Placental Trophoblasts Resist Infection by Multiple Human Immunodeficiency Virus (HIV) Type 1 Variants Even with Cytomegalovirus Coinfection but Support HIV Replication after Provirus Transfection

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Although the rate of vertical transmission of human immunodeficiency virus (HIV) varies among different populations, it is generally accepted that, on average, 20% of HIV-positive pregnant women will vertically transmit the infection to their infants (51). Infection can occur during gestation (41), near the time of delivery (11), or through breast milk (26). Between 20 and 50% of vertical transmissions to live births occur during gestation (8, 12).

Anatomical considerations argue that in utero infection involves the passage of virus or virus-infected cells across the placenta into the fetal compartment (4), although this remains to be established. The expression of viral gene products in the villi of placenta from HIV-positive women has been documented (6, 14, 45), although there is disagreement regarding the type and distribution of the placental cells involved. In the villous placenta, the syncytiotrophoblast (ST) is a multicellular cellular structure of epithelial origin that arises by fusion of underlying cytotrophoblasts (CT) (4, 36). Located at the boundary between the maternal circulation and the fetal mesenchyma, the ST is a central component of the placental barrier, imposing physical and possibly immunological constraints to the passage of microbial pathogens and/or maternal cells into the fetal compartment. Thus, the prolonged and direct contact of the ST with maternal blood makes it a primary target for maternal blood-borne infections.

However, there is conflicting evidence regarding the susceptibility of the ST to HIV infection. HIV gene products have been both localized to the trophoblastic layer of the placenta by some authors and found only in placental macrophages of the villous stroma (5, 43, 44). Controversy extends to in vitro investigations in which different approaches have been used to demonstrate HIV infection of trophoblasts. Primary trophoblast preparations and trophoblast cell lines have been transiently infected by free virus with restricted permissiveness and with enhancements by coinfection with cytomegalovirus (CMV) or coincubation with infected cells or with enhancing antibodies (21, 22, 65, 70). In contrast, other investigators have been unable to demonstrate any trophoblast infection with cell-free virus, even at a high multiplicity of infection (MOI) (10, 24, 47). Thus, whether the ST is a barrier that prevents entry of free virus or actively promotes its dissemination into fetal tissues is not clear. Conclusively determining the contribution of ST infection by free virus is necessary for the evaluation of alternative routes (e.g., cell-mediated trophoblast infection) through the trophoblast barrier.

Several experimental variables may account for the different results. The degree of purity of primary trophoblast preparations is a crucial factor because placental macrophages, which comprise 1 to 10% of semipurified populations, support HIV replication (34, 47). Since immature CT are isolated and then differentiated into ST in culture for all studies, conflicting reports of trophoblast susceptibility may be due to maturation...
TABLE 1. Number of contaminating (vimentin-positive) cells as a function of preparation lot and time of culture

| Prepn date | Day of culture | No. of vimentin-positive cells/106 seeded
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug. 31, 1995</td>
<td>1</td>
<td>13.3 ± 5.6</td>
</tr>
<tr>
<td>10</td>
<td>5.5 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>4.0 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Jan. 16, 1996</td>
<td>1</td>
<td>5.0 ± 2.5</td>
</tr>
<tr>
<td>10</td>
<td>2.2 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.7 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Feb. 22, 1996</td>
<td>1</td>
<td>32.3 ± 12.3</td>
</tr>
<tr>
<td>10</td>
<td>32.2 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>41.4 ± 6.4</td>
<td></td>
</tr>
</tbody>
</table>

a Purified CT were cultured in 96-well dishes as described in Materials and Methods. There were no significant differences in the number of contaminating cells after culture in IMDM with and without EGF or with KGM.

b Single cells unless otherwise indicated.

c Macroscopic colonies.

d State differences, as reported for monoclonal phagocytes (58). Mononuclear phagocytes from different individuals also vary in the ability to support HIV replication in vitro (15). If this is true for placental trophoblasts, analyses of cells from multiple donors are necessary to reveal differences. The importance of the viral phenotype in the outcome of HIV-host interactions is well recognized (35, 57); thus, differences in viral strains used in particular studies may explain observed variations. Vertical transmission is associated with a selective transmission of macrophage-tropic, non-syncytium-inducing (NSI) HIV variants (66), and therefore trophoblast-HIV interactions should be analyzed in this context. Significantly, the ability of primary HIV isolates recovered from infants to infect placental trophoblasts has never been examined.

Defining the exact role of the placental trophoblast in vertical transmission of HIV has become imperative in view of the recent demonstration of HIV provirus in >70% of placentas from HIV-infected women (23). We have therefore addressed, in a single report, the possible confounding issues of trophoblast purity, maturation state, and culture conditions as well as possible differences between individual placental preparations in sensitivity to HIV-1, and differences between HIV-1 variants in ability to infect cultured trophoblasts, especially regarding clinical isolates from infants. Thus, we examined whether five different virus strains, three of which are NSI macrophage-tropic isolates, could productively infect 99.98% pure villous trophoblasts isolated from 11 different placentas and cultured to a variety of differentiation states for up to 3 weeks. By several criteria of detection, including cocultivation with highly susceptible target cells and PCR of HIV DNA, trophoblasts were completely resistant to infection but could support production of infective virus after transfection with provirus-containing plasmids.

