Thus, unlike the animal lentivirus infections in which the immune response is relatively normal, immunodeficiency is the hallmark of AIDS. However, immunopathology cannot account for all of the pathology seen in lentivirus infections. While, there is an inflammatory component seen in the progressive encephalopathy in this diseases, this is overshadowed by the vacuolation and degenerative changes in these individuals. The development of brain lesions seen in AIDS patients has been associated with the enhanced production of cytokines that could be produced both by macrophages and T lymphocytes. A number of groups have identified the monocyte/macrophage as the primary cellular target for HIV-1 infection in the CNS (79-82). Since HIV-1 has not been shown to directly infect neurons, it is likely that the neurological changes in AIDS patients are mediated by substances released from HIV-1 infected mononuclear cells (83). A recent study suggests neurotoxins released from HIV-1 infected monocyte/macrophages but not T lymphocytes act as agonists for N-methyl-D-aspartate (NMDA) receptors (84). NMDA agonists disrupt the nervous system in several ways including degeneration of neurons.

The Immune Response. The fact that the T cells and the monocytes are involved in the primary immune response has a major effect in the pathogenesis of AIDS. In order to understand the potential mechanisms involved in HIV pathogenesis, it is necessary to consider the functions of

these two cell types in the normal immune response (85). The T cells are the orchestrators and regulators of the immune response. T cells ordinarily recognize antigen only together with major histocompatibility complex (MHC) antigens present on antigen presenting cells (APC). CD4+ T cells interact with targets bearing MHC class II molecules, whereas CD8+ T cells interact with targets bearing MHC class I molecules. Although most somatic cells express class I MHC proteins, relatively few cell types express class II proteins. Those that do include macrophages, dendritic cells in lymphoid tissue, Langerhans cells in the skin, Kuppfer's cells in the liver, and microglial cells in the central nervous system. Interestingly, all of these cell types can be infected by HIV-1.

Helper T (T_H) cells are the principal cell type involved in the initiation of the primary immune response. They are involved in the activation of the major effector cells in the immune response, ie, cytotoxic T(T_c) cells and antibody producing B cells, as well as the activation of the inflammatory cells: macrophages and neutrophils. This is accomplished in part by the production of cytokines. The activation of T_H requires at least two signals. One signal is provided by the binding of the T cell antigen receptor to the class II MHC-antigen complex on the APC. The second signal derives from interleukin-1 (IL-1), produced by the APC. Macrophages produce large amounts of IL-1. These two

signals induce the expression of IL-2, as well as a number of other cytokines, including IL-3, Il-4, IL-5, TGFβ, IFNα, MIP-1α, IFNγ and TNF that are important for triggering the growth and differentiation of B cells into antibody producing cells and activation of macrophages. Activated macrophages in turn produce a variety of cytokines and inflammatory mediators such as IL-6, IL-8, and prostaglandins. IL-2 is a growth factor for T_H cells and thus, the result is to amplify the response initiated by contact of T_H with APCs. The activated T_H cell is the key to further steps along the immune pathway including the triggering of cytotoxic T cells.

Monocytes and macrophages can serve as APC for T lymphocytes by ingesting and degrading foreign antigen in phagolysosomes into peptides that bind to MHC glycoproteins for presentation on the cell surface. Human monocytes express both class I and class II MHC glycoproteins and thus, are able to present antigen to CD8 and CD4 T lymphocytes. Activated lymphocytes then secrete factors that affect monocyte and macrophage function and differentiation. Monocyte and macrophage development is affected by the secretion of cytokines, lymphokines, and interferons by activated T lymphocytes. Cytokines produced by monocytes and macrophages similarly affect the response of lymphocytes. Prostaglandin E, for example inhibits lymphocyte function and proliferation, whereas an inhibitor

of IL-1, called contra-IL-1, produced by infected macrophages may contribute to the impairment of T cell proliferative responses (86). Both IL-1 and TNF increase the adhesiveness of endothelial cells by inducing the expression of cell membrane adhesion molecules such as ICAM 1 (intrinsic cell adhesion molecule type 1).

HIV-1 Pathogenesis: Potential mechanisms. Because T-helper responses require a minimum of three components for the initiation of T_H cell function, one possible mechanism that could account for an early defect seen in T_H function in HIV infected individuals is that the APC necessary for processing and presenting recall antigens to the T_H are functionally defective (87). Several studies have reported defects in monocyte/macrophage functions in cells isolated from AIDs patients (88,89). A defect in APC function could prevent T_H function in the absence of a concomitant reduction in CD4+ T cell numbers. However, other studies in monozygotic twins, only one of whom had AIDS, demonstrated that the APC from the twin with AIDS plus T_H from the uninfected twin gave a normal proliferative response to tetanus toxoid while the converse experiment did not suggesting that the early defect in helper function is a defect in the T_H population itself (90). Because the early T_H defect is not a critical reduction in the number of CD4+ T cells, it has been suggested that disregulation of soluble factors, such as TGFβ, in AIDS patients disrupts normal T_H

function (91). At picomolar concentrations, TGFβ has been reported to inhibit murine MHC self-restricted proliferative responses (92) and recently TGFβ has been shown to be involved in selective loss of T_H responses to recall antigens in tumor bearing mice (92). In addition, it has previously been shown that IL-2 activated NK cells will recognize and kill HIV-1 infected cells (76). Recently, it has been suggested that TGFβ could inhibit cytolysis of HIV-1 infected cells by NK (93) and CD8+ LAK cells (94) thus preventing the function of this arm of the immune response.

