High Level of Coreceptor-independent HIV Transfer Induced by Contacts between Primary CD4 T Cells*

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Julia Blanco‡§, Berta Bosch‡, Maria Teresa Fernández-Figuera§, Jordi Barretna‡, Bonaventura Clotet‡, and José A. Esté‡

From the §Retrovirology Laboratory, Fundación irisiCaixa and the ‡Department of Pathology, Hospital Universitari Germans Trias i Pujol, Badalona 08916, Barcelona, Catalonia, Spain

Cell-to-cell virus transmission is one of the most efficient mechanisms of human immunodeficiency virus (HIV) spread, requires CD4 and coreceptor expression in target cells, and may also lead to syncticum formation and cell death. Here, we show that in addition to this classical coreceptor-mediated transmission, the contact between HIV-producing cells and primary CD4 T cells lacking the appropriate coreceptor induced the uptake of HIV particles by target cells in the absence of membrane fusion or productive HIV replication. HIV uptake by CD4 T cells required cellular contacts mediated by the binding of gp120 to CD4 and intact actin cytoskeleton. HIV antigens taken up by CD4 T cells were rapidly endocytosed to trypsin-resistant compartments inducing a partial disappearance of CD4 molecules from the cell surface. Once the cellular contact was stopped, captured HIV were released as infectious particles. Electron microscopy revealed that HIV particles attached to the surface of target cells and accumulated in large (0.5–1.0 μm) intracellular vesicles containing 1–14 virions, without any evidence for massive clathrin-mediated HIV endocytosis. The capture of HIV particles into trypsin-resistant compartments required the availability of the gp120 binding site of CD4 but was independent of the intracytoplasmic tail of CD4. In conclusion, we describe a novel mechanism of HIV transmission, activated by the contact of infected and uninfected primary CD4 T cells, by which HIV could exploit CD4 T cells lacking the appropriate coreceptor as an itinerant virus reservoir.

For many viruses, cell-to-cell virus transmission is the most efficient mechanism of viral spread because of the extremely low infectivity of free viral particles (1). In the case of human immunodeficiency virus (HIV), free viral particles are infectious but show a short life span at 37 °C (2) and lower infective capacity than cell-to-cell transmission (3). This latter phenomenon is favored by the polarization of viral production in the infected cell (4) and the viral receptors and coreceptors in the target cell leading to the formation of a functional (infectious) virological synapse (5, 6). Cell-to-cell virus transmission is probably involved in the spread of HIV among different populations of CD4+ cells in vivo and seems to play an essential role in sexual or vertical transmission through epithelia (7, 8).

The process of membrane fusion induced by the envelope glycoprotein complex of HIV is independent of pH, and therefore endocytic internalization and endosomal acidification are not required to activate HIV entry into the cytoplasm (9–12). Instead, viral entry involves direct fusion of viral and plasma cell membrane that allows for the delivery of the viral core into the cytoplasm of target cells (13). First, HIV envelope (gp120/gp41, Env) binds to CD4 and then to a chemokine receptor (CCR5 or CXCR4 for HIV strains and CCR5 or R5 strains), which is used by HIV particles to activate the gp41-mediated membrane fusion.

A variety of cell types such as macrophages, endothelial and epithelial cells, and also lymphoid cells are able to bind and to internalize HIV particles into vesicular structures coordinately or independently of HIV receptors (7, 14–18). Once internalized, HIV particles may follow different pathways: they can be secreted (19), as in the case of transcytosis (7), degraded (20), or they can fuse with vesicular membranes to inject the viral core into the cytoplasm and initiate the infectious viral cycle (16, 17). Consistently, pharmacological stabilization of the endosomal compartment increases HIV infection (18, 20). Nevertheless, the exact contribution of endocytic pathways to the infection of CD4 T lymphocytes and to HIV pathogenesis in vivo is mostly unknown, and the mechanisms involved in endocytosis are unclear. The term endocytosis includes at least four mechanisms: phagocytosis, macropinocytosis, clathrin-mediated and caveolin-mediated endocytosis (21), showing different properties such as vesicular size, markers, and regulation. Macropinocytosis, which is characterized by big sized vesicles, and clathrin-coated pit-mediated endocytosis seem to be the major nonfusogenic HIV entry pathway in most cell types (15, 16).