MATERIALS AND METHODS

Isolation and purification of placental cells. Placenta were obtained after normal delivery or elective cesarean section from uncomplicated pregnancies. Villous CT (99.97% pure [Table 1]) were isolated by trypsin-DNase digestion of microchroric tissue and immuneabsorption onto immunoglobulin (Ig)-coated glass bead columns (Biotex, Edmonton, Alberta, Canada) as previously described (69), with the modification that in addition to anti-CD9 antibody for immunoneutralization, anti-major histocompatibility complex (MHC) class I (W6/32; Harlan Sera-Lab, Crawley Down, Sussex, England) and anti-MHC class II (clone 50H19) antibodies were added. The purified cells were routinely cryopreserved and after thawing were washed twice in Iscove’s modified Dulbecco’s medium (IMDM; Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Gibco) and in some experiments 20% human serum, plated in the same medium or in keratinocyte growth medium (KGM; Gibco) at a cell density of 10^6 cells/ml, incubated for 4 h at 37°C in a 5% CO2 humidified atmosphere, then washed with prewarmed IMDM to remove nonadherent cells and debris, and replenished with growth medium in both variants.

SyncytIALIZATION of cultured CT was induced by treatment with 5 ng recombinant human epidermal growth factor (EGF; Prepro-Tech, Rocky Hill, N.J.) per ml for 4 to 7 days (49). SyncytIALIZATION was assessed by immunostaining fixed cells with an anti-CD68 monoclonal antibody (Sigma) to visualize desmo-some-containing tight junctions (25) as previously described (69). CT and ST were cultured with and without EGF (5 ng/ml), interleukin-2 (IL-2; 20 U/ml), gamma interferon (IFN-γ; 100 U/ml; Collaborative Biomedical Products, Becton Dickinson, Bedford, Mass.), recombinant human tumor necrosis factor alpha (TNF-α; 10 ng/ml; Hoffmann-La Roche, Basel, Switzerland), recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 5 ng/ml; San-doz, Basel, Switzerland), and recombinant human colony-stimulating factor 1 (CSF-1; 10 ng/ml; provided by P. Ralph, Cetus, Emeryville, Calif.) before and after HIV type 1 (HIV-1) challenge as described in Results.

Cell lines. HeLa cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS. HeLa cells expressing CD4 (CD4-Hela, clone 1022) were propagated in DMEM supplemented with 10% FBS, and AA2 cells, derived from the human B-lymphoblastoid line WIL-2 (13), were obtained from the AIDS Research and Reference Reagent Program of the National Institute of Allergy and Infectious Diseases and propagated in RPMI 1640 medium (GIBCO) supplemented with 10% FBS, nonessential amino acids, and 0.1 mM sodium pyruvate.

Primary blood cells. Human serum and buffy coat for blood cell preparations were obtained from the Canadian Red Cross Blood Centre, Edmonton, Alberta, Canada. Human peripheral blood leukocytes (PBL) from HIV-seronegative donors were stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 1 day and maintained in RPMI 1640 medium supplemented with 10% FBS and 20 U of IL-2 (Boehringer Mannheim) per ml for another 2 days prior to use as previously described (16). Human monocyte-derived macrophages (MDM) were prepared from HIV-seronegative donors by adherence of PBL to tissue culture flasks as described previously (32), with slight modifications. PBL were resuspended in RPMI 1640 medium supplemented with 20% heat-inactivated human serum, 5 × 10^6 cells were incubated in a T-75 flask overnight at 37°C and then washed three times with phosphate-buffered saline (PBS) to remove nonadherent cells. Adherent cells were lysed by incubation with 0.02% EDTA in PBS for 5 to 10 min at 37°C, collected with a cell scraper, and plated into 96-well tissue culture dishes at 5 × 10^5 cells per well.

CVM infection. A169, a laboratory strain of human CMV, was obtained from J. Preiksaitis (Department of Medical Microbiology and Immunology, University of Alberta) and used at MOIs from 0.3 to 1.0 in individual experiments. Coinfection with HIV strains and CMV was carried out at 37°C in IMDM or KGM for 15 h, after which time the cells were washed five times with medium and fresh 10% FBS-IMDM or KGM was added. The culture was continued for various periods of time before termination of parallel cultures to determine CMV immediate-early (IE) antigen expression and assessment of productive HIV infection as noted below. Nuclear expression of IE antigen expression was determined immunohistochemically (described below for p24), using an IgG2a human CMV IE antibody from Specialty Diagnostics (Duport) and an IgG2a control antibody from Zymed, Inc.