In addition, the release of TGFβ has been implicated in the neurotoxicity seen in AIDS patients by a mechanism in which a few infected monocytes release factors which trigger uninfected astrocytes to secrete TGFβ. This potent chemotactic factor may recruit HIV infected monocytes thus, increasing viral spread within the CNS as well as augment the production of neurotoxic cytokines (95).

It has also been suggested that autoimmune responses could interrupt T_H-APC interactions. Autoantibodies have been detected in approximately 35% of AIDS patients and asymptomatic seropositive individuals (96). The mechanism envisioned is that viral envelope glycoprotein is shed and binds to uninfected cells making large numbers of uninfected T cells targets for an autoimmune response directed to viral antigen bound to the CD4 receptor. Another one of these autoantibodies reacts with HIV-infected CD4+ T cells and

inhibits mitogen induced T-cell proliferation (97). Another mechanism of T cell destruction involves the syncytia formation in which surface gp120 from a single infected CD4+ T cell can bind CD4 molecules from uninfected T cells. In this way, one infected cell can form syncytia involving as many as 500 uninfected cells causing lysis and cell death.

An additional mechanism could be the over-production of humoral factors by the infected cell. Infection of monocytes has been suggested to cause increased production of the cytokine TNF. This cytokine is known to cause many immunologic and inflammatory responses leading to wasting, septic shock and ultimately tissue necrosis.

In considering HIV-1 pathogenesis, one must take into account the long clinical latency observed between seroconversion and development of overt disease. Several studies have been reported which show the monocyte/macrophage as a major site of virus infection during the subclinical phase of HIV infection. The ability of the host to escape immune surveillance mechanisms of the host can be explained by either antigenic variation or the "Trojan horse" mechanism. In both visna and HIV, antigenic variants can be isolated in many infected hosts. These occur by point mutations in the envelope of the virus (20). In theory this could provide temporary escape from immune surveillance as the variant strains would not be recognized

by neutralizing antibodies (98,99). However in the case of visna evidence suggests that this antigenic variation plays little role in the pathogenesis of the virus (100). Alternatively, persistence could occur by the latent infection of mobile cells, known as the "Trojan horse " mechanism. This theory links viral persistence to restricted viral gene expression (15). Support of this theory is provided by studies of the pathogenesis of visna. These have shown that monocytes in the lungs and CSF harbor the visna genome and have a low level of gene expression. Visna gene expression could also be demonstrated in epithelial cells (101) and glial cells (102). In these systems, differentiation of monocytes latently infected with visna virus to macrophages increases viral expression and in turn, disease progression (5). Similarly, HIV can be cultured from monocytes obtained from blood (103,104),and organs of patients with AIDS (105,106). As previously stated, increasing evidence suggests that the monocyte/macrophage is mainly responsible for the neurological effects seen in AIDS (82-84). Importantly, in contrast to the CD4+ lymphocyte, the monocyte/macrophage is generally resistent to the cytopathic effects of HIV. Thus, there is strong evidence that the monocyte/macrophage can be a reservoir of HIV in AIDS, although its importance has been questioned (107). Several studies have suggested that a persistent state of latent or chronic low level productive

infection exists in fresh (108, 109) and cultured (110, 111) cells. More important, there are several cases of asymptomatic HIV seropositive men who converted to a seronegative status over a 2-3 year period while remaining positive for provirus (112). These data imply that viral latency and persistence can be a component of the long clinical latency observed before the onset of overt AIDS. The mechanisms involved in developing these latent or restricted states of HIV infection are not well understood. Elucidation of the viral life cycle during the subclinical phase of infection is critical to the understanding of the pathophysiology of AIDS.

GOALS

The state of the virus in the patient during the long clinical latency remains unknown. In particular, it is not known if there are cells in the patient that are truly latent: cells that contain integrated provirus but are producing no virus. Such cells could contribute to the emergence of the disease state if certain events in the patient induced activation of latent virus to a productive state. To examine the question of viral latency in the patient it is first necessary to have a model of latency in a cell line which could be used to address these questions. Information provided by this model concerning mechanisms involved in establishing and overcoming latency may then be extended to the HIV infected individual, thus, establishing the relevance of this model as well as the role of latency in the pathogenesis of AIDS.

