabbit M cells[24], but not confirmed by morphological studies on intestinal biopsy from seropositive patients[1]; (2) by transport through stratified loose epithelium by Langerhans cells[20] that have been found infected 2 days after cell-free SIV infection in the macaque model[7]; or (3) through trauma that gives infected cells or cell-free virus direct access to the blood. It is also clear that *in situ*, the access of transmitting HIV-infected cells to the apical surface may be complicated by the specific luminal microenvironment (mucus, glycocalix, and so on). However, mononuclear cells are well equipped with various cell adhesion molecules to bypass these obstacles[25].

The working model is summarized in Fig. 4. HIV, generated by cell–cell contact from either one of the two major cell types that is a vector for HIV, is able to cross an intact, polarized human epithelial monolayer. Transcytosis occurs in less than 1 hour, a time much shorter than that required for epithelial infection[7,18]. Moreover, downstream of the transcellular transport step, cell-free transcytosed HIV can still propagate. Molecularly, HIV gp120 on the viral side and GalCer on the target cell side modulate transcytosis. If one could demonstrate HIV transcytosis using PBLs from HIV-seropositive patients, more work under conditions closer to those found *in vivo* would be desirable to validate the data presented here.

Methods

Cells. *Epithelial cell lines.* Intestinal cell lines I407 (fetal) [obtained from the

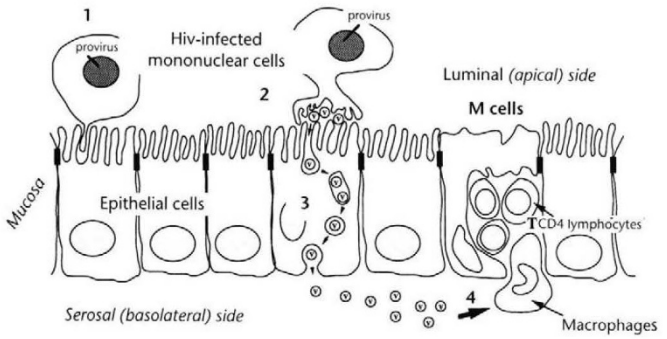

Fig. 4 Working model for HIV entry across an intact mucosa. *Step 1*, HIV-infected mononuclear cells, major HIV vectors in various secretions or semen, adhere to the apical luminal surface of the epithelium. *Step 2*, Cell–cell contact induces an extensive release of HIV particles polarized toward the epithelium without rupture of the monolayer integrity. Consequently, epithelial cells internalize viral particles by a mechanism involving both gp120 HIV envelope and the apical galactosyl-ceramide but not CD4. *Step 3*, From endosome-like structures, HIV crosses the epithelium and, at 30 min, starts to be released into the basolateral medium as a result of transcytotic vesicle fusion with the basolateral membrane. Infection of epithelial cells leading to virus production is not shown in this drawing as it is detected much later, that is, several days after cell–cell contact, a time frame often longer than the epithelial cell half-life *in vivo*[32]. *Step 4*, The released HIV particles, which are infectious, are able to productively infect macrophages or intraepithelial lymphocytes from the submucosa. These cells could therefore become new vehicles for viral dissemination.

American Type Culture Collection (ATCC), Rockville, MD], HT-29-INO and Caco-2 (carcinoma) (gifts from P. Codogno, Paris, France) or endometrial cell line HEC1 (carcinoma) (ATCC) were grown on filters [polycarbonate, 12-mm diameter, 0.45-μm pore size (Costar, Cambridge, MA)] as described elsewhere[4,8]. Briefly, 2×10^5 cells were seeded per filter insert and grown for 5 days (I407), 7 days (HEC1, Caco-2) or 14 days (HT-29-INO) with daily change of medium, resulting in a tight monolayer as monitored both by transepithelial resistance (TER) and routinely by overnight "leakage tests"[9,14,26]. No differences concerning transcytosis were found by growing the cells on a 3-μm pore size filter.

Mononucleated cells were grown in RPMI 1640, with glutamine 1 mM and 10% heat-inactivated fetal calf serum (FCS), and adjusted at 1 million cells/ml 18 h before the onset of the assay.

Uninfected cells used were as follows (1) $CD4^+$ T-lymphocytes: CEM, H9 or Sup T1 cell lines; (2) monocytes: U937 cell line; (3) adherent macrophages purified from normal human PBLs on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden); and (4) HIV-nonpermissive HeLa cells[27] as control. Chronically HIV-infected cell lines used were as follows (1) HIV-1 $CD4^+$ T lymphocytes, CEM-NDK; (2) HIV-2 $CD4^+$ T lymphocytes, CEM-ROD; and (3) HIV-1 monocytes, U1-LAI.

