NEGATIVE REGULATION OF HUMAN IMMUNE DEFICIENCY VIRUS REPLICATION IN MONOCYTES

Distinctions between Restricted and Latent Expression in THP-1 Cells

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Human immunodeficiency virus 1 (HIV-1) is a retrovirus that has many similarities with members of the nontransforming and cytopathic animal lentivirus family (1). These viruses, including HIV-1, cause slowly progressive, chronic, and, in some instances, fatal diseases in their hosts. The time from initial viral infection to clinically observed symptoms of disease is usually measured in years (2). Elucidation of the viral life cycle during the subclinical phase of infection is critical in gaining a clearer understanding of the pathophysiology of AIDS. Several studies have suggested that a persistent state of latent or chronic low level productive infection exists in fresh and cultured cells (3–7). These data imply that viral latency can be a component of HIV infectivity. The mechanisms involved in developing these latent or restricted states of HIV expression are not well understood.

Animal lentiviruses show a tropism for cells of the monocyte/macrophage lineage during viral latency and persistence (8). There is now substantial evidence suggesting that the monocyte/macrophage also serves as a reservoir for HIV infection (9). Fresh monocytes can be infected in vitro with HIV. Also, HIV can be cultured from monocytes obtained from blood and organs of patients infected with HIV (9). In the brain, the macrophage is the major infected cell type and is associated with the development of neurologic symptoms seen in AIDS patients (10, 11).

This study was initiated to develop HIV-infected monocyte cell lines that could be used to understand mechanisms of viral latency and restricted low level chronic expression. THP-1, derived from a patient with acute monocytic leukemia, possesses morphologic, histochemical, phenotypic, and functional properties of monocytes (12, 13). Analysis of THP-1 cultures after an initial productive HIV infection revealed THP-1 cultures with either latent or restricted HIV expression as well as cultures

remaining productively infected. In cells with restricted HIV expression, viral expression is negatively regulated in such a manner as to escape immune surveillance and still be capable of transmitting virus to T cells. In cells with latent virus, no viral expression is seen but infectious virus can be activated by a mechanism distinct from the upregulation of viral expression in cells with restricted expression.

Materials and Methods

Cell Lines.   THP-1 cells were maintained in RPMI with 10% FCS, penicillin (100 μg/ml), streptomycin (100 μg/ml), and glutamine (300 μg/ml); cells were subcultured 1:5 every 4–5 d. HELA and murine CB2MX3 cells producing HIV-1 tat, obtained from G. Pavlakis (BRI, Frederick, MD), were grown as monolayer cultures in DMEM as previously described (14).

Viruses and Infections.   All viruses were isolated from PBMCs. HIV-1 strain BP-1 was grown in HUT-78 (15), strain ADA was grown in U-937 (16), and HIV-2 Rod was grown in CEM (17). 10^7 THP-1 cells in log phase growth were infected with 5×10^5 tissue culture infectious doses at a 50% endpoint $(TCID_{50})[1]$ harvested from cell-free supernatants of each virus after 5 d of growth. Infections were done in 1 ml serum-free RPMI with 2 μg/ml of polybrene for 1–2 h at 37°C in a shaking water bath. Cells were washed twice to remove unabsorbed virus and subcultured for growth. For the multiplicity of infection (MOI; number of $TCID_{50}$ U/cell) study, concentrated viral stocks, made using a tangential flow Millitan Apparatus (Millipore Continental Water Systems, Bedford, MA), were used to give the indicated MOI.

Virus Detection.   Viral p24 antigen was determined on tissue culture supernatants or cell pellets lysed with 1% Triton X-100 by ELISA (Cellular Products, Inc., Buffalo, NY). As previously shown, HIV p24 ELISA kits do not discriminate between HIV-1 and HIV-2 p24 (18). $TCID_{50}$ was determined using microtiter wells of HUT-102B2 with serial dilutions of cell-free virus. Electron micrographs of HIV-infected cells were prepared by OsO_4-fixed, rapidly dehydrated THP-1 or HUT-102B2 cells and embedded in epoxy resin using standard procedures. Thin sections were mounted, stained with uranyl acetate and lead citrate, and viewed in a microscope (H-7000; Hitachi, Tokyo, Japan) as previously described (1). For syncytia formation, 200 infected THP-1 cells were incubated with 10^6 HUT-102B2 cells. HIV-1-induced syncytia were recorded by microscopic analysis. Reverse transcriptase (RT) activity was measured in cell supernates pelleted by high-speed centrifugation using poly(rA)-oligo (dT12-18) template primer, 20 mM Mg^{2+} as cofactor, and appropriate deoxynucleotide triphosphates as previously described (19). Results were adjusted to cpm of [^{3}H]TTP-incorporated [^{3}H]/ml.

Phenotypic Analysis of HIV-Infected THP-1.   Cytofluorometric analysis was performed as described (15). Cells washed in PBS were fixed in -10°C absolute methanol for intracellular p24 antigen determination. The cells were not fixed for all other assays. mAbs used were directed against HIV p24, HIV gp 160:41, and HIV-1 gp 120 (Cellular Products, Inc.). Other monoclonals against Leu-3A (CD4), Leu-2 (CD8), Leu-M3 (CD14), and HLA-DR were purchased from Becton Dickinson & Co. (Sunnyvale, CA).

Functional Assays.   For viral-mediated cell cytotoxicity, HIV-infected cultures are tested either by titering out 5-d supernatants against 10,000 MT-2 cells per well in a round-bottomed 96-well plate or by coculturing with infected cells. After various days of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) is used to measure in vitro growth by cell-mediated reduction of tetrazolium (20). The OD at 540 nm is directly proportional to the number of viable cells. Macrophage functional assays were performed plus or minus PMA (50 ng/ml) treatment for 24 h. Phagocytosis was performed by incubating 10^6 cells with 100 μl zymosan for 1 h. Cell smears were then stained with Jenners, and 1,000 cells were examined microscopically. For accessory cell function, monocyte-free lymphocytes obtained by elutriation were separated into T4 and T8 populations as previously described (15). THP-1- and HIV-infected THP-1 irradiated at 8,000 rad were added to 10^5 T8 cells in a culture volume of 0.2 ml at serial dilutions of THP-1 cells with or without Con A (5 μg/ml).

[1] *Abbreviations used in this paper:* LGL, large granular lymphocyte; LTR, long terminal repeat; MOI, multiplicity of infection; PCR, polymerase chain reaction; RT, reverse transcriptase; $TCID_{50}$, tissue culture infectious dose 50% endpoint.

Stimulated cultures were incubated 72 h at 37°C. MTT assay was performed as previously described (20).