MATERIALS AND METHODS

Cell Lines. THP-1, HUT-78, HUT-102, 8E5, U1, CEM-SS, MT-2 and U937 suspension cell lines 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. Murine CB2MX3 cells producing HIV-1 tat (113,114) obtained from B. Felber and G. Pavlakis (BRI, Frederick Cancer Research Facility), were grown as monolayer cultures in DMEM supplemented with 10% FCS, streptomycin (100 μg/ml), penicillin (100 μg/ml), gentamicin (50 μg/ml), and 20 μm $CdCl_2$. HELA cells were grown as monolayer cultures in the same medium minus $CdCl_2$.

Viruses and Infections. All viruses were isolated from PBMCs. HIV-1 strain BP-1 was grown in HUT-78 (76), strain ADA (monocytotropic) was grown in U-937 (115), and HIV-2 Rod was grown in CEM (116). 10^7 THP-1 cells in log phase growth were infected with 5 x 10^5 of $TCID_{50}$ U from cell-free supernatants of each virus harvested 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, Medford, MA) were used to give the indicated MOI.

Virus Detection. Viral p24 antigen was determined on

tissue culture supernatants or on cell pellets lysed with 1% Triton X-100 by ELISA (Cellular Products, Buffalo, NY). As previously shown, HIV p24 ELISA kits do not discriminate between HIV-1 and HIV-2 p24 (117). Tissue culture infectious dose 50% endpoint ($TCID_{50}$) was determined using microtiter wells of HUT-102B2 with serial dilutions of cell-free virus. Electron micrographs of HIV infected cells were prepared on OsO_4 fixed, rapidly dehydrated THP-1 or HUT-102B2 cells embedded in epoxy resin using standard procedures. Thin sections were mounted, stained with uranyl acetate and lead citrate and viewed in a Hitachi H-7000 as previously described (4,118). For syncytia formation, 200 infected THP-1 cells were incubated with 1 x 10^6 HUT-102B2 cells. HIV-1 induced syncytia were recorded by microscopic analysis. In these syncytia, the nuclei were polarized to one side of the cell, leaving a ballooning membrane bound cytoplasm on the other and eventually fused into a multinucleated giant cell. 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 (3). Results were adjusted to cpm of [^{3}H]TTP incorporated [^{3}H] per ml.

Phenotypic Analysis of HIV-Infected THP-1. Cytofluorometric analysis was performed as follows: Cells washed in PBS were fixed in -10°C absolute methanol for intracellular

p24 antigen determination (119). The cells were not fixed for all other assays. Cells incubated with saturating amounts of test or control antibodies for 30-60 min at 4°C in PBS, 1% BSA, .1% azide, were incubated in FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). After two more washes, cells were analyzed with an Ortho Cytofluorograph System 50 with an argon ion laser at 488 nm. Live cells were gated. The percentage positive cells was calculated against a background of nonspecific labeling by using normal IgG (1-3%) (120). The appropriate isotope negative controls were used. Monoclonal antibodies used were directed against HIV p24, HIV gp 160:41 and HIV-1 gp 120 (Cellular Products, Inc., Buffalo, NY). Other monoclonals against Leu-3A (CD4), Leu-2 (CD8), Leu-M3 (CD16), and HLA-DR, were purchased from Becton-Dickinson (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 bottom 96-well plate or by co-culturing 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 (121). 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 (76). THP-1 and HIV-infected THP-1 irradiated at 8,000R were added to 1 x 10^5 T8 cells in a culture vol of 0.2 ml at serial dilutions of THP-1 cells with or without Con A (5 μg/ml). Stimulated cultures were incubated 72 h at 37°C.

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) as previously described (76). Target cells were labeled with 100 μCi [^{51}]chromium (New England Nuclear, Boston, MA) for 1 h at 37°C and washed extensively before use. Target cells, 5 x 10^3 in 0.1 ml, and effector cells in 0.1 of medium were plated in microtiter plates at several effector to target ratios (50:1, 25:1, 12:1, 6:1, 3:1). After 4 or 8 h of incubation at 37°C, the supernatants were harvested and counted in a gamma scintillation counter. Cytotoxicity was determined by the amount of [^{51}]chromium released from dead target cells. Three replicates per experimental group were employed and the percentage of specific lysis was calculated as mean ± SD according to the formula: % specific lysis =

$$\frac{\text{cpm in experimental wells - cpm in wells with target cells alone}}{\text{cpm incorporated in target cells}} \times 100$$

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 (122). Polyadenylated RNA was prepared by oligo(dT)-cellulose column chromatography (123). RNA pellets were twice precipitated with ethanol and quantitated by absorbance at 260 nM ethidium bromide staining was used to equal 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 (124). The probes were used at a concentration of 4 x 10^6 cpm/ml of dCTP[^{32}]-labeled pHXBΔ11 (a gift of George Pavalakis, LBI-FCRF, Frederick, MD). Hybridization conditions were as follows: prehybridization was performed in 50% deionized formamide, 0.2% polyvinylpyrrolidone, 0.2% BSA, 0.2% Ficoll (mol wt 400,000), 0.5 M Tris-HCl (pH 7.5), 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate (mol wt 400,000) and denatured salmon sperm DNA (100 μg/ml) for 24 h at 42°C. The probe was added in 1 ml of fresh prehybridization buffer without NaCl and hybridized

for 24 h. The membranes were washed twice in 2x SSC (1x SSC, 0.15 M NaCl, 0.0125 M Na Citrate, pH 7) for 15 min at room temperature with constant agitation, then twice in 0.1x SSC, 0.1% SDS for 30-60 min at 65°C with constant agitation, air dried, and exposed to Kodak X-ray film.

