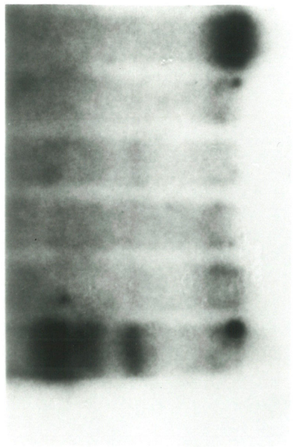


Figure 18. Northern Analysis of latently infected THP-1 following activation by 5-azacytidine. Lane 1, THP-1 with latent HIV virus 72 hours after 5-azacytidine (10 μM) treatment; lane 2, THP-1 with latent HIV-1 virus 72 hours after 5-azacytidine treatment cocultured with HUT-102B2 for 10 days; lane 3, THP-1 with restricted HIV-1 expression.

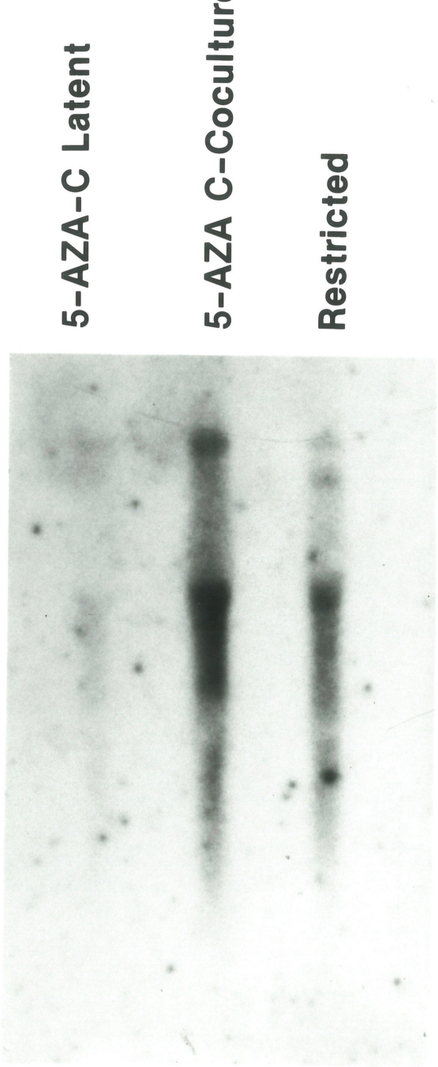
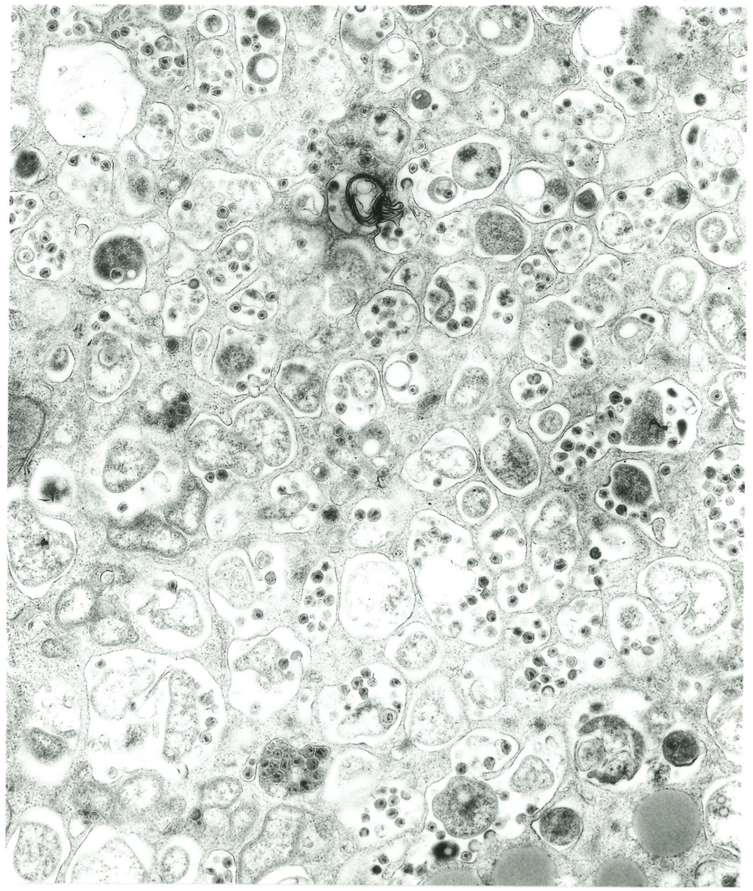


Figure 19. EM of transmitted HIV-1 from latently infected THP-1. After treatment with 5-azacytidine (10 μM) for 48 hours, latently infected THP-1 cells were cocultured with HUT-102B2 cells. After 10 days, electron micrographs of HUT-102 cells were prepared as previously described (4, 118). Shown are intracellular virions in HUT-102B2 (mag = 24,000x).

Figure 19. EM of transmitted HIV-1 from latently infected THP-1. After treatment with 5-azacytidine (10 μM) for 48 hours, latently infected THP-1 cells were cocultured with HUT-102B2 cells. After 10 days, electron micrographs of HUT-102 cells were prepared as previously described (4, 118). Shown are intracellular virions in HUT-102B2 (mag = 24,000x).



strain, ADA, which is highly cytopathic was able to kill T cells as efficiently as virus released from continuously productive cultures (Figure 20). In addition, ADA maintains its tropism for monocytes, while the BP-1 strain which can infect both T cells and monocytes, equally maintains this cellular tropism following activation from latency.

