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Differentiation of Human Embryonal Carcinoma Cells Induces Human Immunodeficiency Virus Permissiveness Which Is Stimulated by Human Cytomegalovirus Coinfection

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Human immunodeficiency virus (HIV) replicates in differentiated but not undifferentiated NTERA-2 human embryonal carcinoma cells; neither cell type expresses CD4. Susceptibility of the differentiated cells is enhanced by coinfection with cytomegalovirus. HIV infection induces lactoseries glycolipids, suggesting a mechanism whereby HIV might interfere with normal embryogenesis.

Human immunodeficiency virus (HIV) is increasingly recognized as a pathogen of the human embryo and fetus (7, 9). About 80% of children infected with HIV are infected prenatally or perinatally (11, 28, 40). Many pregnant women with AIDS are also infected with human cytomegalovirus (HCMV) (41, 43), which itself causes fetal damage (44). Since the HCMV immediate-early protein enhances HIV replication by acting as a *trans* activator of the HIV long terminal repeat (LTR) promoter (12, 17, 19, 20, 36–38) and, reciprocally, HIV (12, 27, 38) and its *tat* gene (27) stimulate HCMV replication, coinfection of the embryo with HCMV and HIV might exacerbate damage caused by either virus alone (see also reference 33).

To determine whether embryonic cells are susceptible to HIV and whether their physiology is altered in ways that may affect developmental processes, we investigated whether HIV can replicate in pluripotent NTERA-2 (clone NT2/D1) human embryonal carcinoma (EC) cells (4) and whether HCMV, which replicates in differentiated NT2/D1 cells (24, 25), alters the course of HIV replication. EC cells resemble early embryonic cells, and their differentiation provides a model of cell differentiation during embryonic development (16, 32). Following induction with retinoic acid (RA), NT2/D1 EC cells give rise to neurons, suggesting their use as a model for development of the nervous system (3, 30), a possible target for damage in HIV-infected fetuses.

Undifferentiated NT2/D1 cells or differentiated NT2/D1 cells obtained by growth in 10^{-5} M RA or 3 mM hexamethylene bisacetamide (HMBA) for 7 days (3, 5) were reseeded at 10^6 cells per 25-cm^2 flask in the absence of RA or HMBA and infected with HIV-1 strain IIIB (2 ml of filtered virus propagated in HUT 78 cells and containing 10^4 50% tissue culture-infective doses [$TCID_{50}$] per ml). After 6 h of adsorption, the cells were thoroughly washed and fed with fresh medium; the medium was subsequently changed on days 5 and 10, and samples were tested for HIV p24 antigen by enzyme immunoassay (Coulter Immunology). No p24 antigen was detected in medium from undifferentiated cells even 15 days later, but it was detectable in cultures of differentiated cells from 5 to 15 days postinfection (Fig. 1A). Expression of the p24 antigen was also detected by immunofluorescence in the cytoplasm of 20 to 25% of RA- and of HMBA-treated cells but not in the undifferentiated cells (not shown). Furthermore, reverse transcriptase (RT) activity (34), assayed with poly(A) · $(dT)_{10}$ (Boehringer Mannheim) and [α-^{32}P]TTP (Amersham), increased (to between 4×10^5 and 18×10^5 cpm/ml) in HIV-infected cultures of differentiated but not undifferentiated NT2/D1 cells (Fig. 1B).

To confirm HIV replication, DNA was isolated (10) from NT2/D1 cells 10 days postinfection, digested with *Sac*I, and analyzed by Southern blotting. HIV DNA was detected as a 9-kb band in DNA from differentiated, HIV-infected cultures (data not shown); uninfected differentiated and infected undifferentiated cells did not exhibit HIV DNA. Production of infectious HIV was shown by the expression of p24 antigen in SUPT-1 indicator cells following exposure to cell supernatants from the infected differentiated NT2/D1 cells (titer, 10^4 $TCID_{50}$/ml, 10 and 15 days postinfection). In an endpoint dilution assay, RA- and HMBA-induced NT2/D1 cells exhibited similar susceptibilities, as indicated by RT production, with a 50% endpoint of approximately 0.001 $TCID_{50}$ per cell, whereas SUPT-1 cells were slightly more sensitive, with a 50% endpoint of approximately one log higher dilution of input virus.

Longer periods of differentiation prior to infection were

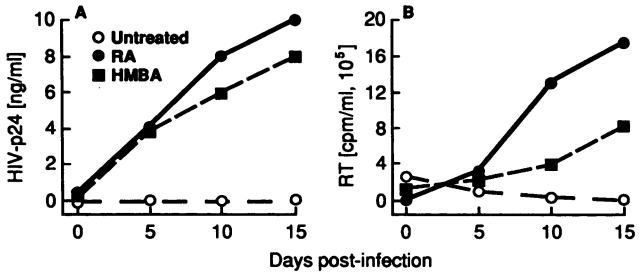

FIG. 1. Appearance of HIV p24 antigen (A) and RT activity (B) in culture supernatants of NT2/D1 cells infected with HIV-1. Mean RT values calculated from duplicate samples were obtained after subtraction of appropriate uninfected-control values; RT activity in uninfected cultures was less than 4,000 cpm/ml at all times tested.