NK Cytotoxicity Assay. For cytotoxicity assays, large granular lymphocytes (LGL) were purified on Percoll gradients and activated overnight at 10^5 cells/ml with 100 U of rIL-2 (Biogen, Cambridge, MA) and cytotoxicity assays performed as previously described (15). A 6% increase in isotope release, above baseline, was consistently statistically significant at $p < 0.05$ (student's t test).

Analysis of Viral Nucleic Acids in THP-1 Cells. Preparation of total cellular RNA for Northern transfer experiments was done by the guanidine thiocyanate CsCl gradient method. Polyadenylated RNA was prepared by oligo(dT)-cellulose column chromatography. RNA pellets were twice precipitated with ethanol and quantitated by absorbance at 260 nM. Ethidium bromide staining was used to equalize amounts of nucleic acids. After equilibration, the RNAs were separated on 0.9% agarose/formaldehyde gels and blotted onto nitrocellulose. Northern blots were probed by primer extension of DNA fragments of the HIV-1 strain HXB2 (21). The probes were used at a concentration of 4×10^6 cpm/ml of dCTP[^{32}]-labeled pHXBΔ11 (a gift of George Pavalakis, Bionetics Research Inc., Frederick Cancer Research Facility, Frederick, MD). Blots were prehybridized and hybridized at 42°C for 24 h each. Hybridization and washings were done as previously described (22, 23).

Long Terminal Repeat (LTR)-directed Nuclear Run-on Competition Experiments. Analysis of RNA transcripts was carried out by nuclear transcription run-on assay. Adaptions of the method of Greenberg and Ziff (22) were made as previously described (23). The ^{32}P-labeled RNA was recovered by treating with a final concentration of 0.2 M NaOH for 10 min on ice, neutralized by acid-free Hepes to a final concentration of 0.24 M. This purified labeled RNA was hybridized at 65°C for 30 h to purified HIV-1 LTR fragment immobilized on nitrocellulose. A recombinant construct pL3CAT consisting of a Bam HI-Hind III fragment of HIV-1 (nucleotides -1068 to +83), which contains the HIV-1 LTR promoter as well as downstream TAR sequences (14), was used to isolate template DNA. pL3CAT was digested with Kpn 1-Hind III, purified on a 1% low melting agarose gel; phenol was extracted and washed with 70% ethanol, and then the dried template was used in the assay. Hybridizations and washings were done as previously described (22, 23).

Mobility Shift Assay. For binding assays, the plasmid pL3-CAT (14) was digested with Hind III, dephosphorylated with CIAP, ethanol precipitated, and then 5'-labeled with γ-[^{32}P]ATP and T4 polynucleotide kinase. The labeled fragment was digested again with Eco RV. The 199-bp fragment was gel purified and recovered as described above. The assay used was a slight modification of the procedure of Kadonaga et al. (24). The assay was done in 20-μl reaction volume containing 20 mM Hepes (pH 7.6), 60 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 1 μg poly dI-poly dC, 0.05% NP-40, 50,000 cpm of end-labeled DNA probe (Eco RV-Hind III of HIV-1 LTR), and nuclear extracts at 5 or 10 μg protein. Nuclear extracts were prepared by the method of Parker and Topol (25). For competition studies, a 25-fold excess of the same unlabeled DNA fragment was added to the reaction mixture. In mixing experiments, a ratio of one part of extract for inhibition to four parts extract to be inhibited was used with the protein content held constant at either 5 or 10 μg. The reaction mixture was incubated at 30°C for 1 h and then subjected to electrophoresis at 10 V/cm through a 4% polyacrylamide (nondenaturing) gel in Tris-EDTA-Borate buffer.

Polymerase Chain Reaction (PCR) Analysis of RNA Products in HIV-1-infected THP-1. 1 μg of DNA or RNA from infected or uninfected cells was amplified as previously described (26) in reaction mixtures containing primer pairs specific to HIV-1 gag region of the HIV-1 viral genome. 50 pmol of each primer was used. For detection of RNA, an additional step of reverse transcription of RNA to DNA by avian myeloblastosis virus RT was incorporated where the amplification primer initiated the reverse transcription (26). PCR products were analyzed by hybridization with specific probes spanning the region between primer pairs followed by analysis on polyacrylamide gels where the expected radiolabeled band for gag is 114 bp (26).

Results

Differential Expression of HIV after Infection of THP-1 Cells. An acute infection of THP-1 cells was established by incubating HIV-1 (strain BP-1) at an MOI of 0.05

with cells in the log phase of growth. In each experiment, productive infection as measured by extracellular virus was not detected at day 7, but was detected between day 14 and 17. Extracellular virus was detected in media by four assays: presence of p24 core antigen, RT, viral-mediated T cell cytolysis, and syncytia formation (Table I). Phenotypic analysis of the cells showed that monocyte surface antigens, such as CD14 (Leu-M3), were unaffected by HIV-1, while CD4 antigen (Leu-3A) could no longer be detected on the cell surface, presumably blocked by the binding of HIV virions (Fig. 1 b). Also, 30–40% of the cells contained surface antigens recognized by anti-gp 160:41 (Fig. 1 b).

In contrast, 45–60 d after the onset of acute infection, entire cultures of these previously productively infected cells produced little or no extracellular virus (Table I, lines 3, 5, and 8–10). Cell surface analysis showed that CD4 antigen could be recognized again while the presence of viral antigens could not be detected (Fig. 1, c). Presence of virus as measured by RT, infectivity, T cell cytopathology, and syncytia formation was not detected in the media of these cells. However, the media of some of these cultures contained p24 core antigen at a concentration of <20 ng/ml, as compared with 200–500 ng/ml for productive cultures. These cultures remained HIV infected as shown by increased expression after treatment of the cells with LPS, (Table I, line 4) or irradiation (Fig. 1 d). 72 h after activation of the cells, extracellular virus, as measured by all the criteria used, was present and viral antigens could again be detected on the cells. These data suggested that viral expression was restricted in these cells. In addition, several nonproducer THP-1 cultures remained nonproducers after activation (Table I). This absence or low level of viral production in infected THP-1 cells was reproducibly seen with two other isolates, HIV-1 ADA, a monocytoid isolate, and HIV-2 Rod (Table I).