Role of cytokines in the regulation of HIV expression: distinctions between Latent and Restricted expression. Many cytokines are produced by monocytes and T cells during an immune response. The role of cytokines in the regulation of HIV expression is bidirectional. HIV expression has been shown to be upregulated in chronically infected T cell and monocyte lines in vitro by cytokines such as GM-CSF (62), TNFβ, TNFα (147), IL-6 (63), IFN (62), IL-1β (148), and CSF-1 (149). Others have reported suppression of HIV-1 replication by cytokines such as GM-CSF (150,151) in cell lines while enhancement of expression several hundred fold is seen in freshly isolated human monocytes (152). Cytokines such as TGFβ (91) and IFNα (153-154) have been shown to suppress HIV-1 expression in chronically infected cell lines.

Thus, the ability of cytokines and small macrophage inflammatory agents to modulate expression in THP-1 with restricted (RTHP-1) and latent (LTHP-1) infection was studied (Table 6). In agreement with previous studies (147-149, 155), several cytokines could increase viral expression

Figure 20. Syncytia and cytolysis of Hut-102 following co-culture with LTHP-1 activated to express infectious virus with 5-azacytidine as described in Materials and Methods. Three days after activation, cells were co-cultured with Hut-102B2 at a 1:10 concentration. Magnification 400X.