HIV infection. Starting stocks of HIV-1 recombinants pNL4-3 (T-cell tropic) (73) and pRRLim (macrophage tropic) (73) kindly provided by R. Montaner (University of British Columbia) were obtained by transfection of HeLa cells as described below and then passaged through AA2 cells and through PBL and MDM, respectively. The titer of virus was estimated by reverse transcriptase (RT) assay as described below and in reference 6, and biological activity was confirmed in all experiments by simultaneous control infections of CD4-Hela cells (for the T-cell-tropic virus) and either MDM or PBL for the macrophage-tropic virus. HIV-1 strains isolated from perinatally infected infants were all NSI macrophage-tropic viruses and were obtained through the AIDS Research and Reference Reagent Program (isolates 2009 from D. Ho, 2450 from J. Sullivan, and 2758 from C. Hutto). The infant isolates were replicated in PHA-stimulated PBL as previously described (16) and assayed as described above for macrophage-tropic virus. CT and ST cultured with and without cytokines, CD4-Hela cells (as a positive control for T-cell-tropic virus), MDM or PBL cultured in T-25 flasks (as positive controls for macrophage tropic HIV-1 and viruses isolated from infants), and HeLa cells (a negative control) were inoculated in 96-well plates with viruses at MOIs between 0.1 and 6. The plates were incubated at 37°C overnight; the cells were then washed five times and fed with fresh medium with or without cytokines as described in Results. Culture supernatants were collected at different time points and frozen at −80°C for later RT measurement. Adherent cells in the plates were immunostained immediately after harvest for HIV p24 as described below. In-fected virus in culture supernatants was assessed by incubating 1 × 10^6 to 3 × 10^6 PBL or AA2 cells with 50 μl of supernatant in 1 ml of growth medium at 37°C for 3 h with occasional shaking as previously described (16); the cells were collected by centrifugation, resuspended in 5 ml of fresh medium, and cultured as described in Results in T-25 flasks. Virus replication was monitored by RT assay (see below). At designated time intervals, 3.5 ml of spent medium was replaced with fresh medium. Cocultivation assays of HIV-1-challenged trophoblasts were carried out by adding 10^6 AA2 cells (for T-cell-tropic virus) or 10^6 PHA-stimulated PBL (for macrophage-tropic virus) on days February 22, 1996.
7 and 10 after infection directly into the trophoblast or control cell culture, coincubating the cells for various periods of time, and replenishing the culture medium every 3 or 4 days. The infection status of the coculture was monitored by RT assay of culture supernatants.

HIV p24 immunohistochemistry of HIV-1-infected cells. Adherent cells were washed three times with PBS, fixed in cold acetone-methanol (1:1) for 2 min, washed three times with PBS, treated with 3% H2O2 at room temperature for 5 min, washed three times in PBS, and incubated in blocking solution (20% FBS, 0.5% Tween 20 in PBS) for 1 h at room temperature. Primary anti-p24 monoclonal antibody clone 183-H12-5C (19) was added at 1:6 μg/ml in blocking solution and incubated for 15 h at 4°C. The cells were then given four 5-min washes with PBS and incubated for 1 h at room temperature with another blocking buffer (Genosys Biotechnologies, Inc.) to reduce nonspecific staining to cultured trophoblasts. Incubation with the secondary antibody, a biotinylated goat anti-mouse antibody (Amersham), was carried out at a 1:1,000 dilution at room temperature for 40 min; the cells washed four times in PBS and incubated in a staining solution containing avidin and biotinylated horseradish peroxidase (Ultra-Sensitive ABC staining kit; Pierce, Rockford, II) at room temperature for 30 min. After four more washes in PBS, the cells were incubated in 3,3′-diaminobenzidine tetrahydrochloride solution (Sigma) containing 0.3% NiCl2 for 2 to 3 min. The reaction was stopped by washing with tap water for 1 to 2 min. p24 staining was scored and photographed with an inverted microscope (Zeiss Telavik). The percentage of p24-positive cells was determined from counts of >1,000 cells. Negative controls comprised staining virus-challenged cells with isotype-matched mouse Ig (DAKO) as the primary antibody and uninfected cells with anti-p24 antibody.

Transfection of HIV-1 plasmids. CT, ST, and HeLa cells were transfected by calcium phosphate-DNA coprecipitation (30), with slight modifications. Briefly, prior to transfection cells were plated into six-well plates cultured with EGF for 4 to 7 days (for ST), 20 h with medium alone, GM-CSF, TNF-α, IFN-γ, CSF-1, and IL-2 (for CT), and medium alone (for HeLa cells). Purified plasmid DNA (T-cell-tropic pNL4-3 or macrophage-tropic pNL4_aDNA) was added in 90 μl of double-distilled H2O and mixed with 10 μl of 2.5 M CaCl2 in a polycarbonate tube; 100 μl of BES-buffered solution [50 mM Na2HPO4, 37°C) followed by washing at 50°C. The hybridization was carried out as described in Materials and Methods. The bar in panel C represents 10 μm and applies to all panels.

PCR analysis of HIV DNA. Trophoblasts were transfected with EGF (5 ng/ml, 4 days), HeLa cells (negative control), and CD4+HeLa cells (positive control) were plated as described above, exposed to T-cell-tropic HIV-1 (pNL4-3) at an MOI of 1 for 1, 3, 6, 9, 12, 15, 18, 21, and 28 days and macrophage-tropic variant pNL4_aDNA for 1 day, washed three times with PBS at room temperature or treated with 0.1% trypsin (GIBCO) plus 0.02% EDTA for 5 min at 37°C, and then washed with PBS (trypsin treatment did not alter the DNA yield). Total DNA was purified from the samples with a Wizard DNA purification kit as instructed by the manufacturer. Adherent cells were fixed and immunostained for HIV p24 as described above.

RT assay. Frozen supernatants were thawed, and 10 μl was added to 50 μl of a reaction mixture containing 60 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM EDTA, 5 μg of poly(T)(A) (Pharmacia, Uppsala, Sweden)/ml, and 0.16 μg of oligo(dT)/ml and then incubated at 37°C for 1 h as previously described (16, 28). Three microliters of the reaction mixture was spotted onto DE81 paper (Whatman), and the radioactivity was quantitated by scintillation counting, both of which gave comparable results.