Conditions for Establishing Restricted Viral Production in THP-1 Cells. To better understand the conditions necessary for the establishment of restricted expression, experiments were done to ask the effect of MOI on subsequent viral expression in THP-1

TABLE I
Analysis of HIV-1-infected THP-1 Cells

Viral strain	Viral expression	Syncytia (percent positive)	p24 antigen	Viral RT
			ng/ml	*cpm/ml*
1. None	None	0	0	0
2. BP-1	Producer	2–5	200–500	30–60,000
3. BP-1	Restricted	0	0.5–20	0
4. BP-1	Restricted (LPS)*	1–2	200–500	15–20,000
5. BP-1	Nonproducer	0	0	0
6. BP-1	Nonproducer (LPS)	0	0	0
7. ADA	Producer	5–10	1,500	50–100,000
8. ADA	Nonproducer	0	0	0
9. ROD	Restricted	>1	0.1–100	5–10,000
10. ROD	Nonproducer	0	0	0

THP-1 cells were grown and infected with HIV strains at an MOI of 0.05, as described in Materials and Methods. Analysis of the viral expression was made 60 d post-infection, as described in Materials and Methods. Values are for at least three separate infections.
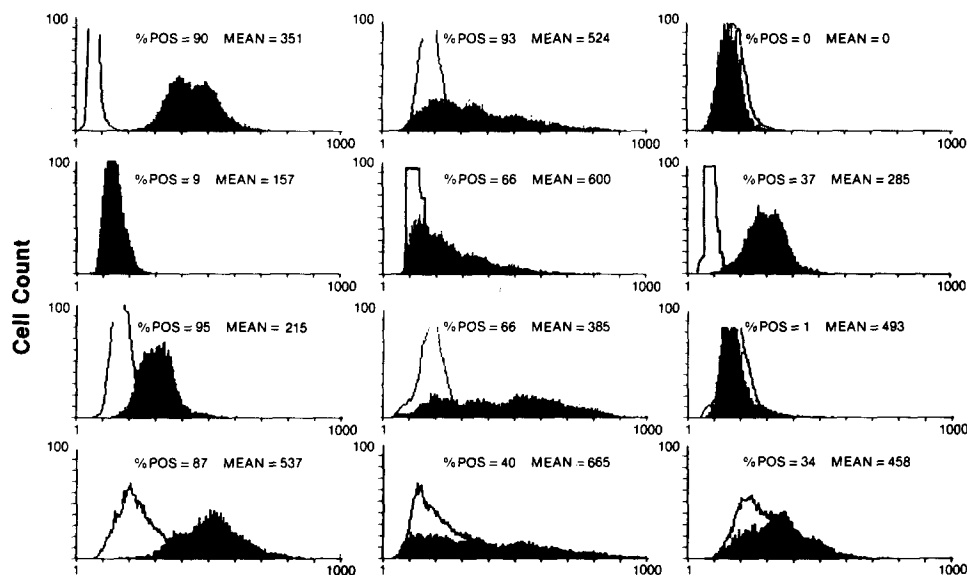* Cells were incubated for 48 h with 10 μg of LPS, and viral assays were performed.

TABLE II

Effect of MOI on HIV Infection of THP-1 Cells

Viral strain	MOI*	Days to infection‡	Cytolysis§	No. productive/ no. total	No. restricted‖/ no. total
BP-1	10	4	+	10/10	0/10
	1	7	+ / −	10/10	0/10
	0.1	14	−	9/10	1/10
	0.01	14–18	−	6/10	4/10
ADA	10	4	+ + +	3/3	0/3
	1	4	+ +	3/3	0/3
	0.1	7–10	+	3/3	0/3
	0.01	14	+ / −	1/3	1/3
ROD	1	14	−	0/2	2/2
	0.1	21–24	−	0/2	2/2

* MOI is the number of $TCID_{50}$ U/cells. THP-1 cells were grown and infected with HIV strains as described in Materials and Methods.

‡ Number of days post-infection that detectable virus was present in extracellular media as assayed by viral p24 antigen.

§ Cytolysis and cell death were observed microscopically. Infected cultures of ADA at high MOI showed complete death.

‖ Analysis of the restricted viral expression was made 60 d post-infection using the criteria of no syncytia formation and low or absent extracellular p24, as in Table I. Restricted and nonproducer cultures are included in total.

presence may be related to the mechanism of restricted expression in these cells. To our surprise, the nonproducer cultures produced no detectable viral RNA (Fig. 2 *A*, lane *5*) even if 60 µg of poly(A)$^+$-selected RNA (Fig. 2 *B*, lane *2*) was analyzed, suggesting that HIV expression in these cells was truly latent (i.e., complete absence of viral expression).

Since some of the nonproductive cultures possessed viral RNA, the status of intracellular viral particles was studied by EM. In those cultures with restricted viral expression, intracellular assembly of numerous viral particles was observed. Most of the virions were seen within intracytoplasmic vacuoles. Many immature and mature forms, as well as virions budding into the vacuoles, could be seen. The viral particles seen on ultrastructural analysis were identical to previous descriptions of HIV (1). As seen in freshly infected macrophages (27), these vacuoles were predominantly found in the perinuclear golgi region. Surprisingly, few if any extracellular virions were seen. Approximately 30% of the cell sections were associated with viral particles. In cultures with latent infection, no intracellular viral particles or viral RNA could be detected.

Negative Regulation of HIV Expression in THP-1 with Restricted Expression. Since the accumulation of viral RNA in the HIV-restricted cells is many fold lower than in the HIV-producing cells (Fig. 2 *A*), the rate of viral transcription was measured using in vitro transcription directed by the LTR in a nuclear run-on assay (Fig. 3). Exogenous LTR was added to the in vitro transcription mixture so that initiation and elongation of viral RNA could be measured. Nuclei from HELA and uninfected THP-1 showed a small basal level of transcription. Nuclei from the THP-1 cells with restricted expression showed a small increase in the rate of transcription over control HELA and uninfected THP-1 (Fig. 3, lane *3*), but the transcription was much re-
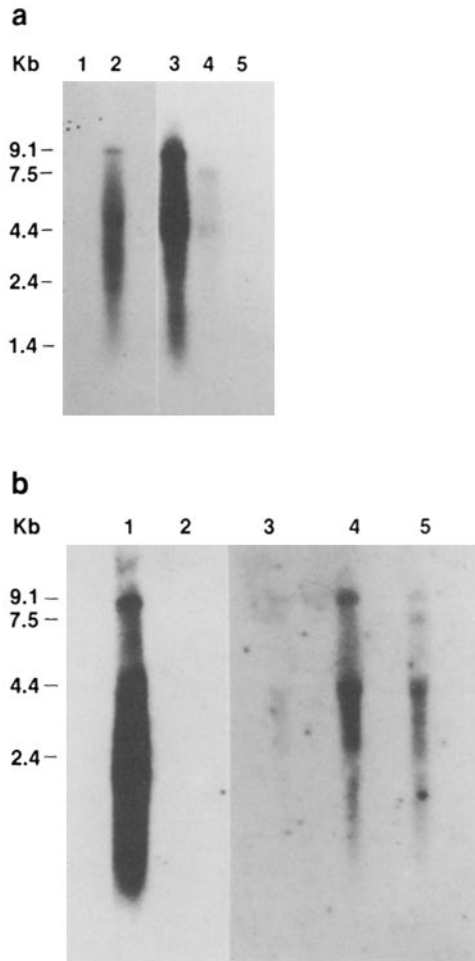
a