Southern analysis of HIV-1 infected THP-1. Preparation of total cellular DNA for Southern transfer experiments was done by the guanidine thiocyanate CsCl gradient method (122). DNA was isolated from the gradient; mixed with an equal volume of water and extracted twice with phenol:chloroform (1:1). The DNA was precipitated with ethanol; treated with RNAse H (8-10 units/400μl) at 37^{0}C for 1-2hrs. At that time 5μl proteinase K (10 mg/ml) was added and the incubation continued for an additional 1-3 hours. The DNA was then extracted with phenol:chloroform and precipitated with ethanol at -20^{0}C overnight. The DNA was quantitated by UV absorbance at 260nm. 20μg was digested with EcoR1 or $BamH_1$ and in some instances followed by Msp1 or Hha1 at 40 units/sample (100μl) at 37^{0}C overnight. Following precipitation, the DNA was resuspended in 20μl sterile water. 5μl of loading dye was added; the sample loaded onto a 1.5% agarose gel and electrophoresed in Tris acetate/EDTA (TAE) at 20 volts/cm overnight. The gel was transferred to nitrocellulose according to standard protocols (123) and prehybridized in 50% deionized formamide,0.2% polyvinylpyrrolidone, 0.2% bovine serum

albumin, 0.2% ficoll (MW 400,000), 0.5M Tris-HCL (pH 7.5), 1.0 NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate (MW 400,000) and denatured salmon sperm DNA (100μg/ml) for 48 hrs. at 42^{0}C. The probe was added in 1 ml of the prehybridization buffer without NaCl and hybridized for 48 hrs. The membranes were washed twice in 2x SSC (1x SSC, 0.15M NaCl, 0.0125M Na. Citrate, pH 7.0) for 15 min. at 20^{0}C with constant agitation; twice in 2x SSC, 0.5% SDS for 30 min at 65^{0}C with constant agitation; air dried and exposed Kodak XAR-5 film for autoradiography. The probe, labeled by primer extension of the HIV-1 LTR strain HXB-2 using dCTP^{32}, was used at a concentration of 4x10^6 cpm/ml.

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 (125) were made. Briefly, after washing three times with ice-cold PBS, a 5 x 10^7 cell pellet was lysed in 4 ml of NP-40 lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 (v:v) NP-40) incubated on ice for 5 min and centrifuged at 500 rpm for 5 min. The nuclei were washed in lysis buffer and immediately resuspended in 100 μl 50 mM Tris-HCl pH 8.3, 40% (v:v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and mixed with 100 μl of reaction buffer (10 mM Tris-HCl pH 8.0; 5 mM $MgCl_2$; 120 mM KCl; 0.5 mM each of ATP, CTP, and GTP; and 150 μCi of [α^{-32}P]UTP (3,000 Ci/mm, Amersham, Arlington Heights, IL)

with 300 ng of HIV-1 LTR and incubated at 30°C for 45-60 min. The reaction was terminated by addition of 50 μg/ml of DNase 1 (RNase free) and incubation at 30°C for 5 min. The [^{32}P]-labeled RNA was then isolated as described (126). The 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 which is a BamHI-HindIII fragment of HIV-1 (nucleotides -1068 to +83) ligated to the CAT reporter gene (113) was used to isolate template DNA. This plasmid contains the HIV-1 LTR promoter as well as downstream tar sequences. pL3CAT was digested with Kpn 1-HindIII, purified on a 1% low melting agarose gel; phenol extracted, washed with 70% ethanol. The dried template was used in the assay. Hybridizations and washings were done as previously described (125,126).

Mobility Shift Assay. For binding assays, the plasmid pL3-CAT (113) was digested with HindIII, dephosphorylated with CIAP, ethanol precipitated and then 5'-labeled with [^{32}P] γ ATP and T4 polynucleotide kinase. Finally, the labeled fragment was digested again with EcoRV. 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. (127). The assay was done in 20 μl

reaction vol 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, 5,000-10,000 cpm of end labeled DNA probe (EcoRV-HindIII of HIV-1 LTR) and nuclear extracts at 5 or 10 μg protein. Nuclear extracts were prepared by the method of Topol and Parker (128). 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 of four parts extract to be inhibited were 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 electrophoresed at 10 V/cm through a 5% polyacrylamide (nondenaturing gel) in Tris-EDTA-Borate buffer.

