Human Immunodeficiency Virus Infection of Monoblastoid Cells: Cellular Differentiation Determines the Pattern of Virus Replication

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Stringent control of human immunodeficiency virus (HIV) replication was observed in the human monoblastoid cell line U937. A low-multiplicity infection of these cells by the LAV1 strain of HIV was productive for 2.5 days; then virus replication became restricted and no further evidence of virion production was observed. The dramatic decrease in HIV production was due in part to reduced accumulation of cytoplasmic viral RNA and occurred in the absence of evident cytopathic effects. In contrast, infected cells induced to differentiate by phorbol ester, vitamin D₃, or lymphokine supernatant did not release markers of HIV despite the accumulation of significant levels of cytoplasmic viral RNA. HIV infection altered the pattern of c-myc RNA accumulation in U937 cells. Expression of this gene changes normally in response to the state of cellular differentiation; in infected cells the level of c-myc expression was correlated to the levels of viral RNA accumulation and not to cellular differentiation. These results suggest that restricted replication of HIV in monocytes might be an important mechanism of virus persistence and demonstrate a relationship between HIV replication and monocyte differentiation.

Accumulating evidence indicates that infection of monocytes by human immunodeficiency virus (HIV) is an important component in the pathogenesis of acquired immunodeficiency syndrome (AIDS). HIV-infected individuals manifest elevated levels of circulating lymphocytes (19), which is released by activated monocytes (8, 18); increased serum lysozyme levels are correlated with progression to AIDS (19). Monocytes derived from AIDS patients have significantly reduced phagocytic (9) and chemotactic (35, 40) activities, and the percentage of these cells that are HLA-DR positive is approximately half the level observed normally (42). The monocyte phenotypes observed in AIDS patients suggest that activation and differentiation of these cells are altered by HIV infection.

In addition to the phenotypic alterations observed in monocyte populations derived from AIDS patients, evidence exists that these and related cells are directly infected with HIV. Viral RNA and antigens have been observed in brain tissue macrophages (14, 26, 43) and in the macrophagelike follicular dendritic cells in lymph nodes (2, 3, 31). Cells with macrophage markers in the skin and lungs of AIDS patients have been shown to contain infectious virus, viral antigens, or viral nucleic acids (6, 23, 31, 34). Peripheral blood monocytes are permissive for HIV in vivo (15, 23, 30), and virus can be isolated from these same cell populations in infected individuals (15, 23). HIV infection of monocytes, and the variety of specialized cell types related to this lineage, constitutes a significant component of virus distribution in the infected individual.

The role of monocytes in AIDS is controversial. It has been suggested that these cells serve as the reservoir for virus in the body (22, 25). The observation of low rates of HIV replication in monocytes in vitro and the absence of cytopathic effects on these cells has been proposed as one possible explanation for HIV persistence in vivo (15, 21). It has also been proposed (33) that infected monocytes serve as the principal agents of HIV dissemination to helper T cells, because these two cell types necessarily come into contact during the course of antigen presentation and the T cell receives activation signals in consequence of this interaction (33). New insights into the role of monocytes in AIDS are especially important in light of the recent discovery that dideoxynucleosides, which constitute an important class of agents for the treatment of AIDS, have reduced antiviral activity in cells of this lineage (39).

The interaction between HIV and the host monocyte is thought to be complex, involving special mechanisms for the control of virus production. Accordingly, we sought to characterize the biology of HIV replication in monocytes. In these initial studies, the human monoblastoid cell line U937 (41) was used as a model for HIV-monoocyte interactions and the relationship between virus production and cellular differentiation. We observed that HIV replication in these cells was regulated stringently. A low-multiplicity infection of U937 cells was productive initially. At 2 days postinfection, we observed that cytoplasmic viral RNA, cell-free reverse transcriptase activity, and p24 gag antigen levels diminished rapidly without evident cytopathic effects. Thus, productive infection had become restricted and virus release ceased. In contrast, infected U937 cells induced to differentiate by treatment with a phorbol ester (tetradecanonylphorbol acetate [TPA]), 1,25 dihydroxy vitamin D₃, or lymphokine supernatants did not release markers of HIV despite significant accumulation of viral RNA in the cytoplasm of TPA-treated cultures.

Direct effects of viral infection on the cellular phenotype were assessed by examining the cellular gene c-myc that is expressed normally in U937 cells. The pattern of c-myc RNA accumulation was altered in infected cells as opposed to uninfected controls, attesting to a direct effect of HIV on the phenotype of these cells. The altered pattern of c-myc RNA accumulation in infected cells indicates that these changes are mediated at least in part by direct effects of HIV on cellular gene expression.