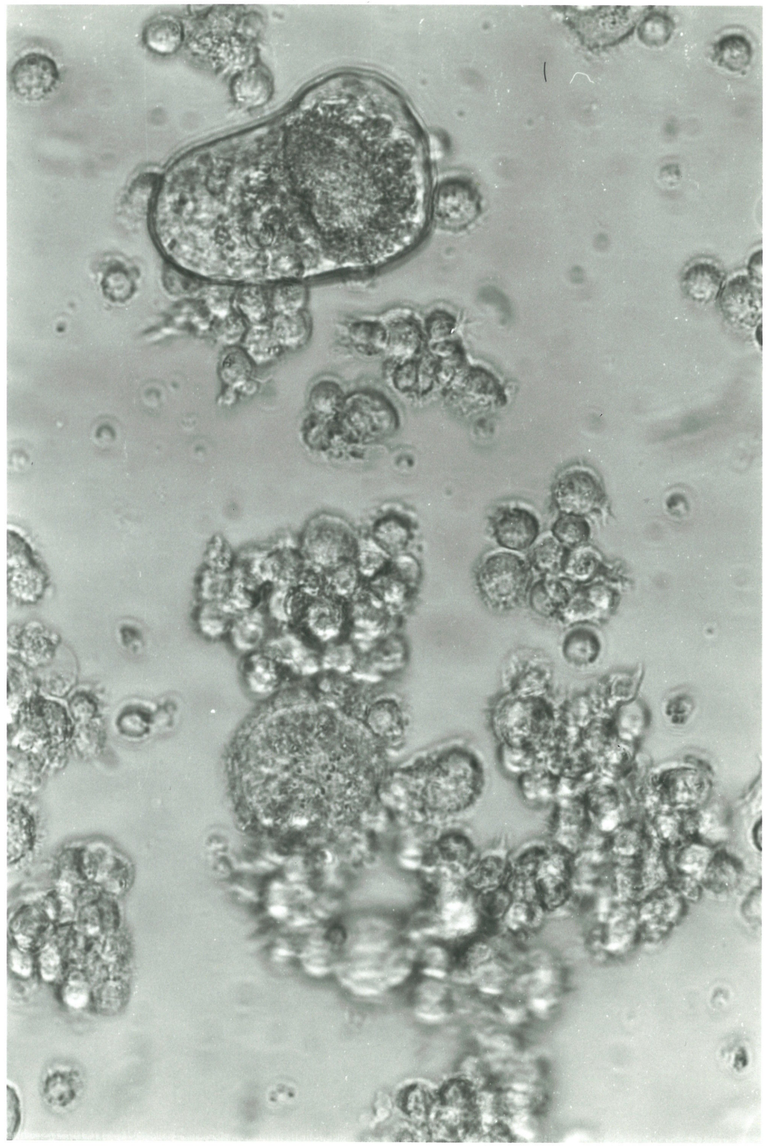


Table 6

Effect of Cytokines on HIV-1 Expression

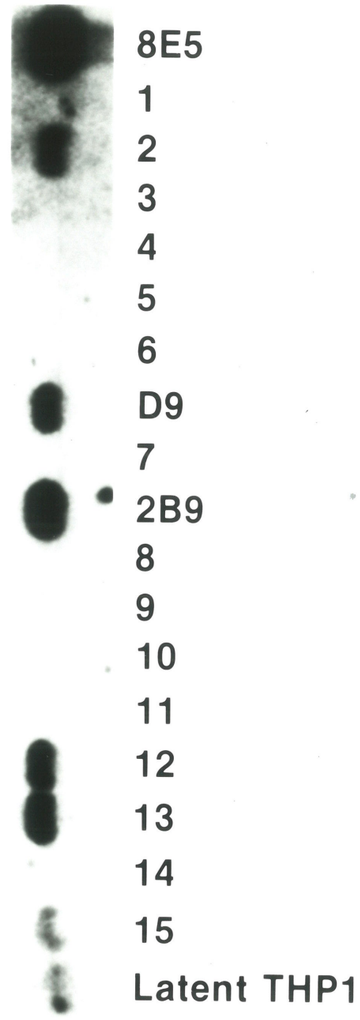
Culture treatment	HIV PCR-RNA		HIV p24(ng/ml)	
	R-THP-1	LTHP-1	RTHP-1	LTHP-1
none	++	-	0.2-20-1	-
TNF	++++	-	0.5-100	-
Il-6	++	-	0.2-20	-
TGFβ	++	-	0.2-20	-
GM-CSF	+++++	-	1.0-500	-
PMA	+++++++	-	10-500	-
LPS	++++	-	1.0-100	-
IL-1	++	-	0.2-20	-
5-azaC	+++	+	.2-20	0.03-.05
LPS/5azaC	+++	++	.2-20	0.05-0.1
PMA/5azaC	+++++	++	1-500	0.05-0.1
M-CSF	++++	-	1-100	-

Nucleic acids were prepared and PCR performed as described in materials and methods. p24 was measured by Elisa (Cellular products Inc., Buffalo, NY). TNF (Biogen 1000u/ml), Il-6, TGFβ, GM-CSF, M-CSF, IL-1 (all used at 20-200ng/ml), PMA (10ng/ml), LPS (10μg/ml), 5azaC (5-10μM).

in RTHP-1. However, these cytokines had no effect on viral expression in LTHP-1. The addition of these stimulating agents to 5 azacytidine activated LTHP-1 increases viral production from these cells. Whether combinations of these cytokines would overcome latency is not known.

Single Cell cloning of THP-1 cells with latent HIV-1 infection. PCR analysis of DNA from THP-1 cells with latent HIV infection revealed the presence of considerably lower levels of HIV specific DNA in these cells. When compared to the T cell line 8E5 which contains one integrated copy of HIV-1 provirus per cell (156), the latently infected THP-1 cell lines were estimated to contain 5-10% the amount of HIV DNA contained in 8E5 (Figure 21). Therefore, in order to further characterize the molecular mechanisms involved in latency, it would be useful to obtain cell lines in which each cell in the population contained at least one copy of the HIV provirus. Because initial attempts at single cell cloning by conventional methods of limiting dilution analysis were unsuccessful, the method of Benveniste et. al (136) was employed. This method improves viability of small cell numbers using feeder layers of sheep choroid plexus cells as a source of nutrients and other unknown factors required for cell survival. The procedure was carried out as described in Materials and Methods using several of the latently infected THP-1 cell lines. Clones were expanded; nucleic acids isolated as described and DNA PCR was used to

Figure 21. PCR analysis of latently infected THP-1 cloning studies. The oligimer hybridization autoradiography of clones containing HIV-1 specific gag DNA sequences is shown. PCR was performed on 1 μg of DNA isolated as described in Materials and Methods. Clones were isolated by limiting dilution as described. Clones were expanded for activation studies according to HIV-1 DNA content compared to that of the cell line 8E5 which has been shown to contain one integrated proviral copy per cell.



screen clones for the presence of HIV specific DNA. As shown in Figure 21 several clones were obtained which contained considerably higher levels of HIV DNA than the parent cell lines, while still less than one copy per cell as demonstrated by comparison with the 8E5 cell line. Two of these clones (D9, 2B9) could be activated by 5-azacytidine treatment to produce infectious virus. These clones, as well as the parent cell line, were used in subsequent studies examining the molecular mechanisms involved in overcoming latency.

Role of viral regulatory proteins in overcoming latency. The ability of 5-azacytidine to induce expression of infectious virus from latently infected cells and studies of Bednarik and his co-workers in which enzymatically methylated HIV-1 LTR was not transactivated in transient transfection assays suggested that methylation is involved in suppression of HIV-1 expression (146,157). However, southern analysis of DNA in latently infected clones 2B9 and D9 using methylation specific restriction endonucleases failed to reveal any differences from bands seen in productively infected THP-1. However, it has recently been demonstrated that the viral regulatory protein, tat, could overcome methylation mediated transcriptional inhibition of HIV-1-LTR-CAT without changing the methylation pattern of the DNA (157). Therefore, viral regulatory proteins were investigated for their ability to directly induce viral

expression from L-THP-1. Expression vectors for tat (157), tev (158), rev and nef (46) were transfected into latently infected THP-1 by electroporation and expression of HIV-1 measured 40-48 hrs later by RNA-PCR. PCR for spliced tat and tev products shows efficient expression in transfected cells (Figure 22). Moreover, this expression leads to production of structural proteins as shown in Figure 23. Interestingly, RNA-PCR for gag protein gave a stronger signal in the tat expressing cells than the in the tev expressing cells (Figure 23). This was surprising in light of the rev function contained in the tev protein. It was expected that tev transfectants would produce higher levels of virus. Furthermore, LPS treatment of latent cells had no effect on the level of gag expression in either case suggesting distinct pathways for LPS and viral regulatory protein mediated activation of HIV from L-THP-1. These results suggest these viral proteins are able to overcome latency by themselves. Because it has been suggested that tat can enter cells directly from the media (159), this could be an important mechanism of viral spread in infected individuals.