Nuclear Extract preparation. Nuclear extracts were prepared by the method of Topol and Parker (128). Briefly, Cells were harvested from cell culture by centrifugation (at room temperature) for 10 min. at 1500 rpm in a Sorvall HG4l rotor. Pelleted cells were then suspended in 4-5 volumes of PBS (4^0C) and collected by centrifugation. All subsequent steps were performed at 4^0C. Cells were washed once in buffer A (15 mM KCL, 10mM Hepes pH 7.6, 2mM $MgCl_2$, 0.1mM EDTA) and then resuspended in 1/10 the original cell culture volume of buffer A with 1mM DTT and 0.5mM PMSF. Cells were lysed in this solution by 15-20 strokes with a dounce homogenizer. Following lysis, the solution was restored to

isotonicity by adding 1/10 vol. of solution B (1M KCL, 50mM Hepes, 30mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 0.5mM PMSF). The crude nuclei were then isolated by centrifugation at 10,000 rpm in a Beckman SW 55.2 rotor. The crude nuclei were resuspended in 9 parts buffer A plus 1 part buffer B (5 mls/10^9 cells). Nuclei were disrupted by the dropwise addition of 4M ammonium sulfate (0.36M final concentration) and gently rocked for 30 min. Nucleic acids were sedimented by centrifugation at 35,000 rpm for 1 hr. Nuclear proteins were precipitated from the supernatant by the addition of 0.25g/ml ammonium sulfate. Precipitated proteins were collected by centrifugation 35,000 rpm for 15 min. Pelleted proteins were resuspended in a minimum volume of buffer C (10% glycerol, 25mM Hepes, 40mM KCl, 0.1mM EDTA, 1mM DTT, 0.5 mM PMSF) and dialyzed against this buffer 2-4 hrs. Extracts were aliquoted and stored at -70°C.

Polymerase Chain Reaction Analysis of RNA Products in HIV-1 Infected THP-1. Cells to be analyzed for HIV RNA or DNA were washed and pelleted in PBS. Cells were lysed in 4M Guanidine thiocyanate, 2% Sarcosyl (in 0.01M Tris, p.H. 7.6, 2% B-mercaptoethanol) with 36% CsCL. Disrupted cells were layered onto a 5.7M CsCL, 0.1 M EDTA gradient and centrifuged for 16.5 hr at 32,000 rpm. The RNA separates to the bottom from the DNA/Protein layer on top. DNA was phenol/chloroform extracted twice, precipitated by ethanol, subjected to RNase treatment at 36°C for 1 hour and

proteinase K treatment (40 ug/sample) overnight at 22°C. The DNA was again phenol/chloroform extracted and ethanol precipitated. RNA was ethanol precipitated overnight at -70°C. Amplification reactions of 1-5 ug of either RNA (129) or DNA (130,131) contained 7mM $MgCl_2$, 10mM Tris-HCl, pH8.8, 50mM KCl, 1mM of each four deoxynucleotides (Perkin Elmer Cetus, Emeryville, CA), 100 units of M-MLV Reverse Transcriptase (BRL, Bethesda, MD) (for RNA only), 2.5 units AmpliTaq DNA polymerase, and 1 unit RNase Inhibitor for RNA (Promega, Madison, WI), and 0.01M DTT, and 1 uM of each primer. Primer pairs used were: SK38/39 HIV gag (1543-1570,1630-1657) (130); Co11/12 HIV tat splice product (8395-8414,5955-5974) (132) and Gap 371/546 GAPDH (371-388,546-565) (133). Amplification was carried out using a thermocycler (Perkin-Elmer Cetus, Emryville, CA), denaturing at 94°C for 1.5 min., annealing at 55°C for 1.5 min. and extending at 72°C for 2.5 min. Aliquotes (10 μl) of the sample were denatured at 94°C for 5 min. and hybridized with 2-5X10^5 cpm of the appropriate oligo for 45 min. at 63°C. Oligos were end-labelled with gamma-P^{32}-ATP and 50 units of T4 kinase (BRL, Bethesda, MD) at 37°C for 60 min. Probes used were SK19 HIV gag (1587-1627) Co11/12 HIV tat (5975-5999) GAP514 GAPDH (514-532). Analysis of the hybridized products were visualized on a 10% non-denaturing acrylamide/1X TAE gel by ethidium bromide and exposure to Kodak XAR-5 film at -70°C for 2-48 hours (with intensifying

screens).