FIG. 1. Differentiation of villous trophoblasts as measured by intercellular boundary staining for desmoplakin when cultured for 10 days in medium alone (10% FBS–IMDM) (mostly mononucleated CT) (A), medium containing EGF (5 ng/ml) (mostly multinucleated ST) (B), and in KGM basal medium, which contains EGF (mostly multinucleated ST) (C). There was some variation in syncytialization between trophoblast preparations: analysis of seven different preparations showed 64.2 ± 10.1% of the cells to have two or more nuclei. In terms of proportions of nuclei in mono- and multinucleated cells, 11% of nuclei were in mononucleated cells, 35% were in cells containing two to five nuclei, and 54% were in cells containing more than five nuclei. Culture and staining were carried out as described in Materials and Methods. The bar in panel C represents 25 μm and applies to all panels.
RESULTS

Properties of cultured trophoblasts. Since the two major contaminating cell types in villous placenta cell preparations (placental macrophages and placental fibroblasts) can be infected by HIV-1 (47, 62), their levels in all preparations of CT were monitored by immunohistochemical staining for the intermediate filament protein vimentin, expressed at high levels in leukocytes, endothelial cells, and fibroblasts but not in primary trophoblasts (36). Unpurified preparations contained between 5 and 30% vimentin-expressing cells (69). After three-antigen (CD9, MHC class I, and MHC class II) immunoelimination purification (69), adherent cells contained between 0 and 40 vimentin-positive cells per 100,000 cells (<0.001 to 0.04%). Table 1 shows the contamination levels of 3 preparations, representative of the 11 preparations used for this study, as a function of culture time. Typically, even after 18 days of culture, <10 vimentin-positive cells were found in the cultures. However, in one preparation (February 22, 1996), a higher level (32 per 100,000) was found initially, and this number increased by day 18 of culture. The data from these cells were excluded from the analysis in this report.

After culture for 10 days in 10% FBS in IMDM, the cells remained predominantly mononuclear (Fig. 1A), as determined by marking cell boundaries with an antidesmoplakin antibody (25), but almost 90% of nuclei were in multinucleated cells after culture with 5 ng of EGF per ml (Fig. 1B) or KGM, which contains EGF (25) (Fig. 1C). Although IMDM-EGF and KGM cultures gave equivalent degrees of syncytialization, the IMDM-EGF cultures were consistently more confluent and remained viable longer (for up to 3 weeks). Pyknotic nuclei (darker stained in Fig. 1) appeared in patches in the KGM cultures by day 10 of culture. Operationally, trophoblasts cultured with EGF for >4 days are called ST and those cultured without EGF are called CT.

Infection challenge of CT and ST with recombinant T-cell- and macrophage-tropic HIV-1. Recombinant variants of both T-cell- and macrophage-tropic variants were used for direct comparison of the results of infection with transfection (see below). Trophoblasts were cultured with and without EGF for 1 or 5 days prior to virus challenge, and repeat experiments were carried out with some preparations of trophoblasts cultured with 20% human serum. The cultures were then inoculated with either the T-cell-tropic (pNL4-3) or macrophage-
tropic (pNL\textsubscript{A2d}) variant at MOIs of 0.1 and 1 (and on some occasions as high as 6). CD4-HeLa cells (18) served as positive controls for T-cell-tropic virus infection, and MDM served as positive controls for macrophage-tropic virus infections. HeLa cells served as negative infection controls. Challenge experiments with different preparations of the two viruses and 11 different placental trophoblast preparations were carried out and analyzed by p24 immunohistochemistry of adherent cells and RT determination of culture supernatants. By these criteria of infection, the results were uniformly negative: the T-cell-tropic virus consistently infected CD4-HeLa cells but never trophoblasts, the macrophage-tropic variant consistently infected MDM (or PBL) but not trophoblasts, and neither virus infected HeLa cells. Depicted in Fig. 2A and B are typical photomicrographs of p24 immunohistochemistry of trophoblasts and CD4-HeLa cells challenged with T-cell-tropic virus; Fig. 2C and D show trophoblasts and MDM challenged with macrophage-tropic virus. Each of the experiments with the 11 different trophoblast preparations contained replicate cultures (up to four) for each infection condition. Table 2 summarizes the results of one representative experiment in this series. The data for PBL infection for both T-cell- and macrophage-tropic virus are not shown. Also not shown are results for other experimental groups in which trophoblasts were treated with cytokines (TNF-\(\alpha\), IFN-\(\gamma\), CSF-1, and GM-CSF) reported to enhance virus replication in other cells (46). Trophoblasts were never infected by the above-stated criteria in any of these experiments.

Infection challenge of trophoblasts with HIV-1 variants isolated from infected infants. If a particular HIV-1 tropism for trophoblasts exists, it is most likely to be found among isolates from infants, 20 to 50% of which are infected in utero, very likely by NSI macrophage-tropic variants (66). Three different infant virus isolates (2758, 2090, and 2450), all of which are NSI macrophage tropic, were propagated in PHA-stimulated PBL. Titers in supernatants were determined by RT assay and used at MOIs of 0.01 to 0.2 to challenge HeLa cells, MDM, and trophoblasts. This experiment was carried out twice for each infant isolate (with replicate cultures as described above).
Table 2. Infection of placental trophoblasts, CD4-HeLa cells, HeLa cells, and MDM with T-cell- or macrophage-tropic HIV-1

