Human Transformed Trophoblast-Derived Cells Lacking CD4 Receptor Exhibit Restricted Permissiveness for Human Immunodeficiency Virus Type 1

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We investigated the nature of interaction of the malignantly transformed cell lines of trophoblast origin BeWo, JAR, and JEG-3 with three different human immunodeficiency virus type 1 (HIV-1) isolates (RF, 3B, and NDK). After inoculation with cell-free virus, the persistence of infection was determined for 1 month by monitoring the presence of viral DNA in the cells by the polymerase chain reaction (PCR). Furthermore, the infectious virus in the culture supernatant was assayed with CEM-SS cells, and attempts to rescue the virus by cocultivation with CEM-SS cells were made. Appraised on the basis of the relative amount of viral DNA and the frequency of positive cocultivation, JEG-3 was the most permissive and BeWo was the least permissive cell line. However, when the cells were transfected with two biologically active molecular clones of HIV-1, the BRU and NDK isolates, all three cell lines turned out to support the production of mature virus progeny to the same extent. The abundance of viral DNA sequences in the infected cells varied with the isolate, showing an overall decline from RF to NDK. The amount of viral DNA in the cells and its expression decreased during the period of observation; this decrease was mirrored in an erosion of the virus recovery rate at cocultivation from 71% recovery on day 8 to failure of isolation on day 32. None of the cell lines expressed detectable amounts of cell surface CD4 molecules when assayed by flow microfluorometry and direct radioimmunoassay. Northern (RNA) blot hybridization analysis of both the total RNA and the mRNA did not reveal any CD4-specific message; nonetheless, by using the PCR, sequences specifically related to the CD4 gene were uncovered. The data demonstrate that the trophoblast-derived cell lines are susceptible to infection with HIV and that they support transient viral replication in the initial phases of infection. However, the latent form of infection may persist over a period of several weeks.

The route by which the prenatal transmission of human immunodeficiency virus (HIV) from mother to infant (14, 29) is effectively accomplished is currently the subject of speculation, although some data suggest that placental tissue may be directly involved in this process (18, 19). The fetally derived trophoblast forms an anatomical barrier which, in itself or through production of the newly described trophoblast interferons (31), may play a crucial role in preventing transplacental infections. Therefore, it is of particular interest to pinpoint the role of this morphological unit in HIV dissemination. In vitro trials with infected placental organ cultures (19) and investigations of placentae of HIV-infected individuals (13a, 18) suggest that the trophoblast lining of the chorionic villi can become infected with HIV, but so far the interaction of the virus with isolated trophoblast cells in culture has not been explored. We used malignant trophoblast-derived established cell lines and thus avoided inherent inconveniences typical of primary trophoblast cultures, such as the limited possibility of continuous propagation, while retaining essential trophoblast properties (33).

In preliminary experiments, we verified that the malignantly transformed human trophoblast cell line JAR is susceptible to HIV replication. Afterwards, we broadened our study with two other choriocarcinoma cell lines, BeWo and

JEG-3, and investigated the HIV-elicited infectious process with respect to its persistence and productivity and the conditions permitting its establishment. Two other cell lines, TE 671 and COS-1, which are susceptible and nonsusceptible to HIV, respectively, were used in order to provide additional controls. All of the cell lines were obtained from the American Type Culture Collection, Rockville, Md. The T-lymphoid cell line CEM-SS, which is highly permissive for HIV infection (23), was used in cocultivation experiments. The HIV type 1 (HIV-1) isolates used were RF, 3B (kindly provided by R. C. Gallo, National Institutes of Health, Bethesda, Md.), and NDK (9). The stock suspensions were prepared by propagating the virus on H9 lymphoid cells, and their viral content was titered by syncytium-forming (23) and reverse transcriptase (RT) (13) assays.

