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Highly Purified Quiescent Human Peripheral Blood CD4$^+$ T Cells Are Infectible by Human Immunodeficiency Virus but Do Not Release Virus after Activation

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Previous studies have suggested that resting CD4$^+$ lymphocytes can be infected by human immunodeficiency virus (HIV), but viral production is inhibited. If these cells are activated, progeny virions are released. The present data indicate that CD4$^+$ lymphocytes in the G_0/G_1 stage of the cell cycle which have been highly purified to remove macrophages and activated HLA-DR$^+$ cells can also be infected by HIV. However, our findings differ from those of earlier reports since in this study, infected quiescent CD4$^+$ cells cannot be activated to produce virus after virus inoculation. PCR analyses indicate that reverse transcription in these CD4$^+$ cells is arrested at a very early step in proviral DNA formation (U3-R region). They do not show any evidence of longer DNA transcripts (e.g., U3-*gag*). When these quiescent infected CD4$^+$ lymphocytes are activated by exposure to mitogens and macrophages and then reinoculated with HIV, the replication of virus takes place. Resting CD4$^+$ lymphocytes are also resistant to infection when they are cocultured with HIV-infected macrophages. Only activated CD4$^+$ cells are susceptible to cell-to-cell transmission. These observations suggest that in vivo tissue macrophages, susceptible to HIV replication, are the major cells initially productively infected by the virus. Then these cells can transfer HIV to activated CD4$^+$ lymphocytes with resultant virus production. The presence of arrested reverse transcription in quiescent cells raises questions about the cellular factors required to permit production of longer HIV proviral DNA copies. Because they can be reinfected once they have been activated, these infected quiescent cells could be a source of recombinant viruses in the host.

Human immunodeficiency virus (HIV) was recovered first from infected CD4$^+$ lymphocytes and was considered a T-lymphotropic virus. Subsequent studies indicated a wider host range for this virus, including macrophages, other hematopoietic cells, and more recently CD4-negative human fibroblasts and brain-derived cells (10). Replication occurs at its highest titer in CD4$^+$ lymphocytes. Which cell type is initially infected in the body is still not known; this information has relevance for approaches that might prevent the early steps in HIV infection. Some studies have suggested that resting CD4$^+$ lymphocytes can be infected by HIV, but there remains an unstable extrachromosomal viral form that is eliminated unless cells are activated within 3 to 14 days (17, 24). These results contrast with earlier studies which suggested that resting lymphocytes cannot be infected by HIV (4).

Human CD4$^+$ lymphocytes are heterogeneous and can be distinguished by cell surface proteins that denote adhesion and activation antigens. The previous in vitro work with quiescent cells did not characterize in detail the exact state of resting CD4$^+$ lymphocytes in the replicative cycle at the time of virus inoculation. In investigating various subpopulations of CD4$^+$ lymphocytes, we considered whether highly purified CD4$^+$ cells with no evidence of activation could be infected by HIV. These cells represent >90% of the CD4$^+$ lymphocytes in peripheral blood. Our results indicate that highly purified human quiescent CD4$^+$ T lymphocytes in the G_0/G_1 state of cell replication which lack an activated-cell phenotype can be infected by HIV but cannot be stimulated to produce virus. Moreover, HIV-infected macrophages pass HIV to CD4$^+$ lymphocytes only if the lymphocytes are activated. These studies suggest that macrophages found primarily in tissues are the preferential target for HIV early in infection and then spread the virus to other cells and tissues in the body. Most importantly, the vast majority of CD4$^+$ cells in blood are not susceptible to productive virus infection.

MATERIALS AND METHODS

Cell culture. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque separation of peripheral blood buffy coats from HIV-seronegative donors (provided by Irwin Memorial Blood Centers, San Francisco, Calif.). Peripheral blood macrophages were obtained from these PBMC by adherence to plastic as previously described (7). They were used 7 to 12 days after plating following two trypsinizations (0.25%) and washings to remove any residual lymphocytes. At the time of inoculation, these cells were >99% pure macrophages as determined by morphological appearance and flow cytometry with CD14 antibodies (14). The MT-4 established T-cell line originally obtained from Naoki Yamamoto was cultured in our laboratory (19). The culture medium for all cells was RPMI 1640 supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum or autologous serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For cultivation of mitogen-stimulated PBMC or purified CD4$^+$ cells, 50 IU of recombinant interleukin 2 (rIL-2) (Electronucleonics, Silver Spring, Md.) per ml was added to the medium. For culture of macrophages, 20% heat-inactivated (56°C, 30 min) normal human AB or autologous serum was used (7).

Virus and virologic assays. The HIV type 1 (HIV-1) strain HIV-1_{SF33} isolated in this laboratory (13, 19) was purified on sucrose gradients (6) to eliminate any cytokines present in the culture medium. This virus is a highly cytopathic strain that grows to a substantial titer in cell culture (19). Other viral strains used for some experiments included HIV-1_{SF2} (a T-cell line tropic, low-level cytopathic strain) and HIV-1_{SF162} (a macrophage-tropic strain) (7, 11). Replicating infectious virus was detected by p25 core antigen enzyme-linked immunosorbent assay (Coulter, Hialeah, Fla.) and particle-associated reverse transcriptase (RT) activity in the culture supernatant (6). In some studies, the MT-4 cell plaque forma-