HIV transmission during cell-to-cell contacts follows the same mechanism as cell-free HIV particles for entry into target cells. However, the potential implications of synaptic structures in the mechanism of transmission are still unclear. We have used previously developed experimental models to study cell-to-cell contacts during HIV infection (22–24). We observed that during cellular contacts primary cells underwent massive cell-to-cell viral transmission. The characterization of this phe-
nomenon showed that in addition to fusion-dependent HIV transmission, high amounts of HIV were reversibly transferred from infected cells to trypsin-resistant compartments of target cells by a mechanism that required gp120 binding to CD4 but not to coreceptor function. CD4 T cells lacking the appropriate coreceptor may therefore become an itinerant virus reservoir that may contribute to the spread of HIV infection.

**Experimental Procedures**

Cells—Peripheral blood mononuclear cells (PBMC) from healthy donors were purified by Ficoll-Hypaque sedimentation. When necessary, CD4 T cells were immediately purified (>95%) from PBMC by negative selection using the CD4 T cells enrichment kit (StemCell Technologies, Vancouver, Canada). Unless indicated, primary cells were used without previous stimulation. The CEM cell line derivative A201 lacking CD4 expression and several clones expressing either wild type CD4 (A201), a truncated form of CD4 at the position 403 which lacks the cytoplasmic tail (A201/403), and a chimeric CD4/CD8 protein (A201/CD8) that contains the gp120 binding site of CD4 were described by Dr. D. Litman (11) and obtained through Dr. M. Biard (Montpellier, France). Primary cells and CEM derivatives were cultured in RPMI. HeLa P455 and H9262/C4/C5R5 (NIBSC and NIH, AIDS Reagent Programs) were cultured in Modified Eagle’s medium. Media were supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and selection antibiotics when required.

**Viruses and Chronically Infected Cells**—Recombinant viruses carrying envelope (Env) sequences corresponding to the X4 HIV-1 strain NL4-3 or the R5 HIV-1 strain BaL were constructed in a HIVHXB2 vector (24), were grown and characterized for Env expression and virus production (24). Uninfected MOLT-4/C5R5 cells were used as negative controls in all experiments. 8E5 cells, which carry one copy of the HIV genome defective for reverse transcriptase activity and produce noninfectious viral particles (26), were cultured in RPMI.

**Cocultures of Infected and Uninfected Cells**—Primary cells (PBMC or purified CD4 T cells depleted of 105 cells) were usually cultured in 96-well plates with effector (uninfected or infected) MOLT-4/C5R5 cells at a ratio of 1:1 in the absence or the presence of the following HIV inhibitors: 10 μg/ml murine IgG (Santa Cruz Biotechnologies), 0.25–5 μg/ml anti-CD4 mAb Leu3a or 10 μg/ml L120.3 (Becton & Dickinson), 10 μg/ml neutralizing anti-gp120 mAb IgGb12 or 2G12, 1 μg CCR5 antagonist TAK779 (all reagents from NIH AIDS Reagent Program and NIH AIDS Reagent Project), 1 μM gp41 inhibitor (Service of Peptide Synthesis, University of Barcelona), and 2 μM reverse transcriptase inhibitor AZT (Sigma). Other drugs used (all from Sigma) were 100 nM bafilomycin A1, 1 nM concanamycin A, 30–100 μM amiloride, and 1–3 μM cytochalasin D.

The A201 cell line and its derivatives were cultured for 6 h in conical bottom wells (for effector (uninfected or BaL-infected) MOLT-4/C5R5 cells at a ratio of 1:1 in the absence or presence of the 0.25 μg/ml of the anti-CD4 mAb Leu3a or 1 μM gp41 inhibitor C34. Target cells were labeled with 1 μM CMFDA (green cell tracker, Molecular Probes) and washed exhaustively before being cocultured with effector cells.

**Evaluation of HIV Transfer**—After coculture, cells were stained with anti-CD8-PerCP and anti-CD4-FITC to identify cellular populations, or stained with Leu3a or L120.3 anti-CD4 mAbs to evaluate CD4 internalization. The action of trypsin was controlled by the disappearance of the Leu3a epitope on CD4 in trypsin-treated cells (25). Trypsin action was stopped by the addition of fetal calf serum. Cells were then washed and stained as indicated above. Quantification of HIV transfer was either assessed by the percentage of p24+ cells (using uninfected cells as control) or by the relative fluorescence intensity (RFI), which reflects the ratio of mean fluorescence intensity (MFI) values obtained from infected and uninfected samples.