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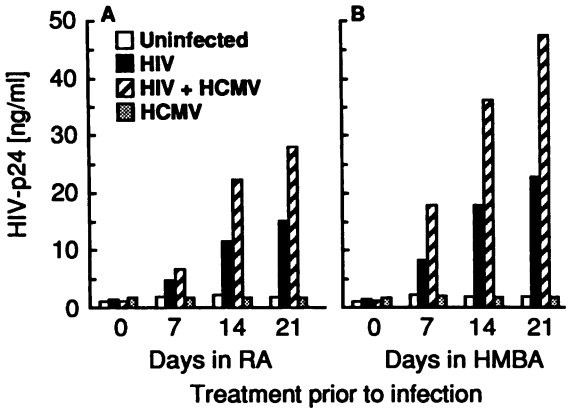

FIG. 2. Appearance of HIV p24 antigen in the culture medium of differentiated NT2/D1 cells derived by pretreatment with RA (A) or HMBA (B) for different periods of time prior to infection with HIV-1 and/or HCMV. The differentiated cells were reseeded and infected simultaneously with either HIV-1, HCMV, or both. HIV p24 antigen expression was assayed in the culture supernatants 7 days postinfection.

associated with higher subsequent yields of p24 protein in the culture medium (Fig. 2). Furthermore, cultures coinfected with HIV-1 and HCMV (Towne strain; multiplicity of infection, 1 to 2; assayed as in reference 45) produced about twofold more p24 antigen than cultures infected with HIV-1 alone (Fig. 2), indicating that HCMV can enhance HIV replication in embryonic cells.

To determine whether lack of a viral receptor could explain the failure of HIV to replicate in undifferentiated NT2/D1 cells, we evaluated the presence of cell-associated virus by assay of RT activity after infection. Undifferentiated NT2/D1 cells were incubated with a virus inoculum for 3 h, washed thoroughly, and refed with fresh medium. A high level of RT activity was associated with the cells 3 h after infection (Fig. 3). This declined sharply by 24 h, presumably due to degradation, since only low activities were detected in the cell-free culture medium at any time.

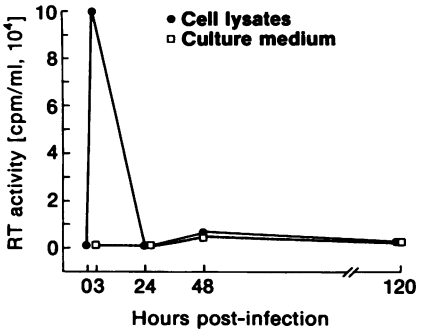

FIG. 3. Entry of HIV into undifferentiated NT2/D1 EC cells, assayed by cell-associated viral RT activity. Cultures containing 2×10^6 cells were incubated for 3 h with 0.4 ml of HIV (10^4 $TCID_{50}$/ml, equivalent to 2×10^5 cpm of RT activity per ml). After being washed thoroughly, the cultures were refed with 5 ml of fresh medium. RT activity was subsequently assayed in lysates of cells harvested by scraping (cell-associated virus, ●) and in the culture medium (cell-free virus, □). Uninfected NT2/D1 cells and washings from infected cultures contained less than 2,000 cpm of RT activity per ml.

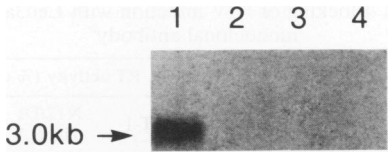

FIG. 4. Northern blot analysis of CD4 expression in NT2/D1 cells. Total RNA was prepared from SUPT-1 cells and included as a control (lane 1) along with undifferentiated cells (lane 2), RA-treated differentiated cells (7 days pretreatment) (lane 3), and HMBA-treated differentiated cells (7 days pretreatment) (lane 4). The hybridization was performed under relaxed conditions as described earlier (35). Size estimation was made by running an RNA ladder (Bethesda Research Laboratories, Bethesda, Md.) in parallel. The autoradiogram shown was exposed for 24 h; further exposure for 1 week did not reveal additional bands.

We concluded that HIV is able to enter the EC cells efficiently but that replication is blocked later in the virus life cycle. Maio and Brown (31) have reported that the HIV LTR is apparently inactive in undifferentiated NT2/D1 cells, but they attributed this to posttranscriptional events. However, since we found no proviral DNA in infected undifferentiated NT2/D1 cells (data not shown), it seems likely that HIV is unable to integrate into the host genome of these cells, as has been suggested in the case of some cells nonpermissive for other retroviruses (22, 42).