<table>
<thead>
<tr>
<th>Culture cell type</th>
<th>T-tropic virus at indicated day after challenge</th>
<th>Macrophage-tropic virus at indicated day after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture conditions</td>
<td>Detection method</td>
</tr>
<tr>
<td>Day 1 trophoblast</td>
<td>IMDM-EGF</td>
<td>p24</td>
</tr>
<tr>
<td></td>
<td>10 days after challenge</td>
<td>RT</td>
</tr>
<tr>
<td></td>
<td>Day 5 trophoblast</td>
<td>p24</td>
</tr>
<tr>
<td></td>
<td>IMDM-EGF</td>
<td>RT</td>
</tr>
<tr>
<td>CD4-HeLa</td>
<td>DMEM</td>
<td>p24</td>
</tr>
<tr>
<td></td>
<td>10 days after challenge</td>
<td>RT</td>
</tr>
<tr>
<td>MDM</td>
<td>RPMI 1640-human serum</td>
<td>p24</td>
</tr>
<tr>
<td></td>
<td>10 days after challenge</td>
<td>RT</td>
</tr>
<tr>
<td>HeLa</td>
<td>DMEM</td>
<td>p24</td>
</tr>
<tr>
<td></td>
<td>10 days after challenge</td>
<td>RT</td>
</tr>
</tbody>
</table>

Scoring for p24: +, none detected (<1 in 10^5); ++, 1 in 10^5 to 10^6; ++++, 10^6 to 10^7; ++++, >10^7.

Each with different trophoblast preparations. By criteria of expression of p24 in cultured cells and RT in culture supernatants, the results again were uniformly negative in that MDM were infected but trophoblasts were not. Table 3 shows data from one representative experiment carried out with two of the isolates. The same experiment on all three isolates was repeated twice with the same negative result.

**Culture supernatants from HIV-1 challenged trophoblasts do not contain infectious virus.** A more sensitive criterion for productive HIV-1 infection involves amplification of released virus by highly sensitive target cells such as AA2 cells (13) and PBL. The sensitivity limits of this HIV-1 bioassay are shown in Fig. 3. AA2 cells and PBL were challenged with known titers of T-cell-tropic and macrophage-tropic variants, respectively, and cultured for various periods of time, during which supernatants were sampled for RT activity. In an assay carried out in this manner, one infectious T-cell-tropic particle added to 10^7 AA2 cells results in a strong RT signal on days 17 and 21 of culture. Similar analysis shows that macrophage-tropic virus produces strong RT signals at one infectious particle per 10^7 PBL on days 13, 17, 21, and 24 of culture. In general, higher virus titers showed peak responses early (days 3 to 10 of culture, always on the same day of culture for a given virus dose in three replicate experiments) and low virus titers showed almost equivalent responses later (days 13 to 24); thus, this assay is very sensitive but rather nonquantitative.

Supernatants from HIV-1-challenged trophoblasts and control cell cultures 10 days after challenge were transferred to the appropriate AA2 and PBL cultures, and RT activity was assessed at various times. This analysis was carried out on all trophoblast cultures challenged with HIV-1 with identical results: positive for control cultures and negative for trophoblast cultures. Representative results of this analysis for two such experiments are shown in Fig. 4. Depicted are peak RT values on the indicated day of culture. Although peak RT responses were observed relatively early for CD4-HeLa and MDM infection supernatants (day 10 [Fig. 4A] and day 3 [Fig. 4B], respectively), at no time during a 21-day culture did supernatants from trophoblast cultures generate measurable RT in target cell supernatants. Supernatants transferred at other times after HIV-1 challenge of trophoblast cultures (1, 7, and 18 days) were also negative in this assay (data not shown).

**HIV-1-sensitive target cells cocultured with HIV-1-challenged trophoblasts are not infected.** Because of the potential to biologically amplify cell-associated virus, or perhaps even activate latent virus, virus rescue by activated PBL or highly sensitive cell lines is a very sensitive assay for HIV-1 presence in a cell population (10, 48, 52). AA2 cells or activated PBL at 10^7 per microwell were added to trophoblast cultures challenged by T-cell- and macrophage-tropic viruses 7, 10, and 18 days earlier. The RT content of supernatants was assessed after a further 4, 7, 10, 14, and 17 days of coculture. Depicted in Fig. 5 are representative results for this data array. ST (challenged after 5 days of EGF treatment), CT (challenged after 1 day of culture without EGF), and control cultures were challenged with T-cell-tropic virus at an MOI of 1 and cultured for 10 days, and then AA2 cells were added for up to 17 days of coculture. The positive control CD4-HeLa cultures showed peak production of RT by day 10 of coculture, the negative control HeLa cultures showed no RT production at any time of coculture, and neither CT nor ST showed RT production at any time of coculture. This result was obtained in two independent experiments using different preparations of trophoblasts for all of the combinations of HIV-1 challenge and coculture times listed above and for both T-cell-tropic virus (using AA2 cells as the target [Fig. 5]) and macrophage-tropic virus (using activated PBL as the target [data not shown]).
### TABLE 3. Infection of placental trophoblasts, CD4-HeLa cells, HeLa cells, and MDM with HIV-1 isolated from infants