In some experiments, up to 1 μg/ml Alexa 488-labeled dextran (Molecular Probes) was added to cells 30 min prior to mixing and maintained during the coculture period (6–24 h). Analysis of dextran uptake was performed by flow cytometry and fluorescence microscopy.

**Fusion Assays**—HIV-mediated fusion was determined in cocultures of uninfected or infected MOLT-4/C5R5 cells with reporter HeLa P455 cells (ratio 1:1) in the absence or presence of the above-mentioned drugs. Reporter cells express β-galactosidase under the control of the HIV-1 long terminal repeat, which is activated after cell-to-cell or virus-to-cell fusion. After 24 h of coculture, syncytium formation was scored by visualizing cultures in a Nikon Eclipse TE-200 microscope, and long terminal repeat transactivation was measured by determining β-galactosidase activity in lysed (0.5% Nonidet P-40) cells as described previously (24).

**Electron Microscopy**—Cocultured cells were washed in phosphate-buffered saline, fixed in 2.5% glutaraldehyde for 1 h, and washed twice in cacodylate buffer before postfixation in 1% osmium (1 h) and final wash in cacodylate buffer. Hot (50 °C) agar was then added to the cells. After solidification, small (1 mm3) blocks were cut, dehydrated in increasing concentrations of ethanol, included in araldite, and incubated at 60 °C for 48 h. Thin sections were cut to select areas of the block to be processed for analysis of ultrastructural sections in a Jeol 1010 electron microscope.

**Infectivity of Captured Virions**—To analyze the fate of captured virions, we purified CD4 T cells after coculture with BaL-infected MOLT-4/C5R5 cells. Briefly, 10 × 106 CD4 T cells were cultured with 10 × 106 BaL-infected MOLT-4/C5R5 in a final volume of 1 ml in the presence or absence of 10 μM TAK779 or 10 μg/ml mAb IgGb12. After 6 h of coculture, cells were recovered, and CD4 T cells were purified again by negative selection using the CD4 T cells enrichment kit. Purified cells were assayed for the percentage of contaminating MOLT-4/C5R5 cells by both morphological parameters and p24 staining and treated with trypsin for 10 min to remove extracellular attached viruses and antibiotics. After extensive washes in phosphate-buffered saline, cells were cultured in RPMI supplemented with 10% fetal calf serum in the absence or presence of 10 μM TAK779 or 5 μM TAK771. Virus infection was evaluated at 12, 24, 48, and 72 h in the supernatant (Innogenetics, pony and trypsin) and/or by flow cytometry as indicated above. Supernatants were recovered at 12 h of culture, and their infective titer was evaluated in U87.CD4/C5R5 cells. Syncytium formation were evaluated at day 6 postinfection by visualizing cultures in a Nikon Eclipse TE-200 fluorescence microscope after staining with 1 μM Hoechst 33342. Wells showing syncytia with more than four nuclei were scored as positive to calculate infectious titer.

**Analysis of HIV Transfer in Primary Cells**—PBMC were stimulated for 48 h with 1 μg/ml phytohemagglutinin and 10 IU/ml interleukin (IL)-2 and then infected with the R5 HIV-1 BaL strain (100 ng of p24 antigen/ml) for 48 h. After removal of extracellular HIV particles, virus was depleted to 106 cells/ml in RPMI containing 20% fetal calf serum and 10 IU/ml IL-2. In the following days, the expression of p24 antigen, CD3, CD4, and CD8 was monitored by flow cytometry as described above. Usually at day 4 after infection, the percentage of CD8 /CD4 / p24 cells reached a plateau (4–6% of total lymphocytes), and CD8+ and CD4+ cells were scored as positive to calculate infectious titer.