Expression of transfected HIV-1 proviral clones in malignant trophoblast-derived cell lines. The pNLBT plasmid, comprising the complete provirus with all the structural genes originating from the HIV-1 BRU (LAV-1) prototype, was prepared by inserting the 8.2-kb *Bss*HII-*Xho*I fragment from plasmid pBT1 (2) into the identical restriction sites of the HIV hybrid infectious molecular clone pNL4-3 (1). The HIV-1 NDK isolate was molecularly cloned and sequenced, and the infectious proviral clone pNDK was constructed as described elsewhere (28). The infectious plasmids were introduced into the cells with the aid of cationic liposomes (10). The cocultivation experiments were controlled by

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FIG. 1. Subcultivation of HIV-infected T-lymphoid cells after cocultivation with target monolayers transfected with complete proviral clones of BRU (pNLBT) and NDK (pNDK) HIV-1 isolates. After the cell monolayers were transfected (10) with infectious plasmids, the cocultivations with CEM-SS cells were initiated and proceeded for 3 days. Afterward, the lymphoid cells were subcultured and analyzed for RT activity (34) at the indicated intervals. In mock transfections, the experiments were performed according to the standard procedure, but the seeding of the monolayer cells was omitted.

performing a complete mock transfection without target cells. Cells were seeded 24 to 36 h before transfection in densities assuring approximately 80 to 90% confluence at the time of the experiment. The uptake of DNA-lipofectin complexes was allowed to proceed for 2 h for TE 671 cells or 8 h for the other cells and was stopped by adjusting the final serum concentration in the medium to 10%. Cocultivations with CEM-SS cells were started 17 h later. The lymphoblastoid cells were left in the coculture for 3 days and were thereafter propagated separately for up to 14 days. The culture media were checked for the presence of virus by a dot blot modification of the RT assay (34) and by an antigen capture enzyme-linked immunosorbent assay (Ag-ELISA) (24).

The transient expression of cloned full-length genomes of BRU and NDK isolates of HIV-1 gave rise to complete progeny virions which were subsequently readily rescued by cocultivation with permissive CEM-SS cells. From the kinetics of virus growth, which culminated on days 3 to 7, it could be deduced that different lines of transfected cells released comparable amounts of infectious virus (Fig. 1). Only slight differences between the profiles of RT activity of the two viruses were found; moreover, this result was corroborated by Ag-ELISA (data not shown). It was difficult to discern on the basis of our data whether observed minor distinctions relied on transfection efficiencies of particular cell types or on the regulatory events governing the virus expression. The virus was also rescued in mock transfection experiments, probably as a result of CEM-SS direct transfection by residual DNA-liposome complexes adsorbed to the surface of cultivation vessels. Nonetheless, the observation of a significant delay period before the virus growth peaked in transfection and mock transfection experiments sufficiently supports the postulated capacity of the cells to express the HIV genes. Moreover, additional evidence for

synthesis of the virus by the transfected cells was furnished by finding the viral antigens directly in monolayer cells and their supernatants 60 h after transfection (data not shown).

Persistence of HIV-1 infection in choriocarcinoma cell cultures. Once we had confirmed that the HIV-1 genome can be effectively expressed in choriocarcinoma cells, we investigated the possibility that the cells can become infected with native virus. Inoculation was achieved with virus stocks at a final input multiplicity of infection of one to five syncytium-forming units per target cell. Filtered virus inoculum was added to the cells 24 h after they were seeded and left in the cultures for the next 24 h. The cultures were split every 4 days, and at the time of splitting a small number of cells was removed and subjected to *in vitro* enzymatic DNA amplification. The employed extension primers were specific for *env* gene sequences homologous in all three strains and bounded a 1,233-bp segment. The specificity of amplified products was verified by Southern blotting using a 5'-terminally 32 P-labeled specific oligonucleotide (21-mer) (32) under stringent conditions.

Using the polymerase chain reaction (PCR), we found considerable differences in the persistence of HIV-1 sequences in the cultures, depending on the virus isolate and cell type (Fig. 2). In BeWo cells, the infection could not be detected after inoculation with HIV-1 3B and was found only at the end of the first testing interval with HIV-1 NDK. After day 27, the viral DNA was still present in infected cultures: HIV-1 3B in JAR cells and HIV-1 NDK in JEG-3 cells. In all other choriocarcinoma cell lines, viral DNA persisted for at least 11 days. As anticipated because of the known susceptibility of TE 671 cells to infection with HIV, the PCR revealed DNAs of all HIV isolates used in the TE 671 cells until the last interval of investigation (data not shown). On the other hand, the COS-1 cells turned out to be universally nonpermissive for HIV.