<table>
<thead>
<tr>
<th>Challenged cell type</th>
<th>Culture conditions</th>
<th>HIV-1 detection method</th>
<th>Infant isolate 2758 at indicated day after challenge</th>
<th>Infant isolate 2090 at indicated day after challenge</th>
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<td></td>
<td></td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Day 1 trophoblast</td>
<td>IMDM-EGF</td>
<td>p24</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>IMDM</td>
<td>RT</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Day 5 trophoblast</td>
<td>IMDM-EGF</td>
<td>p24</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>IMDM</td>
<td>RT</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CD4-HeLa</td>
<td>DMEM</td>
<td>p24</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>RPMI 1640</td>
<td>RT</td>
<td>&lt;10</td>
<td>1,092±1,204</td>
</tr>
<tr>
<td>HeLa</td>
<td>DMEM</td>
<td>p24</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>RPMI 1640</td>
<td>RT</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

a Scoring for p24: −, none detected (<1 in 10^5); ±, 1 in 10^5 to 1%; +, 1 to 10%; ++, 10 to 30%. RT values represent net counts per minute ± standard deviation of triplicate assays. −, only background counts observed (background counts averaged 93 cpm).
blasts cultured in KGM could be infected by HIV-1 by criteria already explored: p24 expression by adherent cells, RT in culture supernatants, presence of infectious virus in culture supernatants, and developing infection during cocultivation with sensitive target cells. The results of one of two experiments with similar outcomes are shown in Table 4: KGM-cultured trophoblasts remain resistant to both T-cell- and macrophage-tropic HIV-1.

To determine whether coinfection with CMV would enhance HIV-1 production as previously described (64), both CT and ST were cochallenged with both T-cell- and macrophage-tropic HIV variants in three independent experiments with strain AD169 at an MOI of 0.35 to 1.0. Two days after challenge, approximately 2% of the nuclei expressed CMV IE antigen by criteria of nuclear staining (Fig. 7A). By all four criteria specified above, CMV coinfection did not result in productive infection of trophoblasts by HIV-1. The data for the ST coinfection studies carried out in both IMDM-EGF and KGM media are summarized in Table 4. However, one well of triplicate cultures of CMV-infected trophoblasts cultured in IMDM-EGF was positive after 10 days of the coculture bioassay. When a parallel culture was routinely examined for presence of vimentin-positive cells, a number of large colonies were detected (coculture detection does not allow localization of the original infection). Further analysis revealed that this particular cytotrophoblast preparation was contaminated with vimentin-positive cells that formed colonies after 10 days of culture (February 22, 1996 [Table 1]). Since HIV-1 infection under these conditions did not occur in two repeat experiments with purer preparations of trophoblasts, all data derived from the February 22 trophoblast preparation were excluded from the analysis.

CT and ST support HIV-1 replication after transfection with provirus. Placental trophoblasts produce antiviral factors such as IFNs and TNF-α (31) and express potent antiviral cytoplasmic proteins such as 2',5'-oligoadenylate synthase (20). To test the possibility that trophoblasts suppress late stages of virus replication, the cells were transfected with provirus-containing plasmids. Plasmids containing either T-cell-tropic (pNL4-3) or macrophage-tropic (pNL_A1Ad) virus were transfected into control HeLa cells, or trophoblasts cultured with EGF for 1 or 4 days, with either CSF-1, IFN-γ, TNF-α, GM-CSF, or medium alone for 1 day. Four days after transfection, adherent cells were stained immunohistochemically for p24 and supernatants were examined for RT. Transfections were carried out in 16 independent experiments on nine different preparations of placental trophoblasts. Photomicrographs of HeLa cells and trophoblasts, previously treated for 1 or 4 days with EGF and transfected with proviral DNA encoding both types of virus, are shown in Fig. 8. The results show that both culture conditions promote replication of transfected proviral DNA in trophoblasts, with p24 expression appearing in the trophoblast cultures in large multinucleated units. The percentages of p24-positive cells in transfected cultures from a representative experiment are given in Fig. 9. In this experiment, both culture conditions before and after transfection (medium alone, EGF, and GM-CSF) and the timing of transfection (1 or 4 days into the culture) were varied. In general, the frequency of successful virus replication after transfection with both virus types is as high in the primary trophoblasts as in HeLa cells (between 5 and 15%). There is a tendency for 1-day treatment with EGF or GM-CSF prior to transfection to enhance the frequency of transfected trophoblasts.

The supernatants from all transfected cultures contained RT activity (data not shown), suggesting that transfected trophoblasts can release active virus. This possibility was confirmed by demonstrating infectivity of supernatants from six different transfection experiments on HIV-sensitive target cells (AA2, cells, PHA-stimulated PBL blasts, or CD4-HeLa cells). Target cell infection was observed in all instances. A representative example is shown in Fig. 10. The results show that all transfected cultures produced active virus, with supernatant from GM-CSF-treated trophoblasts and HeLa cells reaching maximal RT accumulation sooner (10 days rather than 13 days), suggesting that these cultures replicated virus somewhat faster.

DISCUSSION

Understanding mother-to-baby transmission of HIV-1 during gestation ultimately requires understanding how HIV en-
trophoblasts are not productively infected with cell-free virus. Viral determinants appear not to play a role in trophoblast resistance to HIV-1 since productive infection was not observed after trophoblast challenge with five different variants and isolates. We also find that trophoblasts are not productively infected by HIV in the presence of cytokines (TNF-α, GM-CSF, and M-CSF) known to activate HIV replication (54) or after coinfection with CMV (64). Together with the observation that coculture of trophoblasts with AA2 cells or activated PBLs does not rescue infectious virus from HIV-challenged trophoblast cultures, these results suggest that nonproductive or latent trophoblast HIV infection, although possible, is unlikely. This premise is supported by further observations that intracellular provirus cannot be detected by PCR and that transfection of HIV provirus plasmids results in virus production. We therefore argue that trophoblasts resist HIV infection by free virus at the stage of plasma membrane passage or shortly thereafter. These results highlight the importance of an intact ST in preventing the access of free HIV to fetal tissues in vivo and suggest that trophoblast HIV infection, if it occurs, must proceed through other mechanisms, possibly involving the close apposition of infected maternal cells with the villous trophoblast.