b

FIGURE 2. Analysis of viral nucleic acids in infected THP-1 cells. Northern transfer and hybridizations on total RNA were performed as described in Materials and Methods. (a) Lane 1, uninfected THP-1; lane 2, THP-1 productively infected with HIV-1 BP-1 from a 30-min exposure; lane 3, THP-1 productively infected with HIV-1 from a 2-h exposure; lane 4, THP-1 with restricted HIV-1 expression from a 2-h exposure; lane 5, THP-1 with latent HIV virus from a 2-h exposure. (b) Lane 1, THP-1 productively infected with HIV-1, poly(A)+ RNA 20 μg; lane 2, THP-1 with latent HIV virus, poly(A)+ RNA 60 μg; lane 3, THP-1 with latent HIV virus 72 h after 5-azacytidine (10 μM) treatment; lane 4, THP-1 with latent HIV-1 virus 72 h after 5-azacytidine treatment cocultured with HUT-102B2 for 10 d; lane 5, THP-1 with restricted HIV-1 expression.
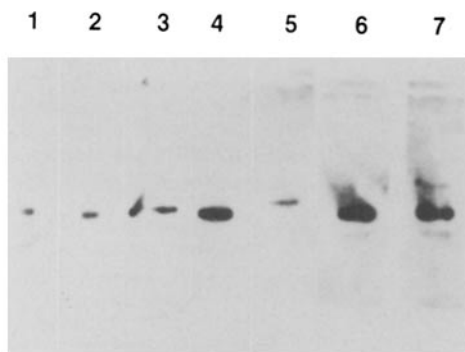
FIGURE 3. HIV-1 LTR–directed nuclear run-on competition experiments. Analysis of RNA transcripts was performed by nuclear transcription run-on assay as described in Materials and Methods. Unless indicated, reactions contained 75 μl nuclei and 25 μl buffer. Lane 1, THP-1 nuclei; lane 2, HELA nuclei; lane 3, nuclei from THP-1 cells with restricted HIV-1 (BP-1) expression; lane 4, nuclei from THP-1 cells productively infected with HIV-1; lane 5, nuclei from productively infected THP-1 (75 μl) and nuclei from THP-1 with restricted HIV-1 expression (25 μl); lane 6, nuclei from productively infected THP-1 (75 μl) and nuclei from THP-1 (25 μl); lane 7, nuclei from productively infected THP-1 (75 μl) and nuclei from THP-1 with latent virus (25 μl).

duced from levels of transcription observed using nuclei from productively infected cells (Fig. 3, lane *4*). To ascertain whether nuclear factors in restricted nuclei were affecting LTR-directed transcription, nuclei from productively infected cells were incubated with various mixtures of nuclei (Fig. 3). Mixing nuclei in a 1:4 ratio from either uninfected THP-1 or THP-1 with latent virus with nuclei from productively infected THP-1 cells did not affect the rate of transcription (Fig. 3, lanes *6* and *7*). However, there was a marked decrease (5-10-fold) in the transcriptional level of nuclei from HIV-producing THP-1 cells when the same ratio (1:4) of competing nuclei from THP-1 cells with restricted expression was added (Fig. 3, lane *5*). This nuclear material would also inhibit LTR-directed transcription of nuclei from HIV-infected T cells but not HTLV-1 LTR-directed transcription in HTLV-1-infected T cells (data not shown).

To determine whether this negative regulation of viral transcription in these cells with restricted expression was at the level of DNA binding complex formation, gel mobility shift assays were performed using the enhancer-TAR region (−117 to +82) of the HIV-1 LTR (Fig. 4). Two concentrations of each nuclear extract were used. Extracts from HIV-1-producing THP-1 cells (Fig. 4, lanes *4* and *5*) and a tat-producing mouse cell line (lanes *8* and *9*) give the same complex formation, while the extract from the restricted cell line (lanes *6* and *7*) did not have any DNA binding in the area where extracts from both the productively infected THP-1 cells or the tat-producing cells bound the DNA. A second (lower) complex was found from both extracts of productive and restricted cells, but not the tat-producing cells. Mixing
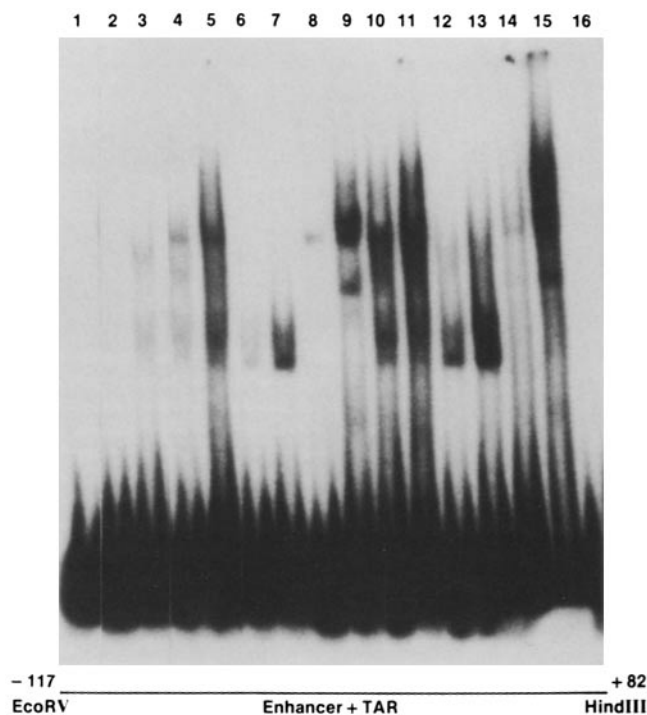

FIGURE 4. Gel mobility shift analysis of protein binding from infected THP-1 cells to HIV LTR. A ^{32}P-labeled oligonucleotide spanning the enhancer-TAR region (−117 to +82) of the HIV-1 LTR was incubated with two concentrations (5 and 10 μg) of nuclear extracts prepared from various cells as described in Materials and Methods. Lane *1*, probe alone; lanes *2* and *3*, uninfected THP-1; lanes *4* and *5*, productively infected (BP-1) THP-1; lanes *6* and *7*, THP-1 with restricted HIV-1 expression; lanes *8* and *9*, tat-producing murine cell line; lanes *10* and *11*, productively infected (BP-1) THP-1 plus uninfected THP-1 (4:1 ratio); lanes *12* and *13*, productively infected THP-1 plus THP-1 with restricted HIV-1 expression (4:1 ratio); lanes *14* and *15*, productively infected THP-1 plus tat-producing murine cell line (4:1 ratio); and lane *16*, productively infected THP-1 with excess cold probe.

an extract from uninfected THP-1 with an extract from productively infected cells did not affect binding (Fig. 4, lanes *10* and *11*). In contrast, when an extract from cells with restricted expression was mixed with an extract from productively infected cells, the binding of productive cell extract to the LTR (Fig. 4, lanes *12* and *13*) was eliminated, suggesting one mechanism of restricted HIV-1 expression was at the level of initiation of transcription by blocking DNA binding complex formation with the HIV-1 LTR. Thus, HIV expression in cells with restricted expression may be actively suppressed by some DNA binding factor. Such expression is not detectable in cells with latent viral infection (data not shown).