Distinct discrepancies in the amount of amplifiable templates were also obvious (Fig. 2). The JEG-3 cells were found to harbor the greatest amounts of viral DNA of all the cell lines irrespective of the virus, whereas the BeWo cells were significantly susceptible only to infection with HIV-1 RF. The JAR cells behaved as an intermediate type. There were also apparent distinctions in the ability of each isolate to infect the choriocarcinoma cell lines. RF was found to be the most efficient virus, and NDK was found to be the least efficient virus. Generally, in the course of observation a decline in the amount of viral DNA, which ultimately resulted in the complete disappearance of any amplifiable sequences in all but two infected cultures, was evident in the majority of the cultures. All of the cells that remained infected until day 27 also scored positive in one more test on day 35, and the amount of virus DNA was apparently unchanged.

Productivity of HIV-1 chronic infection. An attempt to correlate the presence of virus genetic material with its expression into complete virions was made by seeking to rescue the infectious virus. The CEM-SS cells were infected with the monolayer culture supernatant or cocultivated with the choriocarcinoma cell monolayers in both the early (days 11 and 8 to 11, respectively) and late (days 27 and 32 to 35, respectively) periods after inoculation (Table 1). For infection, CEM-SS cells were inoculated for 24 h with an appropriate culture medium, and for cocultivation, the protocol applied to the transfections was observed. In the early period, viral DNA was found in seven choriocarcinoma cell cultures. In five of these cultures, the virus was successfully transmitted to CEM-SS cells by cocultivation. Interestingly,

FIG. 2. Persistence of viral DNA in choriocarcinoma cell lines BeWo, JAR, and JEG-3 inoculated with the RF, 3B, and NDK isolates of HIV-1. The trypsinized cells were analyzed by PCR for viral *env* gene sequence at 4-day intervals. Aliquots from amplification reactions were analyzed by Southern blot hybridization with a ^{32}P -labeled specific oligonucleotide.

cocultivation on day 32 failed to rescue any virus, although the two cultures (JAR cells with HIV-1 3B and JEG-3 cells with HIV-1 NDK) still contained significant amounts of HIV DNA on day 35. Similarly, the attempts to infect the CEM-SS cells with filtered media of the choriocarcinoma cell cultures on days 11 and 27 after inoculation failed. The rate of the early and late positive cocultivations with TE 671 cells was substantially higher (100%). In this case, all three isolates were successfully rescued. Conceivably, only negligible amounts of infectious virions were released, even from TE 671 cells, while only two out of three cell-free filtrates transmitted infection to CEM-SS cells when early supernatants were tested. It is therefore not surprising that virus production could not be detected in any of the culture media that were screened for viral proteins by Ag-ELISA (detection limit, 50 pg/ml).

The persistence and productivity of infection is of particular interest from the point of view of viral dissemination. We conclude from our data that upon in vitro infection, cells may initially enter a stage of limited virus shedding, which subsequently becomes a silent, latent infection. This may be considered a general phenomenon, while the continuous decline in HIV gene activities after infection, resulting in latency or even in sequestration of provirus, was also described for other semipermissive systems (7, 17, 22). Nonetheless, in our investigation only two out of eight cell lines initially harboring provirus retained the DNA for longer than 35 days. The gradual segregation of viral genetic material from the cell culture could be explained by restricted virus spread, presumably confined chiefly to cell-to-cell transfer and dilution out of infected cells during sequential subcultures.