Immune activation of THP-1 with latent HIV-1 infection. Since direct, HLA-DR-restricted interactions between T cells and monocytes are involved in many aspects of the immune response, we determined if T cells could induce HIV expression from latently infected THP-1 cells. THP-1 cells

Figure 22. RNA-PCR analysis of transient expression of RNA of viral regulatory proteins in latently infected THP-1. The oligomer hybridization autoradiography of HIV-1 specific tat RNA sequences from clones of LTHP-1 containing transiently transfected with expression vectors of viral regulatory genes tat and tev as described in Materials and Methods. PCR was performed on 1 μg of RNA isolated as described in Materials and Methods.

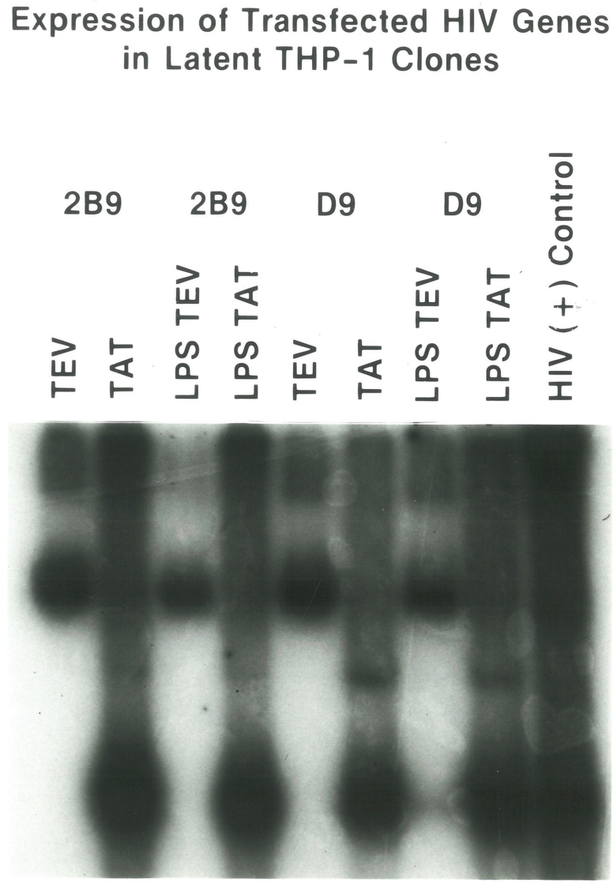
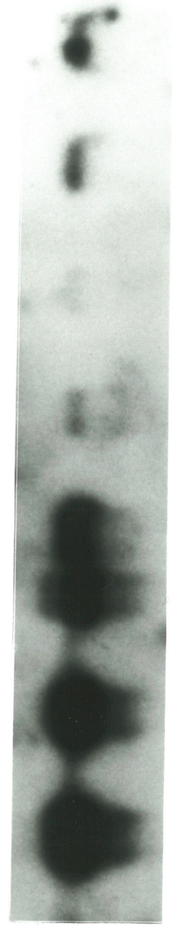


Figure 23. Activation of HIV-1 expression by transient expression of viral regulatory proteins in latently infected THP-1. The oligomer hybridization autoradiography of HIV-1 specific gag RNA sequences from LTHP-1 clones containing transiently transfected with expression vectors of viral regulatory genes tat and tev as described in Materials and Methods.



have accessory cell function (142) in that they can replace the requirement for monocytes in stimulating the proliferation of Con A-activated T cells, a response which is not HLA-DR restricted. Thus, L-THP-1 were co-cultured for 7 days with purified normal (HIV negative) donor T cells activated with Con A 48 hours prior to co-culture. Viral expression was measured by gene amplification using polymerase chain reaction (PCR) methodology (129-133). Normal Con A-activated T cells induced HIV RNA expression in LTHP-1 cells (Figure 24). HIV specific RNA was seen using nucleotide primers to the gag (Figure 24) and spliced tat products of HIV-1 (Table 7). In contrast, co-culture of L-THP-1 with unactivated T cells, supernatants from Con A-activated T cells, or T-cell lines (HUT 102, CEM) yielded no activation of viral expression (Figure 24). Viral p24 antigen production was also seen (Table 7). Using separated T cell subsets, CD4+ but not CD8+ T cells activates latent HIV in L-THP-1 (Table 7). After seven days, the T cells remaining in suspension were removed, cultured in IL-2 and found to be infected with HIV which then could be transmitted to Hut-102. Viral activation and spread to T cells can be seen by PCR-RNA by day 3 reaching a peak by day 7.

Characterization of HIV-1 expression in monocytes from asymptomatic seropositive individuals. Since the latent HIV-1 infection established in THP-1 could be induced to

Figure 24. Activation of HIV-1 RNA expression in latently infected THP-1 cells. RNA from LTHP-1 ($1x10^5$/ml) cocultured 7 days with equivalent numbers of Con A-activated T cells was isolated as dscribed. 1μg and 5μg (equivalent to 150,000-750,000 cells) were added to the amplification reaction following an initial reverse transcription step and subjected to 30 cycles of PCR amplification. The oligomer hybridization autoradiography demonstrating the presence of amplified HIV-1 gag RNA (4 hours exposure). Lanes are 8E5; 1μg, Lane 1; LTHP-1 cells; 5μg, lane 2; LTHP-1 co-cultured with normal Con A-activated T cells; 1μg, lane 3; LTHP-1 treated with LPS/ 5-azacytidine; 1μg, lane 4; LTHP-1 co-cultured with Hut-102; 5μg, lane 5; LTHP-1 cultured with Con A-activated T cell supernatant; 5μg, lane 6.