Reactivation of Virus from Cultures with Latent Infection. Since THP-1 cells with latent virus exhibited no detectable viral expression and did not negatively regulate viral transcription, we asked if, using PCR, we could detect viral expression in the cells with latent virus (Fig. 5). Using primer pairs to the gag region, which has been shown to be the most sensitive region for HIV detection (26), two latently infected cultures (Fig. 5, lanes *3* and *4*) showed no detectable viral RNA after 2 h (Fig. 5 *A*) or 24 h (Fig. 5 *B*) of gel exposure. For comparison, productively infected T cells
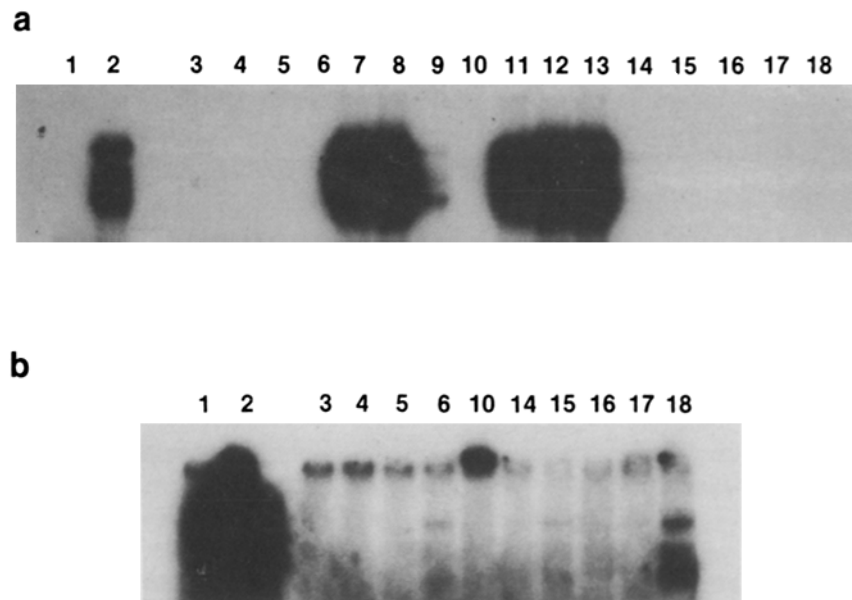

FIGURE 5.   PCR analysis of RNA from infected THP-1 cells under various conditions of RNA isolated, as described in Materials and Methods, was amplified using gag primers. At the end of 35 cycles, aliquots were hybridized to a ^{32}P-end-labeled oligonucleotide probe spanning the region between the primer pairs. PCR products were analyzed on a 20% polyacrylamide gel using the gag probe. (*a*) Lane *1*, uninfected H9; lane *2*, HIV-IIIB H9; lane *3*, latent THP-1 ADA; lane *4*, latent THP-1 BP-1; lane *5*, latent THP-1 BP-1; lane *6*, THP-1; lane *7*, BP-1 HUT-78; lane *8*, BP-1 THP-1; lane *9*, restricted THP-1 BP-1; lane *10*, THP-1; lane *11*, restricted THP-1 LPS treated; lane *12*, restricted THP-1 PMA treated; lane *13*, productive THP-1; lane *14*, latent THP-1 ADA; lane *15*, latent THP-1 ADA with 5-azacytidine; lane *16*, latent THP-1 BP-1 LPS; lane *17*, latent THP-1 BP-1 with 5-azacytidine; lane *18*, latent THP-1 BP-1 with 5-azacytidine plus LPS. Results from 2-h exposure. (*b*) Same lanes 24 h after exposure.

(lane 7), productively infected THP-1 (lane 8), and THP-1 with restricted expression (lane 9) are shown. We next studied under what conditions the virus could be reactivated. Neither LPS (Fig. 5, lane 16) nor IUdR (Fig. 5, lane 17) could induce viral expression from cells with latent virus. Since previous work has shown that the HIV-LTR stably transfected in fibroblasts was methylated (28), 5-azacytidine was used and found to be an inducer of virus expression (Fig. 5 B, lane 18, or Fig. 2 B, lane 3), even after the virus was latent for 10 mo. We cannot be sure whether the level of RNA is due to activation of a small population of cells or to a general lower level of transcription. However, sufficient amounts of infectious virus were produced to get a massive infection of the T cells line, HUT-102B2, within 10 d of transmission (Fig. 2 B, lane 4). Electron micrographs of reactivated latent virus transmitted into HUT-102B2 show typical morphological characteristics of HIV assembly and budding (Fig. 6).

Viral and Cellular Biology of Restrictedly and Latently HIV-infected THP-1. The morphological, phenotypic, and functional characteristics of THP-1 cells with restricted and productive expression of HIV-1 were essentially the same as uninfected cells. Phagocytosis of yeast particles, accessory cell function for T cell activation, and development of anchorage dependence were all normal before and after PMA-induced differentiation.

Since these cells were functionally normal, we next asked whether they were immunologically normal. As we had previously shown (15), IL-2-activated LGL can recognize and lyse HIV-infected cells. Specific LGL-mediated cytotoxicity was observed only on the HIV-1-producing THP-1 cells. IL-2 stimulation increased the magnitude of LGL-mediated cytotoxicity. No cytotoxicity was seen on the cells with restricted HIV expression, suggesting that such cells can avoid recognition by specific immune mechanisms.

The biological characteristics of the virus residing in these functionally and immunologically normal THP-1 cells were determined. T cell cytopathology was measured using an assay where virus-producing cells cause cytolysis of MT-2 target cells (Fig. 7 A). Using HIV-1-infected HUT-78 cells, 50% of the target cells are killed by 100 cells, while all the cells are killed by 250 cells. Using the THP-1 cells with restricted expression, 50% of the target cells are killed by 200 cells. This cell cytotoxicity is blocked when azidothymidine (AZT) is added to the cultures, showing that it is HIV mediated. The supernatant of these cells with restricted expression cannot induce MT-2 cytotoxicity (Fig. 7 B), while those from cells productively infected kill very efficiently. Supernatant from irradiated cells with restricted expression will now kill MT-2. Consistent with this is the observation that THP-1 cells with restricted expression will not kill MT-2 cells when they are separated by a permeable barrier (data not shown). Virus reactivated from latently infected THP-1 cells after 10 mo of culture was able to kill T cells as efficiently as virus released from continuously productive cultures.