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MATERIALS AND METHODS

Cell culture and preparation of virus stocks. U937 cells (41) were maintained at densities of between 1 × 10^6 and 5 × 10^6 cells per ml in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, supplemented with penicillin-streptomycin-glutamine. The cells were passaged on the day of before infection. Cell-free stocks of the LAVIstrain, strain of HIV (1) were prepared in the T-lymphoblastoid cell line, CEM. The titers were adjusted to 10^5 tissue culture infective doses (TCID_{50}) per ml, as determined by syncytium formation in a terminal dilution assay with MT-2 cells (20).

Virus production in differentiated and undifferentiated U937 cells. Cultures of U937 cells were infected at a multiplicity of 0.2 TCID_{50} per cell. At 8 h later, the culture was split and TPA was added to half, at a final concentration of 1 μg/ml. After 1 h, cells were recovered from both cultures by centrifugation, washed once in phosphate-buffered saline (PBS), and suspended in fresh medium to a final concentration of 5 × 10^6 cells per ml. The flasks, each containing 20 ml of culture, were then incubated at 37°C in an humidified incubator with 5% CO₂ in air and were harvested at 0, 1, 2, 5, and 5 days after TPA treatment. The cultures were supplemented with an equal volume of fresh medium at 2.5 and 4 days postinfection. The effects of 1,25 dihydroxy vitamin D₃ or lymphokine supernatant treatments were assessed in a similar manner; vitamin D₃ was added to infected cell cultures at a final concentration of 10⁻⁹ M (provided by Milan Uskokovich, Hoffmann-La Roche Inc.), and commercially available deceptinated supernatant form photheamagglutinin-activated human peripheral blood lymphocytes (Advanced Biotechnologies) was added as a 20% volume supplement to infected U937 cells in culture.

At each time point, the cells were collected by centrifugation. A 1-ml sample of the cell-free supernatant was retained and stored at −70°C for assay of reverse transcriptase activity and quantitation of p24 gag antigen (p24 enzyme-linked immunosorbent assay, Abbott Laboratories, Chicago, Ill.). The flasks were harvested with 5 ml of PBS containing 0.5 mM EDTA to recover adherent cells. The harvested cells were washed again with cold PBS and suspended in 2.5 ml of 15 mM NaCl-15 mM Tris hydrochloride (pH 7.5). Then, 100 μl of 20 mM vanadyl ribonucleoside and 200 μl of 20% Nonidet P-40 were added. Nuclei were removed by a 2.5-min spin at 2,500 rpm in a Beckman TJ-6 centrifuge, and the supernatant was combined with an equal volume of 150 mM NaCl-150 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. Protease K (Boehringer Mannheim) was then added to a final concentration of 200 μg/ml and incubated for 45 min at 56°C and then extracted with an equal volume of 1:1 phenol:chloroform containing 0.1% hydroxyquinoline-0.5% 2-mercaptoethanol. The aqueous phases were recovered, and RNA was precipitated by the addition of sodium acetate (pH 5) to a final concentration of 0.3 M plus 2.5 volumes of ethanol (10). Samples were stored at −20°C overnight, and the nucleic acid precipitates were recovered by centrifugation.

The RNA pellet was purified further to remove contaminating vanadyl ribonucleoside (10). The dried pellet was suspended in 250 μl of 3 M sodium acetate (pH 5), and the RNA was recovered by brief centrifugation in an Eppendorf microcentrifuge. The pellet was rinsed in 70% ethanol, suspended in water, and precipitated again. The resulting material was of sufficient purity that the concentration of RNA could be determined from the optical density at 260 nm. Samples of each preparation were also run on agarose gels as an additional check on RNA content and integrity.

The reverse transcriptase activity of cell-free supernatants was determined by a modification of the method of Popovic et al. (36). After a 2-h incubation at 37°C in the presence of 5 mM MgCl₂, poly(A) template-oligo(dT) primer, with [³²P]dTTP as label, portions of the reaction mixture were spotted on DE81 paper and allowed to dry briefly. The paper was washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), rinsed once in 95% ethanol, and then exposed to film for 3 to 5 h. Individual sections were then cut out, and the incorporated radioactivity was determined by Cerenkov counting.