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Plasma Membrane Isolation. The procedure was modified from one described by Ruscetti et. al. (134). 48-60 hr. Con A activated normal T cells were washed twice in lysis buffer (10 mM Tris/HCL, pH 7.4, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$) and broken by dounce homogenation in lysis buffer equal to 1% the original volume. The cell free lysate was centrifuged at 700g for 10 min. to remove nuclei and unbroken cells. The pellet was washed once in lysis buffer; the two supernatants combined then centrifuged at 5000g for 10 min. Most of the plasma membranes remain in the supernatant fluid. This was applied to the top of a discontinuous sucrose gradient of 0.25M, 1.37M, 1.48M, 1.60M and centrifuged at 25,000 rpm in the beckman SW rotor for 4 hrs. The plasma membranes are found at the 0.25-1.37M interface. The membranes were removed, diluted 1:4 with TK buffer (50 mM Tris/HCl pH 7.4; 5mM $MgCl_2$; 5 mM $CaCl_2$; 25 mM KCl) and centrifuged at 70,000g for 90 minutes. The pellet was resuspended in TK buffer and protein concentration determined by the Biorad method to be 1.5mg/ml. Membranes were used in the activation studies at a final concentration of 100ug/ml.

Electroporation transfection of latent THP-1 cells. Electroporation protocol was modified from a technique of Cann et. al. (135). THP-1 cells cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 ug/ml

streptomycin, 2mM glutamine, were washed twice with fresh ice cold complete medium, and resuspended at a density of 2 x 10^7 viable cells/ml. All subsequent steps were performed on ice. DNA (10-20 μg) was added to 0.25 ml of the cell suspension, mixed gently, and kept on ice for 10 min. The mixture was then transferred to an ice cold cuvette (0.4 cm.) and subjected to a single pulse of 600μF, 200 V from a BTX Electro Cell Manipulator 600 apparatus. After shocking, the cuvette was incubated on ice 10 min. further. The cells were transferred to 10 ml of growth medium and incubated at 37°C for 36-60 hrs. At this time cell pellets were prepared for RNA and DNA extraction and supernatants tested for HIV p24 antigen by Elisa.

Isolation of single cell clones of latent THP-1 cells. Limiting dilution single cell cloning of HIV infected THP-1 cells was performed according to the method of Benveniste et. al. (136). Briefly, sheep choroid plexus cells (CRL 1700), American Type Culture Collection (ATCC), Rockville, MD were grown in DMEM medium supplemented with 2.0 mM glutamine and 10% heat inactivated FCS. A confluent 75 cm^2 flask of cells was trypsinized and seeded into eight 96 well flat-bottom microtiter plates (Costar, Cambridge, MA). In this way each well was 50% confluent with sheep cells. THP-1 cells latently infected with HIV-1 were plated on these microtiter plates in 200μl aliquots at a density of 5-10 cells/ml (30-50 viable cells per plate) in RPMI complete

media. After 7-10 days of growth at 37^{0}C, individual clumps containing 20-50 cells could be seen in some of the wells. These wells were trypsinized and both THP-1 cells and sheep cells transferred to 24 well plates. Cells were expanded to 25 cm^2 flasks; freezes made and cells expanded for DNA analysis by PCR. The sheep cells did not survive after 4-5 passages of the cells.

MTT and XTT assay for HIV-1 cytopathic Effects. MTT and XTT assays were performed according to the method of Alley (121) and Weislow (137). Briefly, MT-2, and CEM-SS cell lines were used as targets in this assay. These were chosen based on their sensitivity to the cytopathic effects of HIV-1 infection as well as their ability to metabolically reduce XTT and MTT to their respective colored insoluble formazan products. Virus stocks were prepared in HUT-78 cells. 5 day supernatants from these cells with a highly productive HIV-1 infection (>500ng/ml p24) were harvested by centrifugation of cells and .45μm filtration of supernatant. Stocks were titrated for cytotoxicity to target cells, aliquoted and stored at -70^{0}C. Infected cells were similarly titered against target cells by serial dilutions across a 96 well microtiter plate in 100μl RPMI complete media. 10,000 target MT-2 or CEM-SS cells were then added in 100μl complete media and the plates incubated 5-7 days. At that time 50μl of XTT (1 mg/ml PBS) is added to each well and the plate incubated at 37^{0}C for 3-6 hrs. The optical

densities of the wells are determined with a plate reader (FisherBiotec, Pittsburgh, PA) at a test wavelength of 450 nm and a reference wavelength of 650 nm. Uninfected cells that have continued to proliferate produce the colored soluble formazan and yield high optical densities, while infected cells are killed by the virus or do not proliferate and yield low optical densities. Data are expressed as % of untreated control formazan = (test OD/control cell OD)x 100.