HIV infection of nontrophoblastic placental cells has been consistently found both in vivo and in vitro (5, 34, 43, 44, 47). Therefore, in determining whether trophoblast preparations support HIV replication, we attempted to exclude the possibility that infection occurred in a small subpopulation of infectable contaminating stromal cells such as placental macrophages (47) or fibroblasts (62). The almost complete absence of stromal cell contamination (<0.01% vimentin positive) was crucial when highly sensitive coculture bulk assays that do not discriminate the cellular source of viral activity were used. The critical role of cell purity is illustrated by our observation that the only instance (out of >30 independent experiments) of even low-level virus detection after HIV challenge occurred with a trophoblast preparation containing ~0.04% vimentin.

### Table 4. Infection of placental ST with T-cell- or macrophage-tropic HIV-1 as a function of basal medium and coinfection with CMV

<table>
<thead>
<tr>
<th>Challenged cell type</th>
<th>Culture conditions</th>
<th>Detection method</th>
<th>T-cell-tropic virus at indicated day after challenge</th>
<th>Macrophage-tropic virus at indicated day after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Day 5 trophoblast</td>
<td>IMDM-EGF + CMV</td>
<td>p24</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sup. infect.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>KGM</td>
<td>p24</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sup. infect.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>KGM + CMV</td>
<td>p24</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sup. infect.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD4-HeLa</td>
<td>DMEM</td>
<td>p24</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sup. infect.</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cocultivation</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>MDM</td>
<td>RPMI 1640 + human serum</td>
<td>p24</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sup. infect.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cocultivation</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a Scoring for p24: –, none detected (<1 in 10^6); +, 1 in 10^6 to 20%; ++, 20 to 40%; ++++, 40 to 60%; +++, >70%. RT values represent net counts per minute ± standard deviation of triplicate assays. –, only background counts observed (background counts averaged 75 cpm). Scoring for supernatant infection (Sup. infect.): +, 1,000 to 5,000 cpm; ++, 5,000 to 10,000 cpm; ++++, 10,000 to 20,000 cpm; +++, >20,000 cpm. ND, not done.
positive cells. These results agree with the finding of McGann et al. (47) that the only cells productively infected in semipurified trophoblast cell populations were those with a macrophage phenotype. Productive infection of primary trophoblasts or choriocarcinoma cell lines after exposure to HIV-1-infected lymphoid cells, but not after exposure to free virus, has been reported by several groups (10, 24, 53). These observations suggest that trophoblasts with even low levels of stromal cell contamination may manifest infection not only because stromal cells may be selective targets for HIV infection but also because, if infected, they may deliver virus efficiently to adjacent trophoblasts in a cell-mediated manner.

Pure trophoblast preparations have been previously reported to be infected by HIV-1, albeit at low levels (27, 64). There are several possible reasons for differences in results. (i) No absolute criterion of purity is given in the above-cited literature; thus, purity may have been excellent but not sufficient. Even low levels of contaminating stromal cells (e.g., >0.035% [see Results]) could either be the source of detected virus or be vectors that, after infection, transfer it to trophoblasts in a cell-mediated fashion. (ii) Slightly different methods of isolation were used, and perhaps different subpopulations of cells were being examined. (iii) Although we have tested the same culture medium (KGM) as described above, subtle differences in culture conditions in different laboratories may lead to different differentiation states and thereby to slight shifts in the balance between susceptibility and resistance. (iv) Some HIV-1 variants have a rather broad host cell specificity (e.g., SF33 infects cells such as B lymphocytes that do not express CD4 [56]); thus, it is possible that viruses used for the earlier studies have a broader host range than any of the five variants tested in this study.

Productive HIV infection depends on the proliferation (T cells or endothelium) or differentiation (macrophages) state of the target cells (60, 61, 72). Donor source also influences the rate of HIV replication in primary cells (15). Since term trophoblasts do not proliferate in vitro (69), our interpretations are limited to nonreplicating cells. EGF treatment of immature, mononucleated CT induces their morphological (29) and

FIG. 6. PCR analysis of HIV-1 provirus expression in cultures of CD4-HeLa and HeLa cells and trophoblasts detected by ethidium bromide staining of amplicon DNA (top panel) and Southern blots probed by interior oligonucleotides (bottom panel). Where indicated, CD4-HeLa cells, HeLa cells, and EGF-treated trophoblasts were challenged as described in Materials and Methods at an MOI of 1 with T-cell-tropic virus pNL4-3 after two passages through AA2 cells to eliminate the original transfection plasmid. After overnight challenge and washing, the cells were cultured for up to 28 days (the results from the 1-day culture are shown), at which times parallel cultures were washed with PBS or treated with trypsin-EDTA and washed with PBS; then DNA was extracted and subjected to PCR amplification, and the resulting 496-bp products were analyzed on agarose gels by ethidium bromide staining and Southern blotting as described in detail in Materials and Methods. Where indicated, cells were treated at 37°C with 5 μM each AZT and 3TC 2 h before and during virus challenge. Positions of molecular weight markers (lane M) are shown on the right. A variation of this experiment was repeated with similar results with the macrophage-tropic virus pNLΔΔM. The PCR analysis of unchallenged trophoblasts is shown in lane 11.