**RESULTS**

**Transfer of HIV Antigens during Cell-to-Cell Contacts**—We studied cell-to-cell virus transmission in cocultures of unstimulated PBMC (target cells) with several MOLT-4/C5R5 cell lines (24) either uninfected or infected with the CXCR4-using HIV isolate NL4-3 or the CCR5-using HIV isolate BaL (effector cells). After 24 h of coculture, staining for HIV antigen p24 (p24) revealed high levels of HIV antigens in PBMC (21 and 55% of primary cells stained positive for p24 after contacting HIV-1 NL4-3- and BaL-infected cells, respectively, Fig. 1A).
Cell-free virus preparations containing amounts of p24 equivalent to infected cells failed to transfer HIV antigens to unstimulated primary lymphocytes 24 h postinfection (3% of p24 cells, Fig. 1B), indicating that p24 antigen uptake occurred during cell-to-cell contacts. Combined CD3 and CD8 staining showed a low level of transfer of p24 (less than 10% of p24 cells) to CD4 lymphocyte subsets (CD3+/CD8+, CD3+/CD8−, or CD3−/CD8−, Fig. 1C), whereas a high percentage of CD3+/CD8+ cells (identified as CD4+ cells, Fig. 1D) were positive for p24 antigen staining.

In all experiments performed, BaL-infected cells induced the highest level of HIV antigen transfer, albeit of low CCR5 and high CXCR4 expression in target cells (10% and 96% of positive cells, respectively; data not shown). Moreover, the transfer of BaL antigens from infected to target cells was blocked by the neutralizing anti-CD4 mAb Leu3a (Fig. 2A) but was unaffected by a coreceptor antagonist, a gp41 peptide or a reverse transcriptase inhibitor TAK779, C34, or AZT, respectively (Fig. 2A). Similar results were observed when NL4-3-infected MOLT-4/CCR5 cells were used. In this case, the blockade of CXCR4 with AMD3100 or gp41 with C34 induced a significant increase in the amount of transferred NL4-3 antigens (Fig. 2B), concomitant to a total inhibition of cell-to-cell fusion and cell death (22). The activity of anti-HIV drugs was evaluated in cocultures of MOLT-4/CCR5 cells with HeLa P4R5 cells and contrasted with the lack of inhibition or enhancing effect on virus transfer (Table I). To confirm that p24 content in CD4 T cells was the result of direct antigen transfer, we cocultured PBMC with 8E5 cells, which produce replication-defective HIV particles (26). Coculture resulted in the transfer of high amounts of HIV antigens to CD4 T cells (Fig. 2C) which was inhibited by Leu3a, unaffected by the reverse transcriptase inhibitor AZT, and increased by the fusion inhibitor C34 (Fig. 2D). Taken together these results suggested that accumulation of p24 in CD4 T cells occurred by fusion-independent transfer of HIV antigens from infected to target cells in the absence of de novo viral production in CD4 T cells.

**CD4 T Cells Internalized HIV Particles and Cell Surface CD4 during Cell-to-cell Contacts**—To evaluate the role of HIV binding to CD4 in viral transfer, we measured the occupancy of the gp120 binding site of CD4 using the Leu3a mAb, directed against this site. Cell surface CD4 expression was monitored using the gp120-insensitive mAb L120.3. Cocultures of HIV BaL-infected cells with CD4 T cells reduced the levels of CD4

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**FIG. 1. Enhanced transfer of HIV antigens to CD4 T cells during cell-to-cell contacts.** A, uninfected or HIV-infected (NL4-3 or BaL) MOLT-4/CCR5 cells (R1) were cultured for 24 h with freshly isolated unstimulated PBMC (R2) and stained for HIV p24 antigen. Effector cells (MOLT-4/CCR5) and target cells (primary lymphocytes) were gated according to forward and side scatter values prior to analysis. B, equivalent amounts of p24 antigen (73 and 35 ng of p24 antigen/well for NL4-3 and BaL viruses, respectively) were presented to PBMC as cell-free virus preparations (empty bars) or infected cells (solid bars). Analysis of p24 in target cells was performed after 24 h of culture as in A. C, in these cocultures, lymphocyte subsets were gated according to CD3 and CD8 expression and then analyzed for p24 antigen staining. D, quantification of the percentage of positive cells in each subset showed the highest levels of HIV antigens in CD3+/CD8− cells, identified as CD4+ T cells (mean ± S.D. of two independent experiments).
expression in target cells as measured by both Leu3a and L120.3 mAbs (Fig. 3A). Reduction of MFI was higher for the Leu3a mAb but was also significant for the L120.3 epitope. Interestingly, the extent of CD4 down-modulation correlated with the extent of HIV uptake (35 ± 14% and 69 ± 4% reduction induced by NL4-3 and BaL-infected cells, respectively, Fig. 3B), suggesting that a fraction of CD4 molecules disappears from the cell surface during cell-to-cell contacts.