Discussion

An acute productive infection of THP-1 cells was established using HIV-1 and HIV-2. Between days 14 and 21, 20-40% of the cells contain antigens recognized by anti-p24 and anti-gp 160/41. These data are consistent with published reports using CD34$^+$ hematopoietic stem cells (29), fresh monocytes (9), and U937 (6, 7).
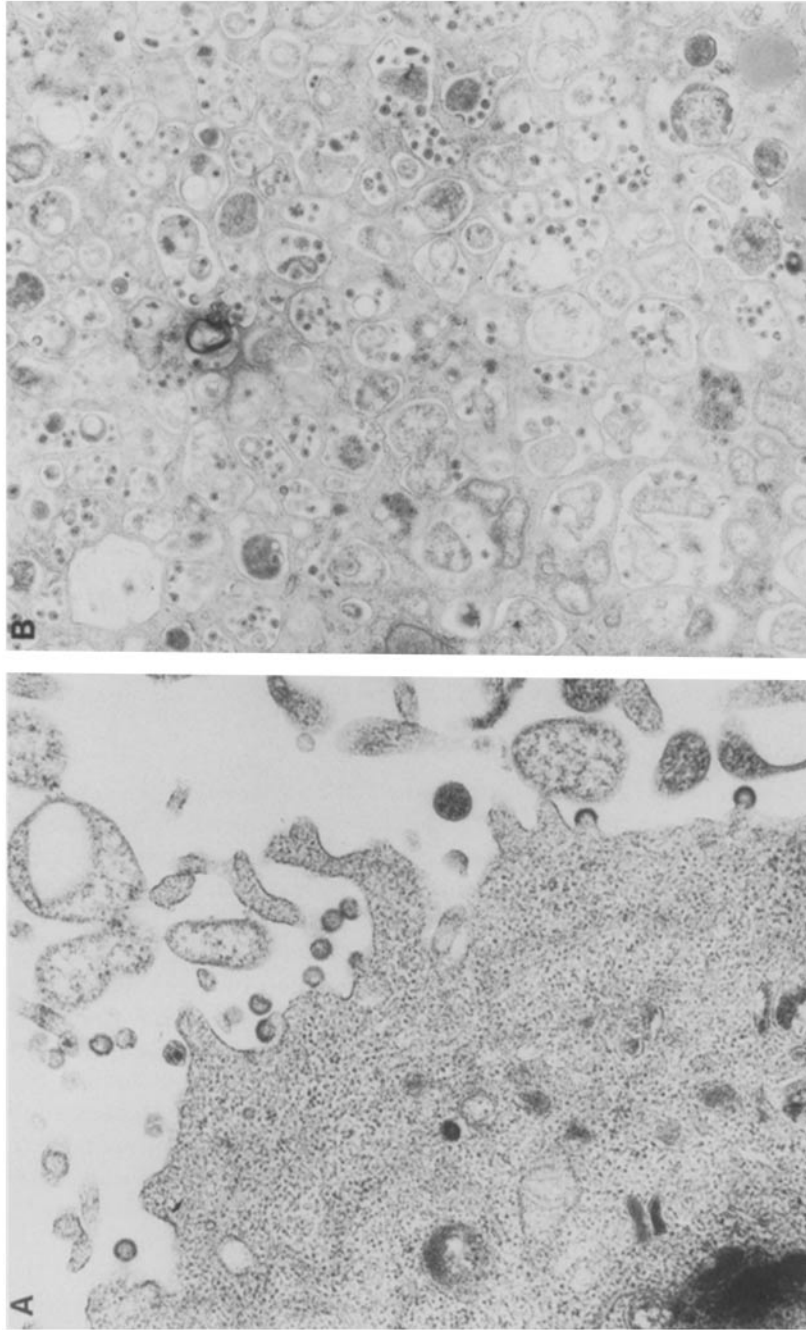
FIGURE 6. EM of transmitted HIV-1 from latently infected THP-1. After treatment with 5-azacytidine (10 μM) for 48 h, latently infected THP-1 cells were cocultured with HUT-102B2 cells. After 10 d, electron micrographs of HUT-102 cells were prepared as previously described (1, 39). (A) HIV budding from HUT-102B2 (×72,000); (B) intracellular virions in HUT-102B2 (×24,000).
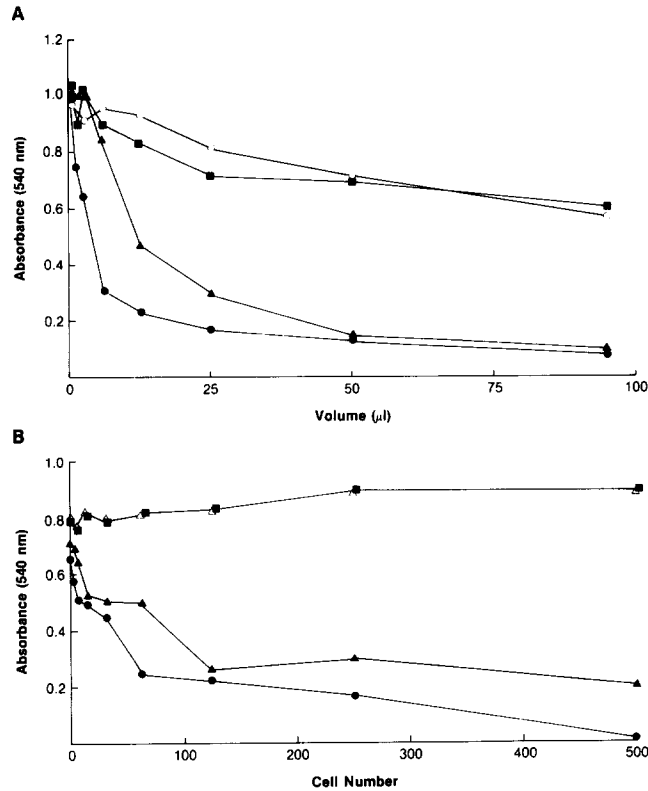
Figure 7. Viral mediated T cell cytotoxicity assays. HIV-infected cultures were tested either by titering out 5-d supernates against 10,000 MT-2 cells per well in a round-bottomed 96-well plates or by coculturing with infected cells at several concentrations. After 5-7 d of incubation, MTT is used to measure in vitro growth by cell-mediated reduction of tetrazolium (20). The OD at 540 nm is directly proportional to the number of viable cells. (A) Ability of extracellular virus to induce cytolysis of MT-2. 5-d-old culture supernatants from THP-1 cells with restricted HIV-1 expression (■), THP-1 cells with restricted expression after irradiation (▲), control THP-1 (O), and HIV-1-infected HUT-78 (●) are shown. (B) Ability of HIV-infected cells to induce cytolysis of MT-2. THP-1 cells with restricted HIV expression (▲), control THP-1 (■), and HIV-1-infected HUT-78 (●) are shown.