Assays for cellular and viral gene expression. Cytoplasmic RNA samples were analyzed to determine the relative contents of c-myc, HIV, and actin RNA. The assays were performed by dot blotting of the cytoplasmic RNA samples onto Schleicher & Schuell, Inc., nitrocellulose filter membrane and hybridization with the appropriate radiolabeled probe.

Samples for dot blotting were made 25 mM morpholinepropanesulfonate (pH 7.0)–5 mM sodium acetate–5 mM EDTA (1× MOPS buffer)–50% deionized formamide-3.7% formaldehyde (vol/vol) in a final volume of 250 μl. The samples were incubated at 56°C for 20 min and then chilled briefly. Nitrocellulose membrane that had been immersed previously in water and again in 20× SSC was placed in the dot-blotting manifold; then 60-μl portions of each sample were placed in wells, and the liquid was drawn through the membrane with gentle vacuum. The membrane was then rinsed once in 2× SSC, baked at 80°C for 2 h in a vacuum oven, and stored until hybridization.

Radiolabeled probe for expression of the c-myc gene was generated by primer extension labeling (11) of a 1.0-kilobase-pair (kb) PstI fragment of the human c-myc gene (37). The radiolabeled HIV probes were prepared by using the 9.5-kb fragment of the plasmid pARV-2 (27), and actin probes were derived from the 0.6-kb PstI fragment of a murine β-actin cDNA clone (28). Prehybridization and hybridization of the membranes were described previously (32).

Analysis of proviral genomes. Pellets containing infected cell nuclei were recovered subsequent to Nonidet P-40 lysis and suspended in 2.5 ml of 25 mM Tris hydrochloride (pH 7.5)–12.5 mM EDTA–140 mM NaCl-0.5% sodium dodecyl sulfate; proteinase K was added to a final concentration of 200 μg/ml, and the samples were incubated at 56°C for 30 min. Then, a second portion of proteinase K was added, and the incubation continued for an additional hour before the sample was extracted with an equal volume of buffered phenol solution. The aqueous phase was collected and dialyzed extensively against 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. The DNA concentration was calculated from the optical density at 260 nm.

Purified cellular DNA was digested with the restriction endonuclease HindIII, and the resulting fragments were separated by agarose gel electrophoresis. The DNA was transferred to Hybond filter membrane (Amersham Corp.); hybridization of the filter membrane was performed as described for the RNA analysis.

RESULTS

HIV production is restricted in U937 cells. The replication of HIV in U937 cells was evaluated by measuring the accumulated reverse transcriptase activity and p24 gag
antigen content of infected-cell supernatants. U937 cells were infected with 0.2 TCID₅₀ per cell; newly synthesized virus was released into the medium on days 1 and 2 postinfection. Thereafter, virus production decreased rapidly and remained at a low level for the duration of the 5-day experiment (Fig. 1). The decrease in supernatant reverse transcriptase activity was due to an effect on virus production and not to a direct inhibition of reverse transcriptase activity. Admixing experiments, in which virus stocks of known activity and culture supernatants were combined, showed no inhibition of enzymatic activity.

The effects of phorbol ester treatment on HIV replication in U937 cells were studied in two different ways. Cells were preinfected in a single flask and then split into TPA-treated and untreated cultures. Preinfection was required because TPA treatment of U937 cells rendered them resistant to infection (7; C.D.P., unpublished data). Infected phorbol ester-treated cells did not release detectable reverse transcriptase or p24 gag antigen (Fig. 1). The results on HIV production by normal and differentiated U937 cells were obtained consistently in more than 15 independent experiments.

We also assessed the accumulation of reverse transcriptase activity in the medium of infected cells that were treated with either 1,25 dihydroxy vitamin D₃ or deoxicitin antagonists from phlytohemagglutinin-activated human peripheral blood lymphocytes. Both treatments are known to induce U937 differentiation (38). In three separate analyses of the effects of both of these treatments, the release of reverse transcriptase activity was similar to that observed with TPA treatment (Fig. 2). Three distinct agents known to induce U937 cellular differentiation thus were able to reduce significantly the release of HIV from infected cell cultures.

The abrupt decline in virus production manifested by undifferentiated U937 cells was not attributable to cell toxicity. No significant differences in the rates of DNA synthesis were apparent between the infected and uninfected cell cultures as determined by the rates of [³H]thymidine uptake (Fig. 3). The percentage of viable cells as determined by trypan blue dye exclusion was greater than 90% in both the infected and uninfected cultures. Some cell death observed in the phorbol ester-treated cultures was attributable to chemical toxicity.