In Vitro Transcription of HIV-1 infected THP-1. Nuclear extracts were made as described above. The protein concentration of the extracts were adjusted to approximately 10 - 15 mg/ml for uninfected and infected cells. The DNA template (0.2 ng/reaction) was incubated with 2-4 ul of each extract. The reaction (123) was carried out in a 10 ul reaction mixture, containing 50 uM NTPs, 15 uCi α-^{32}P-UTP, 8 mM Hepes (pH 7.9), 0.4 mM $MgCl_2$, 0.8 mM DTT, 8 mM KCL, and 2 mM spermidine. The reaction was incubated for 90 min. at 30°C. 5 ul of tRNA (1 mg/ml) 15 ul of 3 molar sodium acetate were added, and the reaction was then stopped with 240 ul of stop buffer (100 mM NaCl, 0.2% SDS, 10 mM Tris-HCl pH 8.0). The samples were extracted twice with a phenol: chloroform mixture (1:1), with chloroform alone and ethanol precipitated. The pellets were resuspended in 98% formamide/2% each xylene cyanol and bromphenol blue. Samples (5μl) were electrophoresed at 550V for 1.5 hrs through a 8 M urea/ 6% polyacrylamide gel in TBE buffer

(0.09M Tris/ 0.09 M borate/ 2mM EDTA) and resolved on a denaturing polyacrylamide gel (6% in 8M urea) and subjected to autoradiography using standard techniques.

Cytoplasmic RNA preparation. Cells were harvested by centrifugation at 1500 rpm for 10 min. Cell pellets were resuspended in ice cold PBS and pelleted. This wash procedure was repeated twice. Subsequent steps were performed at 4^0C. Cells (2x 10^7) were resuspended in 450μl membrane lysis buffer (10mM Tris pH 7.9, 10mM NaCl, 5mM $MgCl_2$) and placed on ice 10 min. Next, 50μl 5% NP-40 (Sigma Chem. Co., St. Louis, MO) was added followed by brief vortexing. The nuclei were collected by centrifugation at 2000 rpm for 10 min. The cytoplasmic supernatant was transferred to a 1.5 ml. Eppendorf tube containing 150μl 0.3M NaOAc, 55μl 3M NaOAc. 500μl Phenol/Chloroform was added and the solution vortexed well. After centrifugation at full speed in an eppendorf centrifuge at 4^0C for 25 min, the supernatant was removed to a new tube and the RNA precipitated with 2 volumes of ethanol. RNA is quantitated by centrifuging the sample for 20 min. at 14,000 rpm and the RNA pellet resuspended in 100μl sterile water. A 5μl aliquot is removed and diluted in a total volume of 100-200μl sterile water and quantitated using A_{260} spectroscopy (1 A_{260} unit = 40μg/ml RNA) (123).

Nuclei Isolation. Approximately 1 x 10^8 cells were prepared by washing twice with ice cold PBS. Cells were

washed twice in Hank's balanced saline solution (calcium and magnesium free), centrifuged at 1500 rpm for 10 min and resuspended in 10 ml of buffer A (10mM Tris pH. 7.9, 10mM NaCl, 5mM Mg Cl_2) and placed on ice 10 min. to allow cells to swell. NP40 is the added to a final concentration of 0.5% and the solution vortexed for 10 seconds. Nuclei were pelleted at 3000 rpm for 10 minutes, resuspended in 1 ml. buffer A containing 15% glycerol and stored in 100 μl aliquots at -70^0C until use (138).

DEAE Dextran Transfection of Suspension Cells. Three hours prior to transfection, pellet and resuspend cells at a concentration of 1 x 10^6/ml in fresh RPMI complete media (138). Warm freshly prepared transfection media (50mM Tris pH 7.8, 200μg/ml glutamine, 200μg/ml DEAE dextran (500,000 MW, Pharmacia, Uppsala, Sweden) to 37^0C. Wash cells once in 37^0C serum free RPMI, pellet cells and add 5ml of transfection media containing 5μg/ml appropriate plasmid. Mix gently with pipetting, transfer to 6 well Plate and incubate at 37^0C with occasional mixing to prevent clumping for 0.5-1 hr. Following incubation, wash cells twice with warm serum free RPMI. Culture in 10 mls. complete media. Harvest at 48 hrs for extract or nucleic acid preparation.

Chloramphenicol Acetyl transferase (CAT) assay. CAT assays were performed according to the method of Gorman et. al. (139). Briefly, whole cell extracts of transfected cell pellets are prepared by 3 cycles of freeze-thawing of cell

pellets resuspended in 0.25M Tris/HCl pH. 7.8. Denatured nucleic acids and other insoluble debris are removed by pelleting at 14,000 rpm in an eppendorf centrifuge for 5 min. Protein concentrations are determined by the method of BIO-RAD, and 5-10 μg of protein is incubated 4-16 hr. in a reaction mixture containing 10mM acetyl CoA, 0.25M Tris/HCl and ^{14}C chloramphenicol at 37^0C. Following incubation, acetylated products are isolated by ethyl acetate extraction. Products are dried; resuspended in 25μl ethyl acetate; resolved by thin layer chromatography (TLC) on silica gel plates in 95:5 chloroform:methanol and exposed to autoradiography. Results are quantiated by densitometric scanning or direct count comparisons of acetylated products.