Several weeks after infection of THP-1 cells by HIV, entire cultures spontaneously became restricted in viral expression, while in the producer cultures, >90% of the cells were viral antigen positive. In some cases, spontaneous nonproducers have been reported for T cells (3, 4) but not for monocytes. Two distinct types of infected cultures with altered expression were identified in THP-1: (a) cells with restricted HIV expression; and (b) cells with latent competent virus.

It is unlikely that these cultures arose due to selection of clonal variants, since with infection at low MOI, no THP-1 cytotoxicity or loss of viability, growth, or function was seen in infected cultures. However, it is not possible to rule out that the cells with restricted or latent infection were present from the first day of infection and eventually overgrew the other cells in the culture. While it is not possible to determine how these cultures arose, their existence has important implications for HIV viral persistence and pathology. Furthermore, in both types of restricted cultures, cells could be induced to produce virus after 10 mo in culture. Thus, the phenotype of these viral cultures was stable.

Characterization of these two types of infected monocytoid cultures has shown that they are clearly different at the molecular and cellular level. In cells with restricted HIV expression, there is a greatly reduced rate of transcription and slower accumulation of viral RNA, as shown by nuclear run-on, PCR, and Northern analysis. This is due at least in part to HIV-specific factor(s) present in the nucleus of

restrictedly infected cells that can negatively regulate transcription of productively infected cells. Gel mobility shift analysis showed that formation of DNA binding complexes associated with tat is eliminated by nuclear extracts of restrictedly infected cells, suggesting that initiation of transcription is being regulated. In addition, production of genomic viral RNA is being reduced in these restricted cells with a concomitant appearance of a novel subgenomic 7.5-kb RNA (Fig. 2). This RNA is not seen when the virus is then reinfected into T cells. In cells with latent virus, viral expression and the ability to negatively regulate transcription of productively infected cells were not observed. By PCR analysis, no viral RNA was found including nef RNA (data not shown), suggesting that nef does not serve as a negative regulator in these cells. This is the first demonstration that HIV infection of monocytoid cells can lead to latency at the molecular level.

In addition, the ability of various agents to activate viral expression in these two infected cell types is distinct. In agreement with several others (30–33), we find various cytokine treatments, LPS, PMA, TNF-α, and GM-CSF, can stimulate expression in restricted cells such that viral levels approach that of a productive cell. However, none of these treatments stimulate detectable production from cells with latent virus. The ability of 5-azacytidine to reactivate virus production suggests that methylation is involved in the regulation of HIV expression in these latently infected cells. Using a stably transfected LTR into fibroblasts, it has been previously shown that methylation of HIV LTR sequences could occur (28).

Treatments that positively regulate HIV transcription can be mediated by cellular factors such as NFκB (34, 35), and AP-1 (36) that bind to specific sequences in the HIV LTR. In addition, that has been postulated to mediate its effects through cellular factors that bind to the sequences responsive to the transactivating response region (37). Viral factors such as the nef gene product, which can repress HIV transcription (38), and vpu, which affects viral release (39), could be important in establishing these states of viral suppression. In addition to the cytokines that upregulate viral expression, IFN-α has recently been shown to restrict viral production in human monocytes (40) and U-937 cells (7). It is clear that multiple pathways regulate HIV transcription, and that negative regulation of viral expression in THP-1 cells probably involves viral and cellular factors.

The biological consequences of these types of restricted HIV expression may be important in the pathogenesis of the disease. In the cells with restricted expression, most if not all infectious virus produced is sequestered intracellularly. The cells eventually store sufficient virus to kill T cells as efficiently as productively infected T cells, probably through cell-cell contact with the uninfected target. Suppression of extracellular virus production and cell surface viral antigen expression allows the monocyte with restricted expression to escape recognition and subsequent lysis by the immune system, which is the fate of productively infected cells (15). In the cells with latent infection, no virus is seen. Infectious virus can be activated and can efficiently kill T cells even after being quiescent for long periods of time. From the molecular and biological aspects, these two states are mechanistically and functionally different. Chronic low level expression is not a model for viral latency at the molecular level, and viral latency has no low level expression (33). However, each of these states provides at least one mechanism of the establishment of HIV viral persistence. It would be important to determine the status of viral latency in HIV-

infected patients. Understanding these diverse interactions between HIV and mono-cytes is important in understanding the nature of viral persistence and its relationship to disease.

Summary

In THP-1 monocytoid cells infected with HIV, viral expression can be regulated in several ways: (a) latency (no viral expression); (b) restricted expression (chronic low-level viral expression with little or no detectable virus released); and (c) continuous production. In cells with restricted HIV expression, nuclear factor(s) were found that blocked tat-associated DNA binding complex formation, suggesting that initiation of transcription was negatively regulated. Also, viral particles were seen budding into and accumulating within intracytoplasmic vacuoles with little virus released, suggesting multiple levels of regulation. These cells with restricted expression had no detectable viral antigens on the cell surface and were not lysed by IL-2-activated large granular lymphocytes. However, they could cause viral-mediated T cell cytolysis in cell-cell assays, suggesting viral transmission through cell contact. In addition, cells with latent HIV were identified and could still produce infectious virus after 5-azacytidine exposure 10 mo later. LPS and other treatments could increase viral production in cells with restricted but not latent expression, suggesting they occur by distinct mechanisms. These infected cells provide a reservoir for viral transmission to uninfected T cells that itself is not detected by immune surveillance mechanisms.

References

1. Gonda, M. A. 1988. Molecular genetics and structure of the human immunodeficiency virus. *J. Electron Microsc.* 8:17.
2. Ranki, A., S. L. Valle, M. Krohn, J. Antonen, J. P. Allain, M. Leuther, G. Franchini, and K. Krohn. 1987. Long latency precedes overt seroconversion in sexually transmitted human immunodeficiency-virus infection. *Lancet.* ii:589.
3. Zagury, D., J. Bernard, R. Leonard, R. Cheynier, G. Feldman, P. S. Sarin, and R. C. Gallo. 1986. Long-term cultures of HTLV-III infected T cells: a model of cytopathology and T cell depletion in AIDS. *Science (Wash. DC).* 231:850.
4. Hoxie, J. A., N. Pillsbury, and J. A. Levy. 1985. Persistent noncytopathic infection of normal human T lymphocytes with AIDS-associated retrovirus. *Science (Wash. DC).* 229:1400.
5. Folks, T., D. M. Powell, M. M. Lightfoote, S. Benn, M. A. Martin, and A. S. Fauci. 1986. In induction of HTLV-III/LAV from a nonvirus-producing T-cell line: implications for latency. *Science (Wash. DC).* 231:600.
6. Folks, T. M., J. Justement, A. Kinter, S. Schnittman, J. Orenstein, G. Poli, and A. S. Fauci. 1988. Characterization of promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.* 140:1117.
7. Mace, K., M. DucDodon, and L. Gazzolo. 1989. Restriction of HIV-1 replication in

promonocyte cells: role for IFNα. *Virology.* 168:399.