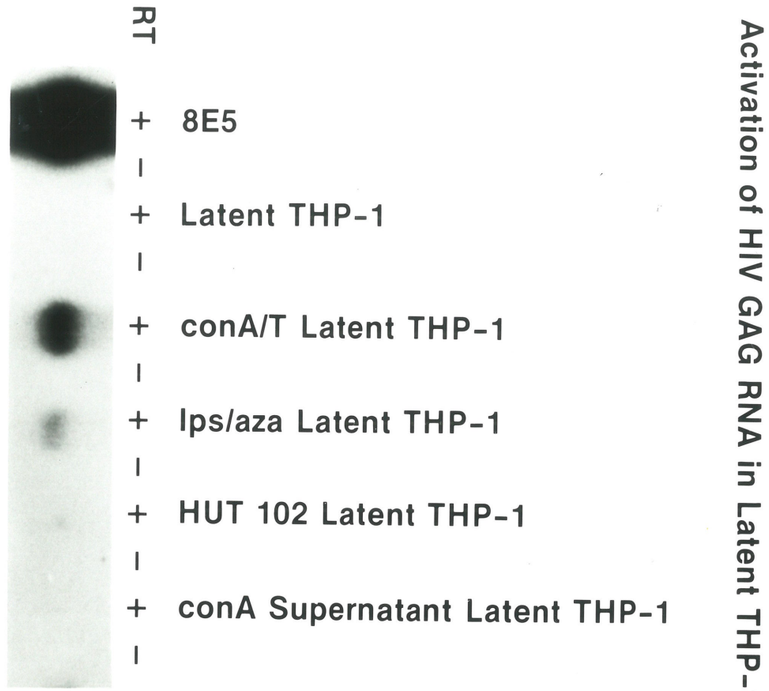


Table 7

Activation of HIV-1 Expression in Latent THP-1 Cells

Culture Treatment	HIV-1 RNA (spliced tat)	p24 Antigen (pg/ml)	Infectivity (Hut-102)
None	-	-	-
T cells	-	-	-
Hut-102	-	-	-
Con A/T cells	+	30-100	+
Con A/CD4+	+	25-50	+
Con A/CD8+	-	-	-

LTHP-1 ($1x10^5$/ml) were cocultured 7 days with equivalent numbers of ConA-activated T cells. At that time nucleic acids were harvested as described. 1 and 5μg were added to the reaction mixture and subjected to 30 cycles of PCR. $2x10^6$ cells were disrupted and HIV p24 antigen by Elisa was performed as described in the Materials and Methods. Viral transmission to Hut-102 was used as a measure of infectivity.

express infectious virus by immune activation, this result afforded us the opportunity to determine the role of latency in HIV-1 persistence and eventual disease progression in HIV-1 infected individuals. To examine this, monocytes were obtained from blood of HIV seropositive asymptomatic individuals by ficoll hypaque density gradient centrifugation, CD3 depletion and overnight adherence as described in Materials and Methods. At 24 hours, non-adherent cells were removed and the monolayer washed twice with PBS to remove loosely adherent cells. Phenotypic analysis demonstrated the monolayers consisted of >95% esterase and Leu M3 positive, and <1% CD3 and CD8 positive cells (Figure 25) characteristic of monocytes. HIV-1 expression as measured by RNA-PCR and intracellular p24, was detected in the monocytes of only 3 of 21 patients cultured in media alone (Figure 26, 27, Table 8). In contrast, HIV-1 expression could be detected in 14/18 of these same patient monocytes following cocuture with normal (uninfected) allogeneic Con A-activated T cells (Figure 26, 27, Table 8). That the RNA from monocytes incubated in media alone was amplified as efficiently as the other RNAs was shown by the amplification of GAPDH (Figure 28).

Detection of HIV-1 specific DNA in monocytes from asymptomatic seropositive individuals. Since these results suggest that HIV can exist in a latent state in monocytes of HIV infected individuals, the HIV-1 DNA status in these

Figure 25. Phenotypic characterization of CD3 depleted monocyte monolayers isolated from HIV seropositive individuals. FACS analysis using Leu-3a (CD4), Leu-M3 (CD11c), T cell receptor (CD3) was performed as described in the Materials and Methods.