Peripheral blood leukocytes from HIV-seropositive patients were purified on Ficoll-Hypaque and cultivated up to 20 days at 2 million/ml in RPMI 1640, with 10% FCS supplemented with interleukin-2 (10 U/ml) and changed twice weekly. Virus production was routinely tested using a p24 ELISA kit (Mediators, provided by the National Institutes of Health AIDS Research and Reference and Reagent Program) according to the manufacturer's instructions, and was always above 1000 pg/ml.

Transcytosis assay. The standard transcytosis assay was performed as follows (see Fig. 1*a*). Epithelial cells were grown on filters as a tight monolayer (step 1). Chronically HIV-infected cells were washed to remove free viruses and added to the apical chamber at a ratio of 1:1, mononuclear-to-epithelial cells in 300 μl (step 2). When used instead of HIV-infected cells, cell-free viruses (corresponding to overnight culture production at 8×10^5 cells/ml of the same amount of cells added for the standard assay) were concentrated by centrifugation (5 min, 180,000*g*) and added apically in 300 μl of medium. Cocultures were recultured at 37 °C in the 5% CO_2 incubator. At each time point indicated, the coculture cell system was removed. Corresponding basolateral media were analyzed for HIV content (step 3) with a bioassay that detected HIV histochemically as blue foci of HeLa-$CD4^+$-LTR-LacZ as originally described[27] or with a more sensitive technique[28] that is, by monitoring *o*-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co.) cleavage. For some experiments, especially when monitoring cell-free virus properties, β-galactosidase activity was also quantified using a second substrate[29], that is, chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim).

For opening the tight junction[14], 5 mM EGTA was added to the culture medium 15 min before addition of HIV-infected cells. For irreversible depolymerization of microtubules, epithelia were pretreated with 10 μM colchicine for 30 min at 4 °C followed by 10 min at 37 °C (ref. 30), and excess drug was removed by extensive washing. The drug was absent during the transcytosis assay itself to preserve HIV-infected cell lines from microtubule depolymerization.

To monitor infectious potential of transcytosed HIV (see Fig. 3*a*), each type of indicated uninfected cells (2×10^5 cells/500 μl) was added in the basolateral chamber at the onset of the assay (step 2). After allowing transcytosis to occur, the basolateral medium was further cultured (step 3). After 24 to 48 h, cells were washed to remove free viruses and analyzed for HIV content as above (step 4).

When PBLs from HIV-seropositive patients were used, the transcytosis assay was performed as above but using a 2:1 HIV^+ cell-to-epithelial cell ratio. Cell-free viruses were prepared accordingly. Transcytosis was estimated 3 h after HIV^+ cell/epithelial cell contact from p24 detection in the basolateral medium using the p24 ELISA kit as described above.

Tightness of the monolayer during the coculture period. This parameter was monitored routinely by TER (ref. 4, 14) or by measuring [^{14}C]inulin diffusion[14]. In some experiments, CEM-NDK cells were loaded with calcein-AM (Molecular Probes, Eugene, OR)[13], which becomes trapped by esterification in the cell cytosol. Calcein concentration was reduced to 30 ng/ml. At higher concentrations (3 μg/ml)[13], the fluorophore may alter mobility but only when fluorescence is excited before the mobility assay (Y.Z. Ji and M.B., unpublished results), a condition that does not apply to the present study. The resultant fluorescent HIV^+ cells were used in the transcytosis assay as described above. Three hours after the onset of cell–cell contact, the epithelial cell monolayer was fixed and labeled for ZO1, the tight junction-associated antigen as in ref. 4, by using rabbit anti-ZO1 (61-7300, Zymed, South San Francisco, CA, 1:200) as primary antibody. Labeled cells were analyzed by fluorescence microscopy. The basolateral medium was directly observed by epifluorescence to detect putative transcytosed HIV-infected cells. In some experiments, calcein labeling was performed at the end of the coculture period, directly on the basolateral medium.

Inhibition experiments. Recombinant glycosylated HIV-1-SF2 gp120 expressed in CHO cells (5 μg/ml, no. 386 from the NIH AIDS reagent program) or antibodies were added apically 10 min before CEM-NDK cells[18] and maintained throughout the 3-h and 30-min experiment. Monoclonal antibodies were as follows: against CD4: sim4 (IgG1) and sim2 (IgG2b) (1:100 ascites), against gp120: F105 (IgG1) (1:200) (all three provided by the NIH AIDS Reagent Program), and anti-GalCer (IgG3)[31] (1:40 cell culture supernatant). By immunofluorescence[4], the last-named antibody positively stains the I407 cell line (data not shown). It was verified that the antibodies added apically did not nonspecifically transcytose during the assay and did not modify HIV quantification with the HeLa-$CD4^+$-LTR-LacZ bioassay (data not shown). Matched appropriate irrelevant isotype antibodies were used as negative controls: IgG1 or IgG3 (10 μg/ml) (Southern Biotechnology Associate, Birmingham, AL). Values are given as percent of transcytosis observed after CEM-NDK cell/epithelium contact for 3 h 30 min, the standard conditions.

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