Because HIV binding has been associated with endocytosis in different cell lines including primary cells (29), we investigated the role of HIV internalization in virus transfer. Trypsin treatment of CD4 T cells cocultured for 24 h with uninfected or BaL-infected MOLT-4/CCR5 cells removed almost completely (more than 90%) cell surface CD4. However, trypsin had a low impact in the percentage of p24" cells (89% versus 86%, Fig. 3C), although the MFI of p24 antigen staining was lowered by 58 ± 4%. Increasing the time of trypsin treatment failed to increase p24 removal (Fig. 3D). Moreover, similar amounts of trypsin-resistant p24 antigen were observed in the presence of the inhibitors TAK779 and C34 (Fig. 3E). Kinetic analysis of trypsinized or untreated cocultures of MOLT-4/CCR5 cells with CD4 T cells showed HIV transfer at short incubation times (2–4 h) that rapidly directed p24 antigen to trypsin-resistant compartments. Longer incubation times (up to 24 h) allowed for the progressive accumulation of extracellular HIV antigens from BaL-infected cells. In contrast, NL4-3-infected cells transferred p24 antigen with a different profile, showing a stable and low level of trypsin-resistant antigen transfer (Fig. 3F).

In fluorescence microscopy analysis of trypsinized cocultures, HIV BaL-infected cells showed homogeneous p24 antigen staining, whereas the smaller target cells showed punctuated trypsin-resistant staining (Fig. 4A, i–iii). We further confirmed HIV internalization by electron microscopy. Effector and target cells were clearly identified by size, heterochromatin structure, and cytoplasm opacity. Ultrastructural analysis revealed strong cell-to-cell contacts between HIV-infected MOLT-4/CCR5 and CD4 T cells in which viruses accumulate. HIV particles were readily identified on the surface of CD4 T cells at the sites of cell-to-cell contacts (Fig. 4B, i) and at sites of membrane appendage (Fig. 4B, ii), inducing lamellipodia that seemed to engulf HIV particles. Moreover, 14% of cells showed large intracellular vesicles ranging from 0.2 to 1.2 μm, with an average diameter 0.7 ± 0.2 μm (Fig. 4B, iii–vi). Most positive cells displayed at least one vesicle that contained 1–14 (8 ± 4) viral particles, confirming that primary CD4 T cells internalize HIV by endocytic mechanisms after viral presentation by infected cells.
HIV Uptake Required Intact Actin Cytoskeleton and Binding of gp120 to CD4 but Not the Cytoplasmic Tail of CD4—

To characterize the requirements for the activation of HIV uptake induced by cellular contacts, we investigated the effect of several mAbs directed against different epitopes of CD4 or gp120. The anti-CD4 mAb Leu3a and the anti-gp120 mAb IgG12 that block gp120-CD4 interaction inhibited HIV uptake by CD4 T cells either measured as the percentage of p24<sup>+</sup> cells (Fig. 5) or as the MFI of p24 staining in CD4 T cells (data not shown). This effect was concomitant with a near total inhibition (80–100%) of HIV envelope-induced fusion (Fig. 5). In contrast, anti-gp120 or anti-CD4 antibodies (2G12 and L.120.3, respectively) shown not to block the binding of gp120 to CD4 (27, 31) showed little or no inhibitory effect on HIV transfer, irrespective of their effect on Env-mediated fusion. A 32% reduction in the transfer of BaL antigens was observed in the presence of 2G12. How-
ever, as noted for C34 and AMD3100 (Table I), a 2-fold increasing effect of 2G12 was observed in NL4-3 transfer, which was associated to the inhibition (71%) of fusion.