The nature of the HIV receptor in both undifferentiated and differentiated NT2/D1 cells is unknown. The CD4 molecule acts as a receptor for HIV in most cell types (15, 29), but certain CD4-negative cells are also susceptible (1, 13, 14, 26, 27, 39). We failed to find expression of CD4 in the undifferentiated or differentiated NT2/D1 cells either with monoclonal antibody OKT4 (Orthodiagnostic) in immunofluorescence assays of cell surface expression (not shown) or by Northern (RNA) blot assay of cellular RNA under relaxed conditions of hybridization as described before (35), with a nick-translated pT4B probe (CD4 cDNA) (Fig. 4). To confirm that CD4 is not the receptor in differentiated NT2/D1 cells, we determined whether the anti-CD4 antibody Leu3a (Becton Dickinson) could block infection (Table 1). Whereas Leu3a completely inhibited infection of SUPT-1 cells, no

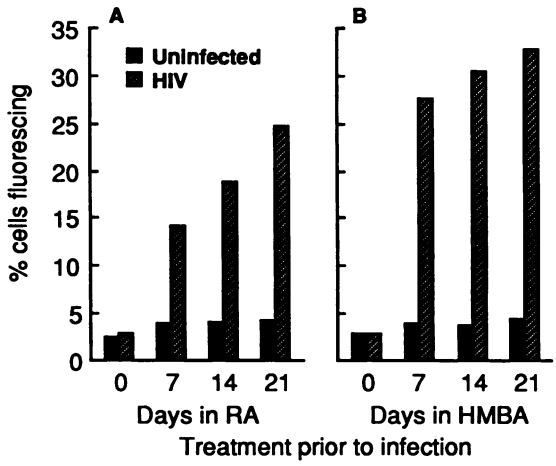

FIG. 5. Expression of Le^y cell surface antigen by HIV-infected, RA-treated (A) or HMBA-treated (B) differentiated NT2/D1 cells 7 days postinfection, assayed by flow cytofluorimetry with monoclonal antibody AH-6 to Le^y (23).

TABLE 1. Blocking of HIV infection with Leu3a (anti-CD4) monoclonal antibody[a]

Preincubation	RT activity (% of control)		
	SUPT-1	NT2/D1 plus RA	NT2/D1 plus HMBA
Control			
Culture medium	100	100	100
Irrelevant antibody (25 μg)	100	100	100
Anti-CD4 Leu3a (25 μg)	<1	98	99

[a] 10^6 cells (SUPT-1) [$CD4^+$ T-lymphoid cells] and RA- and HMBA-induced NT2/D1 cells) were preincubated for 1 h with culture medium or with 25 μg of Leu3a or an irrelevant antibody (rat monoclonal antibody to mouse immunoglobulin G; Gibco-Bethesda Research Laboratories) at 37°C before infection with HIV-1 (2×10^4 $TCID_{50}$). After 6 h, the cultures were washed and refed with fresh medium; RT activity was assayed in duplicate cultures 5 days later and expressed as a precentage of the activity in the control culures preincubated with culture medium alone (SUPT-1, 121,000 cpm/ml; NT2/D1 plus RA, 99,240 cpm/ml; NT2/D1 plus HMBA, 87,760 cpm/ml). RT activity in the final wash after infection and in mock-infected control cells was <600 cpm/ml.

inhibition was seen in the case of either RA- or HMBA-induced NT2/D1 cells. Evidently another virus receptor must be active in these cells.

If HIV infects embryonic cells, it might exert effects on embryo physiology other than by cytotoxicity. When differentiated NT2/D1 cells were infected with HIV-1, cell surface expression of the fucosylated lactoseries glycolipid antigen Le^y (Fucα1→2Galβ1→4[Fucα1→3]GlcNAcβ1→R]) was induced (Fig. 5); expression of the related, monofucosylated Le^x epitope (Galβ1→4[Fucα1→3]GlcNAcβ1→R) also increased slightly (10% positive cells) (data not shown). HIV-1 similarly induces Le^y in T lymphocytes (2). Fucosylated lactoseries glycolipids have been implicated in various morphogenetic cell-cell interactions during development; e.g., Le^x carbohydrates block compaction of the morula during early mouse development (8, 20), while lactoseries carbohydrates might play a role in axon homing in the developing nervous system (18). Thus, the inappropriate expression of these carbohydrate structures occasioned by viral infection could disrupt normal development, as we have also postulated in the case of HCMV-induced embryopathy (6).

The identification of HIV-permissive cells in differentiating teratocarcinomas suggests that some embryonic cells are indeed susceptible to HIV infection and replication. This supports the contention that intrauterine infection has the potential to cause an embryopathy. Furthermore, differentiating EC cells provide a model for investigating the mechanism of such embryopathy, as well as for studying the mechanism whereby some embryonic cells but not others restrict virus replication.

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The first three authors contributed equally to the experimental work.

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