Viral RNA and provirus accumulation. Levels of HIV RNA in the cytoplasmic RNA fraction were studied on the levels of virus production in undifferentiated U937 cells. Accumulation of HIV-specific cytoplasmic RNA was observed on days 1 and 2 postinfection; the RNA decreased in abundance on day 5 when virus production was also at its lowest (Fig. 4). This correlation between cytoplasmic RNA levels and virus production was not observed in the differentiated cell cultures. Differentiated (TPA-treated)
cells contained significant amounts of cytoplasmic viral RNA even though viral reverse transcriptase and p24 antigen were not released into the medium. The level of viral RNA remained constant in differentiated U937 cells over the course of the experiment. Hybridization with the mouse β-actin gene probe demonstrated that equal amounts of RNA had been applied to all positions of the filter and that the levels of actin RNA were not affected significantly by infection or differentiation (Fig. 4). Similar results were obtained in three additional experiments.

The relative accumulation of HIV provirus was assessed by DNA blot analysis (Fig. 5). The hybridization with samples from days 2.5 and 5 postinfection indicated a relatively constant amount of proviral DNA. In this particular experiment, the sample in lane b contained less DNA, as judged by ethidium bromide staining of the gel, and this probably accounts for the difference in hybridization intensity between lanes b and c. There was little difference in the relative amounts of the provirus between differentiated and undifferentiated infected cell cultures, and this result was consistent in three separate infection experiments. Therefore, neither the decrease in virus production during restricted replication nor the absence of particle release in differentiated cell cultures could be attributed to selective depletion of the infected-cell population.

Expression of the c-myc gene in infected U937 cells. Expression of the c-myc gene is a marker of cellular differentiation in U937 cells (28). Accordingly, we examined the accumu-

FIG. 4. Cytoplasmic RNA dot blots showing the levels of HIV (A) and actin (B) RNA in untreated and TPA-treated U937 cells. The blots were prepared as described in the text and hybridized with a radiolabeled 9.8-kb DNA fragment of the cloned genome of the HIV isolate ARV-2 (27) or an EcoRI fragment of mouse β actin. In panel A, only the samples from infected cells are shown. There was no detectable hybridization to uninfected cell RNA. In panel B, the upper section shows the actin RNA content of uninfected cells and the lower panel shows the actin content of infected cells. The amount of total cytoplasmic RNA per dot is shown at the right, and the day postinfection is shown at the bottom. These RNA samples were prepared from the same cell cultures represented in Fig. 1. No RNA was loaded in the day 2, uninfected TPA-treated lane, and this accounts for the absence of hybridization at this position.

FIG. 5. Southern blot analysis of HIV proviral DNA in untreated and TPA-treated U937 cell cultures. Nuclear DNA was prepared as described in the text, digested with the restriction enzyme HindIII, and fractionated on a 0.8% agarose gel. After transfer to nitrocellulose and hybridization with a probe from the plasmid pARV-2 (27), the hybridization pattern of the provirus was revealed. Only an upper section of the hybridization pattern is shown. Lanes contain the following samples: a, uninfected U937 cell DNA; b, day 2, infected, no TPA; c, day 5, infected, no TPA; d, day 2, infected, TPA-treated; and e, day 5, infected, TPA-treated. The sizes of the bands are shown at the right.

FIG. 6. Relative content of c-myc RNA in infected and uninfected U937 cells with and without TPA treatment. The upper section shows the levels of c-myc RNA in infected cells, and the lower section shows the levels in uninfected cells. The RNA samples are identical to those used in Fig. 4, thus the β-actin control can be compared directly with the c-myc hybridization pattern.
species in infected cells superimposed on the normally constant RNA levels in uninfected cells. We have not yet examined the pattern of c-myc gene expression in cell cultures containing 100% infected cells because at higher multiplicities of infection the property of restricted replication is not observed (C. D. Pauza, unpublished). In contrast to the undifferentiated infected U937 cells, the abundance of c-myc RNA remained nearly constant in the infected TPA-treated cells throughout the 5-day time course. In this case, the altered regulation of c-myc RNA accumulation is especially apparent. The uninfected differentiated cell cultures would normally be expected to contain little or no c-myc RNA after 2 days of TPA treatment. Thus, the relative abundance of c-myc RNA in the infected differentiated cells was clearly evident.