Disruption of actin function by cytochalasin D treatment reduced by near 80% the amount of transferred NL4-3 or BaL antigens, either total (not shown) or trypsin-resistant (Fig. 5), suggesting that cytoskeleton rearrangements are required not only for viral internalization but also for the massive HIV binding to CD4 induced by cellular contacts. Conversely, cytochalasin D increased the infection of HeLa P4R5 cells induced by NL4-3 and BaL-infected MOLT-4/CCR5 cells as described previously (32), highlighting the different mechanisms operating cell-to-cell HIV transmission in primary cells. A similar effect was found when different inhibitors of vesicular trafficking were used (bafilomycin A1 and concanamycin A). Both drugs failed to increase HIV transfer to CD4 T cells at 24 h (Fig. 5) or shorter time points (not shown). In contrast, both drugs enhanced HIV infectivity in HeLa P4R5 cells (Fig. 5) as described previously (14, 20). Amiloride, a classical inhibitor of macropinocytic entry, failed to modify either transfer or fusion at the highest nontoxic concentration tested (Fig. 5).

The key role of CD4 in HIV binding and internalization may suggest a mechanism of CD4-dependent endocytosis of HIV particles. However, this mechanism is known to be mediated by clathrin-coated pits (33, 34), a phenomenon seen only sporadically in the electron microscopy analyses of HIV-loaded CD4 T cells (data not shown); in our cocultures, most HIV particles appeared to be endocytosed by membrane invaginations (Fig. 4B). To clarify the role of CD4 in the endocytosis process, we used several cell clones either lacking CD4 and CCR5 expression (A201), expressing CCR5 (A201/CCR5), or expressing wild type CD4 (A301), a truncated form of CD4 at position 403 (A201/403) or a chimera CD4-CD8 (A201/CD8). The inability of truncated or chimeric form of CD4 to be endocytosed was analyzed after treatment of cells with phorbol 12-myristate 13-acetate, which resulted in the exclusive down-regulation of wild type CD4 (data not shown). Conversely, all three clones were able to bind HIV during cell-to-cell contacts, as measured

Fig. 4. CD4 T cells internalized HIV. A, CD4 T cells cocultured for 24 h with uninfected (i) or BaL-infected MOLT-4/CCR5 cells (ii and iii), treated with trypsin, and examined by fluorescence microscopy after p24 antigen (red) and nuclear (blue) staining. Effector cells were identified by size and intense homogeneous p24 antigen staining (asterisks). Images show the phase contrast and the overlay of nuclear and p24 antigen staining. B, untreated cocultures of BaL-infected MOLT-4/CCR5 cells and primary CD4 T cells were also analyzed by electron microscopy. i, cellular contacts showing trapped viruses between MOLT-4/CCR5/BaL cells (asterisk) and a CD4 T cell. ii, viral particles bound to the surface of CD4 T cells at sites of membrane invaginations (asterisks). iii–vi, CD4 T cells showing large intracellular vesicles containing several HIV particles, indicated by arrows. Scale bars are 1 μm.
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**Fig. 5. Coreceptor-independent HIV transfer requires binding of gp120 to CD4 and an intact actin cytoskeleton.** Purified CD4 T cells or HeLa P4R5 cells were cocultured for 24 h with uninfected, NL4-3- or BaL-infected MOLT-4/CCR5 cells in the absence (Control) or the presence of the following inhibitors: irrelevant IgG (IRR), anti-CD4 mAbs Leu3a and L120.3; anti-gp120 mAbs IgGb12 and 2G12, cytochalasin D (CYTO), bafilomycin A1 (BAF), concanamycin A (CONC), amiloride (AMILO), and AZT. The amount of p24 transferred to primary cells was calculated after trypsinization of cells and p24 staining as the percentage of p24. These data confirmed that HIV envelope-mediated cell-to-cell fusion in HeLa P4R5 cells was evaluated in cell lysates by determining the presence of the p24 antigen to trypsin-resistant compartments by a mechanism requiring gp120 binding to CD4 but not gp41-mediated fusion (Fig. 6A). Very low levels of attachment and endocytosis were observed in A201 control cells and in A201/CCR5 cells (Fig. 6B). Analysis of cells by fluorescence microscopy showed that p24 were not the result of cellular aggregates but individual cells displaying punctuated trypsin-resistant p24 staining. These data confirmed that HIV endocytosis required extracellular but not intracellular moiety of CD4.