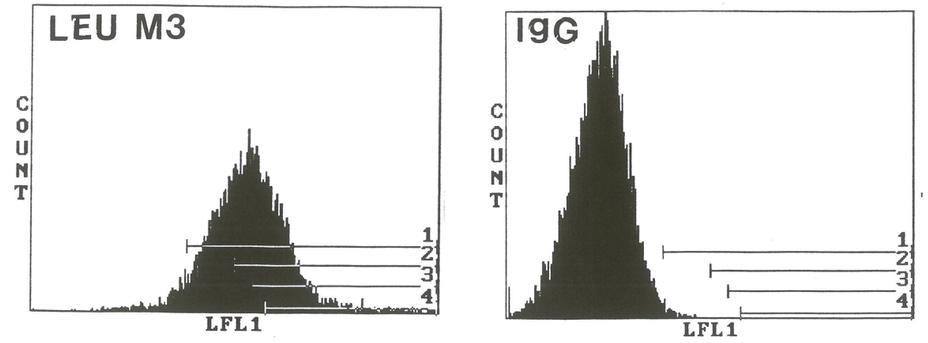


Figure 26. Detection of HIV RNA expression in fresh isolated adherent monocytes from HIV seropositive asymptomatic individuals. RNA from monocytes (1-5x10^6 cells) cultured 7 days as adherent monolayers in the presence and absence of equivalent numbers of normal Con A-activated T cells was prepared as described. 1μg and 5μg of total cellular RNA were added to the reaction mixture following an initial reverse transcription step and subjected to 30 cycles of PCR-amplification. The oligomer hybridization autoradiography detecting the presence of amplified HIV-1 spliced <u>tat</u> RNA product (4 hour exposure) is shown. Longer exposures of 24 and 48 hrs yielded the same results.

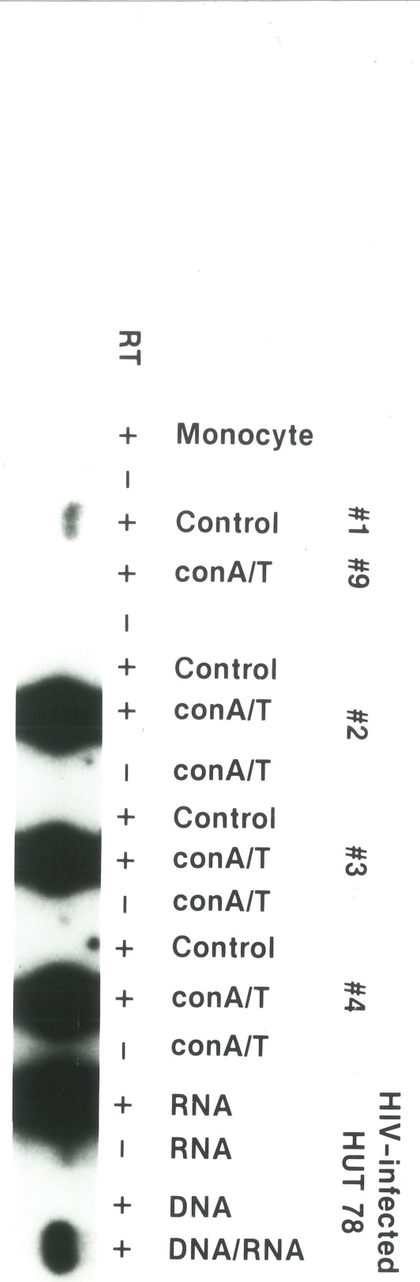


Figure 27. Detection of HIV RNA expression from monocytes isolated from previosly frozen PBMCs from HIV seropositive individuals. RNA from monocytes cultured 7 days as adherent monolayers in the presence and absence of normal Con A-activated T cells was prepared as described in Materials and Methods. The oligomer hybridization autoradiography showing the presence of amplified HIV-1 spliced tat RNA product (4 hour exposure) following an initial reverse transcription step of 1 ug and 5μg of RNA isolated from $2x10^6$ monocytes performed as described in Materials and Methods. After reverse transcription, cDNA was amplified by PCR. Detection of HIV tat RNA product (4hr exposure) is shown. Longer exposures of 24 and 48 hrs yielded the same results.