Isolation of Normal Peripheral Blood Cells. Buffy coats from normal healthy donors were separated as previously described (140). Briefly, mononuclear cells were separated by centrifugation on a Ficoll-Hypaque gradient and depleted of monocytes by elutriation. These non-adherent cells were applied to a nylon-wool column and the eluted B-cell depleted cells were fractionated on a seven-step discontinuous Percoll gradient from which highly purified T cells (> 95%) were collected from the high density bottom fractions (93). T cells were either activated with Con A (5 ug/ml) or PHA (1 ug/ml) for 48 hrs or further separated into subsets as previously described (94). Briefly, T cells were labeled by incubation with OKT4 or OKT8 for 30 min. on ice.

The PBS washed cells were then mixed with goat anti-mouse IgG-coated magnetic beads (Advanced Magnetics, Cambridge, MA) at a bead to cell ratio of 20 to 1. and gently rotated for 30 min. at 4°C. By using a magnet, the beads were collected at the side of the tube, unbound cells were collected, placed in a new tube and the separation was performed three times. Phenotyping showed that the cells were <5% contaminated with the depleted subset (94).

Isolation of Monocytes from peripheral blood of HIV infected individuals. These patients have CD4+ T cell counts and immunologic profiles within normal ranges and can be either virus culture positive or negative. PBMC were obtained from 30-50 mls of peripheral blood of HIV seropositive asymptomatic individuals (141) by ficoll hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. PBMC, were resuspended in 1.5 mls (10^7 cells/ml) and incubated on ice 45-60 min. with OKT_3 antibody (1 $\mu g/10^6$ cells). Cells were pelleted, and supernatant aspirated to remove unbound antibody. Cells were then resuspended in 3 mls of rabbit complement and incubated at 37^oC for 35 min. Cells were washed twice with cold balanced saline solution; resuspended in RPMI supplemented with 5% human AB serum (Irvine Scientific, tested HIV negative, Santa Ana, CA) at 2-4 x 10^6/ml; and adhered to T-25 tissue culture flasks (Costar, Cambridge, MA) or 6 well plates. At 24 hrs, non-adherent cells were removed and saved for

coculture and HIV analysis by PCR and P24 antigen. The adherent monolayers were washed twice with PBS to remove loosely adherent cells. The cells in the monolayers consisted of >95% esterase and Leu M3 positive, and <1% CD3 and CD8 positive cells.

Reagents. The following reagents were generously provided for these studies: Cem-SS cell line; Peter Nara, NCI-FCRDC, Frederick, MD; U1 and 8E5 cell lines Tom Folks and Dan Bednarik, CDC, Atlanta GA; ADA strain of HIV-1 and HIV-2 ROD, Scott Koenig, NIH, Bethesda, MD; CB2MX3, BF-24, and H938 cell lines, pHXB2 and pL3CAT plasmids; Barbara Felber and George Pavlakis, BRI, NCI-FCRDC, Frederick, MD. Tev expression vector, Donna Benko; nef, rev expression vectors S. Venkatesan, NIH, Bethesda, MD; tat expression vector, Dan Bednarik, CDC, Atlanta, GA; PCR primers SK38/39, Co11/12 and Gap 371/546; Martin Ruta, Div Blood prod., FDA, Bethesda, MD.

RESULTS

Differential Expression of HIV after Infection of THP-1 Cells. Due to the difficulty of growing normal human monocytes in long-term culture, a tissue culture model of HIV-1 infection in monocytes was established to obtain sufficient numbers of replicating cells in which to investigate the molecular mechanisms of HIV-1 persistence. The cell line, THP-1, was chosen because in addition to morphological, phenotypic and functional characteristics of normal mature monocytes it was the only monocytoid cell line that had accessory cell function (the ability to replace monocytes in mitogen stimulation of purified T cells) (142,143). An acute infection of THP-1 cells was established by incubating HIV-1 (strain BP-1) at a multiplicity of infection (MOI) of 0.05 with cells in log phase growth. In each experiment, extracellular virus was not detected between days 4 and 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 1). Phenotypic analysis of the cells showed that monocyte surface antigens such as CD14 (Leu-M3), were unaffected by HIV-1 while CD4 antigen, as measured by Leu-3A, could no longer be detected on the cell surface presumably blocked by the binding of HIV virions (Figure 3). Also, 21 day post infection, 30-40% of the cells contained internal antigens recognized by anti-p24

TABLE 1

ANALYSIS OF HIV-I INFECTED THP-1 CELLS

	Viral strain	Viral expression	Syncytia (% pos.)	p24 antigen (ng/ml)	Viral RT (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	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 the Materials and Methods. Analysis of the viral expression was made 60 day 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.

Figure 3. Phenotypic characterization of HIV infection of THP-1 FACS analysis using Leu-3a (CD4), LM1 (CD15), and HIV-1 gp 160:41 was performed as described in the Materials and Methods. Panel 1, uninfected THP-1; panel 2, productively infected THP-1; panel 3, infected THP-1 with no expression; panel 4, infected THP-1 with no expression 48 h after irradiation. Antibodies used were A-CD4, B-CD15, and C-HIV-1 gp 160:41. Analysis was performed as described in Materials and Methods.