**DISCUSSION**

Our initial observation on the high level of p24 antigen content in CD4 T cells after contacting infected cells (Fig. 1) was puzzling for several reasons. First, we used unstimulated target cells; therefore only low levels of p24 antigen production should be expected. Second, HIV binding to primary lymphocytes appeared to be mostly independent of CD4 (35) and could not explain the specificity of transfer to CD4 T cells. The higher transfer of HIV by BaL-infected cells compared with NL4-3-infected was consistent with the expression of HIV coreceptors by PBMC, which were 98% CXCR4, but less...
than 10% CCR5 (data not shown). After characterizing the mechanisms of HIV capture by CD4 T cells, we concluded that infected cells rapidly transferred HIV antigens to CD4 T cells during cellular contacts. Transfer could be observed at short times (Fig. 3), in the absence of functional coreceptors (Fig. 2) but showed an absolute dependence of CD4 engagement by gp120. In turn, transfer was dependent of actin polymerization (cytochalasin D-sensitive, Fig. 5) and was reversible because HIV particles were rapidly released by CD4 T cells (Fig. 7).

Although these data may suggest a massive HIV binding to CD4, HIV particles did not seem to be just bound to CD4 T cells but appeared to reside transiently in trypsin-resistant compartments (Fig. 3F). Therefore, we postulate that cell-to-cell contacts between HIV-infected (Env-expressing) and target (CD4-expressing) cells concentrate viral particles in contact areas, increasing the binding of virus to CD4 and, in the absence of coreceptor, inducing the endocytosis of bound particles (Figs. 1–4).

Several cell types such as macrophages or endothelial cells use macropinocytic mechanisms to internalize cell-free viral particles (15, 16). A compensatory mechanism for HIV fusion and endocytosis has been described recently after presentation of cell-free virus particles to CD4+ cells (18). The presence of HIV antigens in CD4 T cells after treatment with trypsin, the concomitant disappearance of CD4 molecules from the cell surface (Fig. 3), and the fluorescence and electron microscopy data (Fig. 4) might suggest the existence of a similar compensatory CD4-dependent endocytic mechanism in primary cells. Further characterization of the endocytic process showed that bafilomycin A1 and concanamycin A, two classical inhibitors of vesicular trafficking, did not modify HIV transfer and that the cytoplasmic tail of CD4 was dispensable for HIV uptake (Fig. 6).

Moreover, based on ultrastructural data (Fig. 4B) and in search of a clathrin-independent mechanism, we explored the possible involvement of macropinocytosis in the endocytic process. The macropinocytosis inhibitor amiloride failed to block HIV capture by CD4 T cells (Fig. 5), and, importantly, HIV capture was not associated with fluid phase uptake by CD4 T cells, as assessed with Alexa 488-labeled dextran (data not shown), ruling out a classical macropinocytic mechanism.

The mechanism of HIV transfer during cellular contacts in the absence of coreceptors clearly differed from that governing infectious HIV transfer to HeLa cells (Fig. 5) and was not mediated by clathrin or macropinocytic pathways. In contrast, our observations are in agreement with the intercellular transfer of antigens described at immune synapses. These synapses are built around a central active zone of exocytosis and endocytosis encircled by adhesion domains. Surface molecules may be incorporated into and around the active synaptic zones and modulate the functional state of the synapse (36) activating a process, recently called trogocytosis (37), which allows one of the cells to transfer up to 20% of specific synaptic surface antigens to its partner (38, 39). Trogocytosis has been suggested to act as a mechanism of control of the length of synaptic contacts and might be used by infected cells to increase viral transfer. Indeed, synaptic structures involving HIV envelope, CD4, and coreceptors have been described for the contact of dendritic cells and CD4 T cells and for infected/uninfected CD4 T cell contacts (6, 40). Consistent with this hypothesis, disruption of actin cytoskeleton by cytochalasin D, which prevents the
formation of synaptic structures between infected and target cells (6), strongly inhibited HIV transfer and endocytosis (Fig. 5).