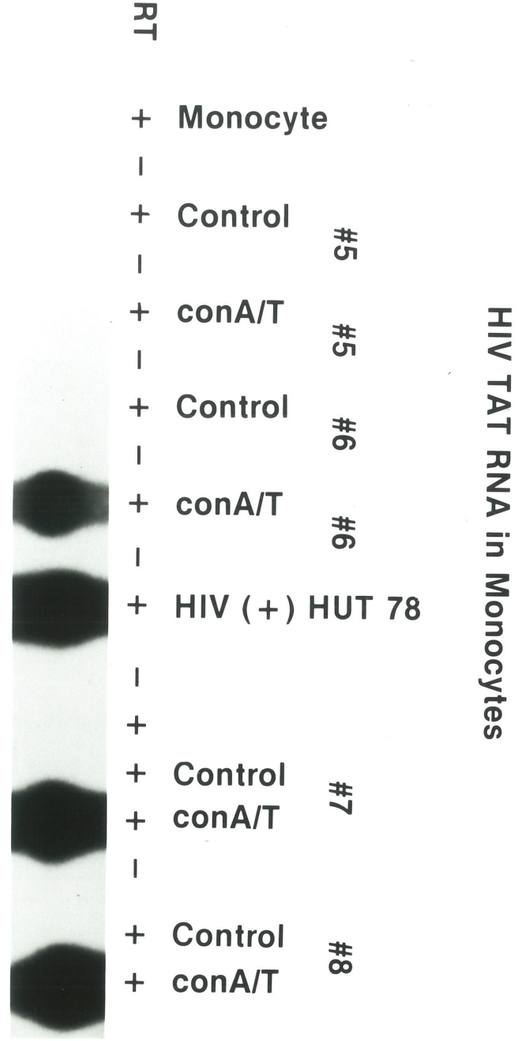


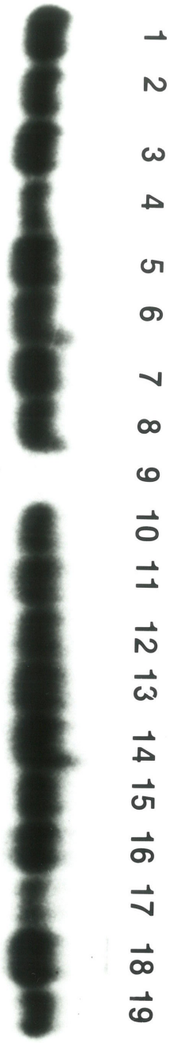
Table 8

Activation of HIV-1 Expression in Primary Monocytes

Sample Totals	Culture Treatment	DNA (# positive)	RNA (# positive)	p24 Antigen (pg/ml)	Infectivity (# positive)
3	None	3	3	100-250	ND
6	None	6	0	0	0/3
6	Con A/T cells	6	6	30-250	3/3
12	None	0	0	0	0/6
12	Con A/T cells	8	8	30-250	6/6

Nucleic acids were detected by PCR as described in materials and methods HIV p24 antigen Elisa (Cellular Products Inc., Buffalo, NY) was performed on cell supernatants according to manufacturers instruction with a sensitivity of 10 picograms/ml p24. Viral transmission to Hut-102 as a measure of infectivity performed using 100 ul of cell-free supernatants was assessed as previously described (4,21).

Figure 28. Detection of the cellular enzyme gylceraldehyde 3 phosphate dehydrogenase (GAPDH). RNA from monocytes cultured 7 days as adherent monolayers was prepared as described in Materials and Methods. The oligomer hybridization autoradiography showing the presence of amplified GAPDH RNA product (4 hour exposure) following an initial reverse transcription step of 1 ug of RNA. RNA from the first 19 samples of cultured monocytes is shown a control of equivalent levels of amplifiable RNA added.



cells was examined. Interestingly, PCR analysis of the DNA isolated from these monocytes revealed HIV-specific sequences for gag and tat in only 9 of 21 individuals (Figure 29, Table 8). However, DNA could be detected in the peripheral monocytes of 17 of 21 individuals following co-culture with normal Con A-activated T cells (Figure 29). The same four individuals were negative for both DNA and RNA. These results suggest that the levels of HIV DNA in the peripheral monocytes in these cultures inducible for viral expression are below the detectable limits of the PCR analysis. As with the RNA, primers for GAPDH show that the amplification of the DNA samples was of similar magnitude. The limit of detection in this system was determined by PCR performed on serial dilutions of the 8E5 cell line, a chronically infected T cell line containing one DNA copy of HIV per infected cell (156), was shown to be one infected cell per one hundred thousand (Figure 30) as previously reported (107). These results indicate that the HIV-1 provirus was latent in the peripheral monocytes of these infected individuals but that levels of HIV DNA in 8 monocyte cultures inducible for viral expression was below the detectable limits of the PCR analysis. Despite the low levels of latently infected monocytes in this cultures, the cells could still be induced by uninfected Con A-activated T cells to produce infectious virus and could be induced by uninfected Con A-activated T cells.

Figure 29. Detection of HIV-1 DNA in adherent patient monocytes by gene amplification. 1μg and 5μg DNA isolated from monocyte monolayers as described in Materials and Methods was added to the reaction mixture and subjected to 30 cycles of PCR. The oligomer hybridization autoradiography demonstrating the presence of amplified HIV-1 DNA (4 hour exposure) was performed as described in Materials and Methods. Longer times of exposure yielded the same results.

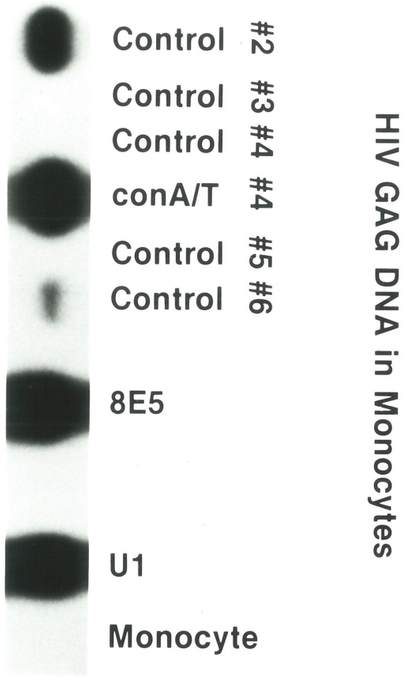


Figure 30. Sensitivity of PCR analysis for HIV-1 specific DNA. The oligomer hybridization autoradiography demonstrating the presence of HIV-1 gag DNA (4 hour exposure) was performed as described in Materials and Methods. Detection of 10 fold dilutions of 1 μg DNA from 8E5 cells representing 150,000 cells containing a single copy of HIV provirus. Longer exposure times yielded the same results.

Detection of 8E5 DNA

Conditions for immune activation of HIV-1 expression from latently infected monocytes. Next, the nature of the immune activation was further studied. For HIV activation from patient monocytes, partially purified plasma membranes from Con A-activated T cells could replace the need for whole cells (Figure 31). In addition, parallel co-cultures of patients' monocytes were set up with either HUT 102 or Con A-activated normal T cells. In several cases, HUT 102 cells were not infected while parallel cultures with activated T cells were virus positive (Figure 31, Table 9). In addition, coculture of L-THP-1 with Hut 102 did not result in viral activation even after six weeks of cocultivation (Figure 24). These results indicate that the detected virus was not passively adsorbed to the surface of these monocytes and infected the T cells during the co-culture period.