Detection of CD4 epitope implicated in HIV binding and

CD4-specific mRNA. Our primary aim was to investigate whether the main HIV receptor, the CD4 molecule, could have played a role in HIV infection. Initial studies identified the CD4 molecule as a main HIV receptor (8, 15), but more recent research has suggested that the CD4 receptor may not be a prerequisite for susceptibility to HIV. This was most typically illustrated in cells of nonlymphoid origin (3, 12, 16, 27, 30). With the aim of evaluating surface CD4 expression, we labeled choriocarcinoma cells with the biotinylated monoclonal antibody (MAb) anti-Leu-3a (Becton Dickinson, Mountain View, Calif.) and streptavidin-phycoerythrin conjugate (Becton Dickinson) and performed surface CD4 expression analysis by flow cytometry. Each cell type, besides being subject to a complete labeling procedure, was processed as a negative control by omitting the MAb. CEM-SS cells expressing abundant CD4 (23) were used as a reference. Superimpositions of plots of the frequency of fluorescent signal intensity were used to discriminate between experiments and corresponding controls. The analysis demonstrated nearly identical experiment-versus-control fluorescence patterns in all monolayer cells, thus clearly excluding the presence of CD4 at the given level of sensitivity (Fig. 3). In order to evaluate the possibility that the anti-Leu-3a MAb reactive epitope, which is topically intimately associated with the gp120-binding site (26), was affected during the release of the cells from the support surface, we used ^{125}I -labeled anti-Leu-3a MAb in a direct radioimmunoassay (20). In this procedure, the cells were processed for evaluation in a counting device only after being labeled. This assay, performed at a level of signal-to-noise discrimination comparable to that of the previous one, did not demonstrate any signal resulting from MAb specifically bound to the surface of the choriocarcinoma cells (data

TABLE 1. Relationship between DNA sequences in monolayer cells, rescue of virus by cocultivation with CEM-SS cells, and infectivity of monolayer culture medium for CEM-SS cells at defined intervals

HIV-1 isolate	Cell line	Virus recovery at indicated interval by indicated method			
		Early ^a		Late ^b	
		DNA analysis ^c	Cocultivation ^d	DNA analysis	Cocultivation
RF	COS-1	+	—	—	—
	TE 671	+	+ ^e	+	+ ^e
	BeWo	+	—	—	—
	JAR	+	+	—	—
	JEG-3	+	+	—	—
3B	COS-1	—	—	—	—
	TE 671	+	+ ^e	+	+ ^e
	BeWo	—	—	—	—
	JAR	+	+	+	—
	JEG-3	+	—	—	—
NDK	COS-1	—	—	—	—
	TE 671	+	+	+	+ ^e
	BeWo	—	—	—	—
	JAR	+	+	—	—
	JEG-3	+	+	+	—

^a HIV DNA was analyzed on day 11, and cocultivation was performed on days 8 to 11 after inoculation. The monolayer cell culture medium from day 11 after inoculation was used to infect CEM-SS cells.

^b HIV DNA was analyzed on day 35, and cocultivation was performed on days 32 to 35 after inoculation. The monolayer cell culture medium from day 27 after inoculation was used to infect CEM-SS cells.

^c HIV DNA was detected by PCR in trypsinized cells at the time of subculture.

^d Cocultivation of the HIV-inoculated monolayers with CEM-SS cells was scored as positive (+) or negative (—) for virus recovery.

^e Recovery of HIV after CEM-SS cells were infected with the culture medium of the monolayer cells previously inoculated with HIV is indicated.

not shown). The pattern of the choriocarcinoma cells matched that of the TE 671 cells, which were previously shown to lack CD4 expression (5, 12).

Because of the possibility that minute amounts of CD4 protein might escape detection because of the limited sensitivity of the immunoassays, we next analyzed the related mRNA. We performed Northern (RNA) blotting (11) on total cellular RNA isolated by the guanidinium thiocyanate-CsCl method (4) and on polyadenylated RNA which was purified by oligo(dT)-cellulose chromatography. We used the 1.7-kb (*EcoRI-BamHI*) coding sequence of the CD4 cDNA clone inserted in the pT4.4 plasmid (generously provided by D. Littman, University of California, San Francisco, Calif.) as a probe. The Northern blot analysis of CEM-SS cells showed significant amounts of a 3-kb CD4-specific species in both the total and poly(rA) preparations, whereas no traces of CD4 message were observable in any of the choriocarcinoma cell lines (data not shown).