Our results suggest that for CD4 T cells, the interaction of HIV envelope with CD4 appears to be sufficient for the establishment of cellular contacts that allow for a massive fusion-independent transfer of virus, adding complexity to the process of cell-to-cell virus transmission. This process begins in the infected partner of the cellular contact, in which cytoskeleton rearrangements polarize cellular adhesion molecules and HIV...
budding to the sites of cell-to-cell contact (4, 5, 41). Then, infectious viral entry during cell-to-cell transmission requires expression of viral receptors on the surface of target cells (42), and this may result not only in viral entry, but also in profound cytopathic effects, as cell-to-cell fusion and cell death (22, 43). Although HIV endocytosis may also occur in coreceptor-expressing cells, it is more evident in cells lacking the appropriate coreceptor (Fig. 1). These cells are protected from cytopathic effects and from fusion-mediated viral entry to the cytoplasm (24). Survival of target cells probably increases the effective time of cell-to-cell contact allowing for the accumulation of viral particles on the cell surface and vesicular structures.

The possible in vivo relevance of the mechanism of cell-to-cell viral transmission described herein is highlighted by early ultrastructural studies of lymphoid tissue of homosexual men with HIV infection (44) describing viral structures (similar to those shown in Fig. 4) inside vesicular bodies of tissular lymphocytes (44). However, to infect the carrier cell (infection in cis), virions require fusion (coreceptor)-mediated injection of the viral core into the cytoplasm (29). The endocytosed HIV (which can be found in CD4 T cells even after 3 days after the end of the synaptic contact, Fig. 7) could reach the cytoplasm solely after changes in chemokine receptor expression, as a consequence of changes in the surrounding chemokine concentrations or in cellular activation that would affect HIV coreceptor expression such as IL-2 (45) or IL-7 (30).

Alternatively, as shown in Fig. 7, the reversibility of the uptake of HIV by CD4 T cells makes possible the release of captured viruses and the transmission in trans to a third cell, providing a novel mechanism of cell-to-cell HIV transmission, by which HIV may exploit CD4 T cells lacking the appropriate coreceptor as an itinerant viral reservoir. This mechanism of viral transmission should be taken into account when developing pharmacological and immunological anti-HIV strategies.

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REFERENCES

1. Igakura, T., Stinchcombe, J. C., Gonn, P. K., Taylor, G. P., Weber, J. N., Griffiths, G. M., Tanaka, Y., Osame, M., and Bangham, C. R. (2003) Science 299, 1713–1716.
2. Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duinjhooven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000) Cell 100, 587–597.
3. Dimitrov, D. S., Willey, R. L., Sato, H., Babcock, G. J., and van Kooyk, Y. (2001) J. Virol. 75, 2027–2037.
4. Deschambeault, J., Lalonde, J. P., Cervantes-Acosta, G., Lodge, R., Cohen, E. A., and Lemay, G. (1999) J. Cell Biol. 146, 501–5017.
5. Fais, S., Capobianchi, M. R., Abbate, I., Castilletti, C., Gentile, M., Cordiali, G., Fagioli, F., and Dianzani, F. (1995) AIDS 9, 329–335.
6. Joly, C., Kashoff, K., Hollinshead, M., and Sattentau, J. (2004) J. Exp. Med. 199, 283–293.
7. Bomsel, M. (1997) Nat. Med. 3, 42–47.
8. Lagoye, S., Berrien, M., Menin, E., Cote, C., Tressoldi, E., Maucclerc, P., Scarlatti, G., Choua, G., Barre-Sinoussi, F., and Bomsel, M. (2001) J. Virol. 75, 4780–4791.
9. Kielian, M., and Jungerwirth, S. (1990) Mol. Biol. Med. 7, 17–31.
10. Schaeffer, E., Soros, V. B., and Greene, W. C. (2004) J. Virol. 78, 1375–1383.
11. Dustin, M. L., and Colman, D. R. (2002) Annu. Rev. Immunol. 20, 323–359.
12. Mellor, A. L., and Popik, W. (1999) Annu. Rev. Immunol. 17, 77–107.
13. Lou, N. Q., Lossinsky, A. S., Popik, W., Li, X., Gujuluva, C., Kriederman, B., Roberts, J., Pushkarsky, T., Bukrinsky, M., Witte, M., Weinand, M., and Fiala, M. (2002) J. Virol. 76, 6689–6700.
14. Marechal, V., Prevost, M. C., Petit, C., Perret, E., Heard, J. M., and Schwartz, O. (2001) J. Virol. 75, 11166–11177.
15. Goto, T., Nakai, M., and Ikuta, K. (1998) Microb. Pathog. 25, 123–138.
16. Hsu, R. Y., and Wiley, D. C. (1999) Annu. Rev. Biochem. 68, 574–597.