On the basis of these experimental observations, we conclude that HIV infection altered the regulation of c-myc RNA accumulation. Surprisingly, a correlation between HIV and c-myc RNA levels was observed. This is different from the pattern of c-myc expression observed in uninfected cells. Altered expression of the c-myc gene was observed in four separate infection experiments; in each case, the changes in c-myc RNA were accompanied by the pattern of viral RNA accumulation. This result appears to be a specific effect of viral infection because expression of the actin gene was not affected by HIV infection.

**DISCUSSION**

HIV replication in U937 cells is productive initially and then becomes restricted. During restriction, the level of viral RNA in the cytoplasm of infected cells decreases substantially. Expression of the c-myc gene also decreases concomitantly with viral gene expression. DNA blot analysis showed that the same amounts of proviral DNA per cell are present during both productive and restricted replication; this observation attests to the fact that the changes in RNA levels indicate altered accumulation of these species in individual cells and not selection against a subpopulation of infected cells. Furthermore, thymidine uptake rates for infected and uninfected cells are comparable, indicating further that the altered behavior of infected cells is not due to a cytopathic effect of the virus. Consequently, the stringent control of HIV replication in U937 cells is an intrinsic attribute of the interaction between the monoblastoid cell and the virus.

Infected U937 cells, induced to differentiate by phorbol ester, release reduced amounts of HIV. In contrast to the restricted replication observed in undifferentiated U937 cells, the differentiated cells were nonproductively infected despite accumulation of significant levels of cytoplasmic viral RNA. In this case, high levels of c-myc RNA were also shown to be present during differentiation of infected cells in contrast to the rapid decline in abundance of this RNA observed in differentiated uninfected cells. Infected U937 cells were also induced to differentiate by the addition of 1,25 dihydroxy vitamin D3 (4, 5) or deleted supernatant derived from PHA-activated human peripheral blood lymphocytes. In all three cases, the infection was rendered nonproductive in the treated cells. Differentiation of infected U937 cells initiated by any of three independent compounds resulted in an abrupt decline in virus production.

The interaction between HIV and the host cell U937 is complex. Undifferentiated cells, after an initial burst of virus production in the first few days after infection, subsequently show restricted virus replication. In contrast, the infection of differentiated cells is nonproductive despite high levels of viral RNA accumulation. In both cases, provirus accumulation is relatively constant. Alterations in the pattern of c-myc but not actin gene expression reveal that HIV infection exerts a specific and selective effect on cellular gene expression. These results demonstrate that the mechanisms controlling HIV replication in monoblastoid cells are specific to the cellular differentiation state. In addition, virus infection itself resulted in phenotypic changes in the monoblastoid cell population which suggest that HIV can alter the capacity of these cells to differentiate.

Others have noted that infected U937 cells manifest altered phenotypes and that their differentiation is somewhat inhibited (7, 21). Here we present molecular evidence in support of this concept and introduce the finding that virus production is diminished in normal and differentiated U937 cells; the operative mechanisms in these two cases are functionally distinct.

It is of interest to compare our results with the observations of Folks et al. (12, 13) on the behavior of a persistently infected cloned cell line (designated U1) derived from the parent line U937. The U1 line showed minimal constitutive expression of HIV, and treatment of these cells with phytohemagglutinin-activated lymphocytes supernatants increased virus production (12). In our hands, this treatment inevitably causes a rapid decline in virus release, which is consistent with the observed correlation between cellular differentiation and reduced HIV production. The differences between our observations on the pattern of HIV replication following acute infection of the U937 cell line and those of Folks et al. (13) on the chronically infected U1 cell line remain to be elucidated.

HIV replication in differentiated and undifferentiated U937 cells is very similar to the pattern of visna virus replication in normal sheep monocytes and macrophages. In the visna virus studies, undifferentiated monocytes were permissive and showed low levels of viral replication. Differentiation of the infected monocytes was induced by an interferon activity (16, 24, 29) and was accompanied by increased viral gene expression (16, 29). Despite the elevated levels of cytoplasmic viral RNA, infectious virus was not released from the differentiated cells (16). Thus, the same relationship between differentiation and viral replication that was documented here for HIV in human monoblastoid cells was observed previously for another lentivirus infection of normal sheep monocytes and macrophages. Visna virus infection of sheep macrophages is associated with the formation of a reservoir of virus (24) and the establishment of a persistent infection (16, 17). These two characteristics, virus persistence and the establishment of a virus reservoir, are also important components in HIV infection. The state of human monocyte differentiation and its impact on virus replication is likely to be central to the mechanisms controlling these two features of HIV infection and of crucial importance in the pathogenesis of AIDS.

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