To demonstrate that this viral activation resulted in production of infectious virus, viral transmission experiments using supernatants from these original cocultures were performed using the cell line HUT 102. The presence of infectious virus was detected in the supernatants of the Con A activated T cell-stimulated monocytes and this virus could be subsequently transmitted from the infected T cells to Hut 102 (Table 9). Thus, these data show that HIV was truly latent in these monocytes and suggests that activated T cells interact with monocytes

Figure 31. Activation of HIV-1 expression from latently infected monocytes by partially purified membranes from Con A-activated T cells. The oligomer hybridization autoradiography demonstrating the presence of amplified HIV-1 spliced tat RNA product (4 hour exposure) following an initial reverse transcription step of 1 ug of RNA performed as described in Materials and Methods.

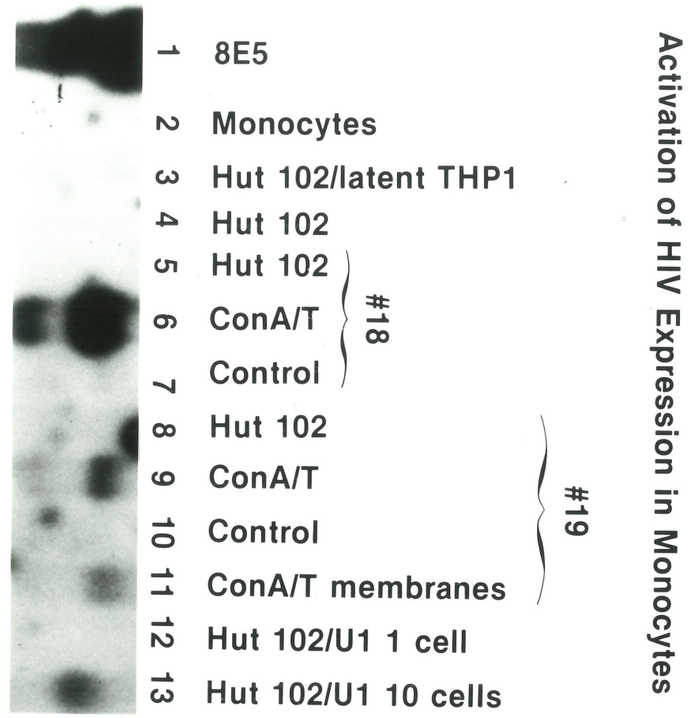


Table 9

Comparison of Latent HIV Transmission to T-cells and Hut-102

Cells	Primary Coculture	Second Coculture T-cells	Hut-102
Cell Lines			
Latent THP-1	None	-	-
Latent THP-1	HUT-102	-	-
Latent THP-1	LPS/5 AZA C	+	+
Latent THP-1	Con A T cells	+	+
U1	None	+	+
Patients # 7, 11 & 12			
Monocytes	None	-	-
Monocytes	Con A T cells	+	+
Monocytes	HUT-102	-	-
Monocyte	Con A Hut 102	_	_

1-5x10^6 monocytes or LTHP-1 cocultured 7 days with the equivalent number of T cells. Viral transmission to Hut-102 and PHA activated T cells as a measure of viral production was performed on cell-free supernatants and assessed as described in the Methods. Infectivity was determined by the presence of HIV nucleic acids detected by PCR amplification of 1 and 5μg of RNA. 2x10^6 detergent disrupted cells were used for HIV p24 antigen detected by Elisa.

differently than the T cell line Hut-102.

The possibility that the HIV expression came from a small percentage of either contaminating T cells or virus-producing cells present in the adherent monocyte monolayers can not be completely ruled out. This is highly unlikely because Con A and PHA will activate virus from T cells and not from monocytes of these donors (Figure 32, Table 10). In addition, LPS and cytokines known to increase HIV production (62,64,110,147,160) did not stimulate viral production in these monocytes (Table 10). Furthermore, in co-cultures using serial dilutions of U1 and ACH2, chronically infected cell lines producing HIV at low level (110,156), with monolayers of normal monocytes for 7 days as in the patient samples, we could consistently detect 10 infected cells per 1x10^7 normal monocytes (Figure 32). Using RNA from 8E5 which contains a single HIV pro-viral copy per cell, we could detect 10-100 femptograms of RNA which a log greater sensitivity than for 8E5 DNA, indicating it is much more difficult to get a false negative for HIV RNA than DNA. These results provide strong evidence that neither contaminating T cells nor virus producing cells are responsible for the HIV expression induced in these monocytes. Furthermore, Con A-activated normal T cells seeded into chambers with 4 um pore filtered bottoms (a transwell barrier to prevent cell-cell contact while allowing exchange culture supernatants and cell products)

Figure 32. Activation of HIV-1 RNA expression from latently infected monocytes requires cell-cell contact. The oligomer hybridization autoradiography demonstrating the presence of amplified HIV-1 spliced tat RNA product (4 hour exposure) following an initial reverse transcription step of 1 ug of RNA performed as described in Materials and Methods. Activation of HIV tat RNA from patients' monocytes. Longer exposures of 24 and 48 hrs yielded the same results.