FIG. 7. CMV infection of ST cultures simultaneously challenged with CMV and HIV-1 as detected by commmunohistochemistry of CMV IE antigen (dark nuclei) and desmoplakin, which marks cell boundaries. ST cultures (trophoblasts treated 5 days with EGF) were simultaneously challenged with HIV-1 variant pNLΔΔM at an MOI of 1 and CMV strain AD169 at an MOI of 0.5 (A) or control medium (B), cultured for 72 h, and stained simultaneously for CMV IE antigen and desmoplakin as described in detail in Materials and Methods. The experiment was carried out in both IMDM–10% FCS and KGM with identical results. The bar in panel B represents 25 μm and applies to both panels.
functional (49) differentiation into mature ST. We found that neither immature CT nor EGF-induced ST supported HIV replication, suggesting that trophoblast resistance to HIV infection is independent of its state of differentiation. Finally, our observation that productive infection was not observed in trophoblasts isolated from 11 different placentas argues that donor source does not play a role in the resistance of villous trophoblasts to HIV infection.

HIV-1 variants have different patterns of transmission, disease progression, and drug resistance in vivo, and viral phenotype is an important determinant of HIV infection in vitro, with isolates and/or variants classified on the basis of their biological behavior (54). In this regard, vertical transmission has been shown to be primarily associated with NSI macrophage-tropic variants (66) as well as with specific minor subtypes within the maternal HIV repertoire as determined by V3 region typing (3). However, there are no reports of in vitro HIV infection of placental cells with primary HIV-1 isolates, and most studies of HIV infection of placental cells in vitro have used well-characterized laboratory strains (24, 47, 64). Here we examined the possibility that differences in viral type accounted for the disparity in reports of trophoblast infection. Productive infection of trophoblasts was never observed after challenge by cloned T-cell- and macrophage-tropic variants or by any of the three infant isolates examined, suggesting that resistance of the ST to cell-free HIV-1 infection is an inherent property of this cell type.
HIV-infected cells can harbor the virus in a state of relative or absolute latency (54), a phenomenon in which both viral and cellular genes have been implicated. Latency is associated with absence of infectious virus but detection of proviral DNA sequences by PCR (reviewed in reference 46). Alternatively, mature virus has been rescued from latently infected cells by coculture with highly sensitive targets or by cell activation. Although trophoblasts are the most abundant fetal cell in the placenta and proviral DNA is detected in fetal cells in 70% of placentas from HIV-infected women (23), we have no evidence that these cells can be latently infected in vitro with HIV. Although DNA PCR resulted in a detectable HIV band, suggesting the presence of viral DNA, the intensity of the band was not diminished by AZT and 3TC (which would inhibit reverse transcription to known latent forms [46]) and was completely abolished by trypsin treatment of the cells (which removes cell surface virions that contain provirus DNA [40]). Therefore, the bulk (all detectable) of HIV DNA associated with trophoblasts in vitro is trypsin accessible on the cell exterior, possibly tightly adherent to the plasma membrane. The HIV DNA on the cell surface appears associated with HIV particles, since DNase pretreatment of virus stocks neither diminished the PCR signal from trophoblast extracts nor decreased virus activity on susceptible CD4-HeLa cells (data not shown).

The resistance of villous trophoblasts to free HIV-1 infection is not unexpected in view of their lack of CD4 expression (38), which absence suggests that the resistance occurs at the cell surface. This supposition is supported by the observation that HIV provirus plasmids are able to produce active virus after transfection, which bypasses any plasma membrane restriction. Transfection of HIV-chloramphenicol acetyltransferase constructs has been previously reported (70). These data argue that trophoblasts can be productively infected under the correct conditions, which may include cell-cell contact or Ig-facilitated transfer (10, 22, 24, 53, 65).

Cytokines play an important role in the host-mediated control of HIV-1 replication (54). Inflammatory cytokines (TNF-α, IL-2, GM-CSF, and M-CSF), for instance, induce HIV expression partly via activation of the cellular transcription factor NF-κB (50). In some cells, TNF-α can rescue defective viruses otherwise incompetent for replication (55). Yet none of the above cytokines had any effect on the ability of trophoblasts to support HIV-1 replication, indicating that resistance is not a function of HIV transcriptional dormancy. Cytokines, on the other hand, regulate varied aspects of trophoblast survival and function. TNF-α induces trophoblast apoptosis in synergy with IFN-γ (29, 69), and IL-1α, TNF-α, and IFN-γ all stimulate the adhesion of monocytes to cultured ST via induction of cell surface ICAM-1 (68). The association between placental inflammation and increased transmission of HIV-1 during pregnancy (42, 63) suggests that inflammatory cytokines enhance infection of fetal tissues. However, our observations suggest that such enhancement may occur not directly by increasing the rate of HIV replication in trophoblasts but rather indirectly by impairing the barrier function of the ST either through its loss or by upregulating adhesion of infected leukocytes.

Although CMV has been implicated as a cofactor in HIV disease (67), there is no reported association between active CMV infection during pregnancy and an increased risk of vertical transmission of HIV-1. In vitro, coinfection with CMV has been shown to upregulate HIV-1 expression in some cell types (39) but not in others (33). Recent studies show that CMV inhibits HIV-1 RNA and protein synthesis in nonlymphohemopoietic cells productively infected by both viruses (67), an effect mediated by interactions between HIV-1 and...
CMV gene products (37). Our results show that CT and ST, although readily infected by CMV, did not become susceptible to HIV-1 when challenged with both viruses. These results differ from those of Toth et al. (64), who found that ST were unable to support late stages of CMV replication but coinfection of ST with CMV and HIV-1 greatly enhanced HIV-1 production. The reasons for these discrepancies are unclear.

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