8. Gendelman, H. E., O. Narayan, S. Kennedy-Stoskopf, P. G. E. Kennedy, Z. Ghotbi, J. E. Clements, J. Stanley, and G. Pezeshkpour. 1986. Tropism of sheep lentiviruses for monocytes: susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages. *J. Virol.* 58:67.

9. Gendelman, H. E., J. M. Orenstein, L. M. Baca, B. Weiser, H. Burger, D. C. Kalter, and M. Melter. 1989. Editorial review: the macrophage in the persistence and pathogenesis of HIV infection. *AIDS (Lond.).* 3:475.

10. Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. DalCanto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci. 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science (Wash. DC).* 233:1089.

11. Epstein, L. G., L. R. Sharer, E.-S. Cho, M. F. Meyenhofer, B. A. Navia, and R. W. Price. 1985. HTLV-III/LAV-like retrovirus particles in the brains of patients with AIDS encephalopathy. *AIDS Res. Ther.* 1:447.

12. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobyashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer.* 23:171.

13. Tsuchiya, S., Y. Kobyashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada. 1982. Induction of maturation in cultured human monocytic leukemia cells by phorbol diester. *Cancer Res.* 42:1530.

14. Wright, C. M., B. K. Felber, H. Paskalis, and G. N. Pavlakis. 1986. Expression and characterization of the trans-activator of HTLV-III/LAV virus. *Science (Wash. DC).* 234:988.

15. Ruscetti, F. W., V. S. Kalyanaraman, R. Overton, J. Mikovits, H. Stevenson, K. Stromberg, R. B. Herberman, W. L. Farrar, and J. R. Ortaldo. 1986. Analysis of effector mechanisms against HTLV-I and HTLV-III/LAV infected lymphoid cells. *J. Immunol.* 136:3619.

16. Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, M. Mitra, T. Phipps, L. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, D. Skillman, and M. Meltzer. 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes *J. Exp. Med.* 167:1428.

17. Clavel, F., D. Guetard, F. Brun-Vezinet, S. Chamaret, M. A. Rey, M. O. Santos-Ferreira, A. G. Laurent, C. Dauguet, C. Katlama, C. Rouzioux, D. Klatzmann, J. L. Champalimaud, and L. Montagnier. 1986. Isolation of a new human retrovirus from West African patients with AIDS. *Science (Wash. DC).* 233:343.

18. Healey, D. S., W. J. Maskill, E. V. Neate, F. Beaton, and I. D. Gust. 1988. A preliminary evaluation of five antigen detection assays. *J. Virol. Methods.* 20:115.

19. Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Isolation of type-C retrovirus particles from cultured and fresh lymphocytes from a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA.* 77:7415.

20. Alley, M. C., D. A. Scudiero, A. Monics, M. I. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker, and M. R. Boyd. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 48:589.

21. Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R.-S. Liou, R. C. Gallo, and F. Wong-Staal. 1987. Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res.* 3:57.

22. Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (Lond.).* 311:433.

23. Sparks, M. C., W. L. Farrar, D. Rennick, and F. W. Ruscetti. 1986. Interleukin-3 regulates expression of IL-2 receptors on IL-3 dependent cells by increased transcription of the IL-2 receptor gene. *Science (Wash. DC).* 233:455.

24. Kadonaga, J. T., K. C. Carner, F. R. Masiarz, and R. Tjian. 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell.* 51:1079.

25. Parker, C. S., and J. Topol. 1984. A drosophila RNA polymerase II transcription factor contains a promoter-region specific DNA-binding activity. *Cell.* 36:357.

26. Hewlett, I. K., M. Ruta, K. Cristiano, C. A. Hawthorne, and J. S. Epstein. 1989. Co-amplification of multiple regions of HIV-1 genome by the polymerase chain reaction: potential use in multiple diagnosis. *Oncogene.* 4:1149.

27. Orenstein, J. M., M. S. Meltzer, T. Phipps, and H. E. Gendelman. 1988. Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. *J. Virol.* 62:2578.

28. Bednarik, D. P., J. D. Mosca, and N. B. K. Raj. 1987. Methylation as a modular of expression of human immunodeficiency virus. *J. Virol.* 61:1253.

29. Folks, T. M., S. W. Kessler, J. M. Orenstein, J. S. Justement, E. S. Jaffe, and A. S. Fauci. 1988. Infection and replication of HIV-1 in purified progenitor cells of normal bone marrow. *Science (Wash. DC).* 242:919.

30. Folks, T. M., J. Justement, A. Kinter, C. A. Dinarello, and A. S. Fauci. 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science (Wash. DC).* 238:800.

31. Koyanagi, Y., W. A. O'Brien, J. Q. Zhoa, D. W. Golde, J. C. Gasson, and I. S. Y. Chen. 1988. Cytokines alter production of HIV-1 from primary mononuclear phagocytes. *Science (Wash. DC).* 241:1673.

32. Hammer, S. M., J. M. Gillia, J. E. Groopman, and R. M. Rose. 1986. In vitro modification of human immunodeficiency virus infection by granulocyte-macrophage colony-stimulating factor and γ interferon. *Proc. Natl. Acad. Sci. USA.* 83:8734.

33. Rosenberg, A. F., and A. S. Fauci. 1989. Minireview: induction of expression of HIV latently or chronically infected cells. *AIDS Res. Hum. Retroviruses.* 5:1.

34. Nabel, G., and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature (Lond.).* 326:711.

35. Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel. 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κβ. *Nature (Lond.).* 339:70.

36. Dinter, H., R. Chiu, M. Imagawa, M. Karin, and K. A. Jones. 1987. In vitro activation of the HIV-1 enhancer in extracts from cells treated with a phorbol ester tumor promoter. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4067.

37. Gatignol, A., A. Kumar, A. Rabson, and K.-T. Jeang. 1989. Identification of cellular proteins that bind to the HIV-1 TAR RNA. *Proc. Natl. Acad. Sci. USA.* 86:7828.

38. Ahmad, N., and S. Venkatesan. 1988. *nef* Protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. *Science (Wash. DC).* 241:1481.

39. Strebel, K., T. Klimkait, and M. Martin. 1988. A novel gene of HIV-1, *vpu*, and its 16 kilodalton product. *Science (Wash. DC).* 241:1221.

40. Kornbluth, R. S., P. S. Oh, J. R. Munis, P. H. Cleveland, and D. D. Richman. 1989. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. *J. Exp. Med.* 169:1137.