To search for CD4 message with maximal sensitivity, we implemented the cDNA PCR technique. MAPPING Amplimers specific for CD4 (Clontech, Palo Alto, Calif.) enabled amplification of the 437-bp stretch located at the 3' end of the open reading frame. The specificity of amplified CD4 sequences was confirmed by standard Southern blot hybridization with the probe derived from plasmid pT4.4 (see above). In all choriocarcinoma cell lines the PCR showed surprisingly distinct signals, which, however, when com-

FIG. 3. Flow cytometric analysis of CD4 expression on the cytoplasmic membrane of choriocarcinoma cells of various lines. The choriocarcinoma cell lines BeWo, JAR, and JEG-3 and the control cell lines CEM-SS, COS-1, and TE 671 were labeled with biotinylated anti-Leu-3a MAb and streptavidin-phycoerythrin as a reporter molecule. At least 3×10^4 cells were analyzed with an Epics Profile Analyzer. Shown in each panel are the fluorescence patterns of labeled cells (—) and the corresponding controls (---), which were treated only with the second-stage immunoreagent. CEM-SS is a cell line positive for CD4 in 96% of its cells, as determined previously (23).

pared with signals from CEM-SS cells, suggested minimal quantities of CD4 message (Fig. 4). Comparison of the intensity of hybridization signals corresponding to CEM-SS cells in Northern blotting and PCR implied that the latter

FIG. 4. PCR analysis of CD4 mRNA. Aliquots (10 μ l) of reaction mixtures from amplification reactions performed on cDNAs (A) or on mRNAs (B) were subjected to Southern blot hybridization with ³²P-labeled CD4 cDNA probe (kindly provided by D. Littman). Equivalent amounts of full-length first-strand cDNAs were amplified as estimated on the basis of trichloroacetic acid-precipitable fractions and alkaline agarose gel electrophoresis. The blots were exposed to film at -80°C with an intensifying screen for 5 h. Lanes 1, CEM-SS; lanes 2, COS-1; lanes 3, TE 671; lanes 4, BeWo; lanes 5, JAR; lanes 6, JEG-3. CEM-SS cells were used as a positive control, as they express the CD4 receptor at a high level (23). In the photographic reproduction, only a faint band appeared in lane 5 (JAR); however, it was clearly discernible in the original autoradiogram.

method yielded at least a 10^3 -fold increase in sensitivity over the former. Although the amplified DNA proved to be CD4 specific, the PCR approach did not allow a characterization of original mRNA. It is therefore unclear whether these CD4-related species present in a very low abundance can code for a biologically active protein or can have any functional meaning with respect to HIV susceptibility. A somewhat similar intricacy was encountered earlier in the case of fetal neural cells (16), which expressed a CD4 message with a rather unusual pattern to which no defined role could have been assigned. In the particular case of trophoblast, results published so far are rather inconsistent. A previous report described the presence of CD4 antigen in locations suggestive of chorionic villous trophoblast in placental explantates (19). However, as we have demonstrated here, and as was reported earlier (18), it was not possible to prove unambiguously the expression of CD4 either at the transcriptional level or as a surface receptor in choriocarcinoma cells or preliminarily in primary cytotrophoblast cultures (35).

The quantitative aspects and the persistence of virus DNA made it possible to establish a scale of HIV-1 infectivity: RF > 3B > NDK. That the RF isolate was more cytopathic than 3B on T- and B-lymphoblastoid cells was already known (6, 22). However, the most intriguing outcome was that NDK, which was previously found to be more than 10^3 times more infectious and cytopathic on T cells than the HIV-1 BRU prototype (25), did not exert any enhanced effect on choriocarcinoma cells relative to other isolates. Accordingly, it appears that particular HIV isolates may differ substantially in their tropism to trophoblast. With regard to the observed rapid genetic perturbations in the HIV virion population (21), it seems reasonable that strains with increased infectivity for trophoblast can be generated. Such virus strains might be of major importance for HIV infection acquired in utero.

In conclusion, previous investigations (13a, 18, 19) and our data, together with some preliminary findings on cultured trophoblasts (35), suggest that the cytotrophoblast layer of the placenta is susceptible to infection with HIV and may play a role in the pathogenesis of fetal HIV infection. However, in vitro the permissiveness is restricted, and we therefore do not expect that the trophoblast can play a major role in promoting transplacental HIV transfer. In light of the known possibility of maternal blood passing into a fetus, the possibility that HIV infection of fetuses might primarily result from circumvention of a protective trophoblast layer arises. The role of the cells of the fetal portion of the placenta in HIV transmission will be the subject of further planned experiments with primary trophoblast cultures.

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