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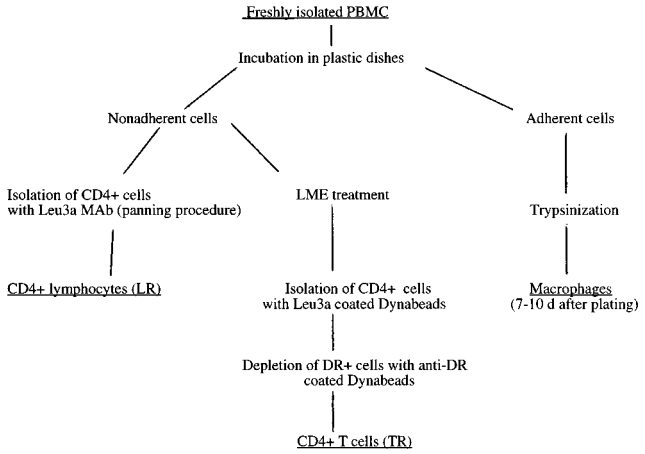

FIG. 1. Isolation of cell populations. See Materials and Methods for details of the derivations of LR and TR cell populations and macrophages. MAb, monoclonal antibody; d, days; LME, leucine methyl ester.

tion assay was used (19). Viral protein expression in cells was detected by indirect immunofluorescence assays (8).

PCR was performed by standard procedures that involved 30 cycles of amplification with primers and probes for segments of the entire HIV-1 genome. Total DNA was prepared by using Trireagent (Molecular Research, Inc., Cincinnati, Ohio) according to the manufacturer's instructions. Each sample was amplified with β-actin primers and an internally conserved fluorescent probe (Taqman β-actin detection kit; Perkin-Elmer, Foster City, Calif.) to verify the presence of amplifiable DNA. An equivalent amount of DNA from each sample was amplified with primer sets for early (long terminal repeat [LTR] U3-R) and late (LTR U3-*gag*) reverse transcription products as previously described (1). The primer sets used in this study were optimized for single-copy detection by using the 8E5 cell line (2). Product DNA was resolved by electrophoresis on a 1% agarose gel and transferred to a nylon membrane by using the Turboblotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.). Product DNA was detected by hybridization with an internally conserved probe 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase (Promega, Madison, Wis.). Membranes were prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% sodium dodecyl sulfate–100 mg of sonicated salmon sperm DNA per ml and hybridized overnight at 56°C in prehybridization buffer with 500,000 counts of probe per ml. Membranes were washed under high-stringency conditions and exposed to Hyperfilm-HP (Amersham, Arlington Heights, Ill.) at −80°C with an intensifying screen for 4 h. Densitometry was performed with a GS 670 imaging densitometer (Bio-Rad, Hercules, Calif.), and data were analyzed with Molecular Analyst software (Bio-Rad).

Individual HIV-1-infected cells were detected by infectious center assays (12) in which 10-fold dilutions of HIV-1_{SF33}-inoculated cells at various times after infection were added in duplicate to MT-4 cell monolayers plated on poly-L-lysine-coated wells. The infected cells under agar were scored as plaques after 5 days (19). To determine HIV-1_{SF33} entry, infection was performed by inoculating the HIV-1_{SF33} purified viral preparation (10^6 PFU/ml [measured in MT-4 cells]) at a multiplicity of infection of 3 in the presence of 2 μg of Polybrene per ml. After 1 h at 37°C, cells were washed twice with Hanks' balanced salt solution and exposed to trypsin (type XII-S; 25 μg/ml) for 5 min to remove residual virus on cell surfaces (18). Then cells were maintained in RPMI 1640 with 5% autologous human serum. HIV-1 replication was also measured in virus-inoculated $CD4^+$ T cells after activation of these cells. The expression of HIV antigens in infected cells or formation of plaques by infected cells provided evidence of viral entry.

Derivation of $CD4^+$ quiescent lymphocytes. Freshly isolated PBMC from seronegative donors were incubated in 30-mm-diameter plastic dishes (Falcon Labware, Lincoln Park, N.J.) for 60 min at 37°C to remove adherent cells. These adherent cells were subsequently used for experiments with macrophages (Fig. 1). Nonadherent cells were harvested and maintained in serum-free RPMI 1640. A $CD4^+$ cell preparation was obtained by panning freshly isolated PBMC with anti-Leu3a monoclonal antibody fixed to the surface of a plastic dish by standard procedures (Fig. 1) (23). The cells removed by vigorous pipetting were $CD4^+$ lymphocytes and some monocytes; they were designated LR cells.

A highly purified resting $CD4^+$ T-cell population, designated TR cells, was obtained by further selection procedures (Fig. 1). Nonadherent PBMC were completely depleted of monocytes/macrophages by treatment with 5 mM leucine methyl ester (LME) (Sigma Chemicals, St. Louis, Mo.) in RPMI 1640 for 45 min at room temperature (20). The remaining cells were shown to be devoid of contaminating macrophages by flow cytometry (14). $CD4^+$ lymphocytes were further purified from these PBMC with monoclonal antibody (Leu3a)-coated magnetic beads (Dynal, Lake Success, N.Y.) as previously described (16). Finally, $HLA\text{-}DR^+$ cells were removed from the $CD4^+$ cell population by a panning procedure with an anti-DR monoclonal antibody-coated plate or mixed with anti-DR-coated magnetic beads for double depletion. The viability of TR cells was >99%, as measured by trypan blue dye exclusion.

For cell activation, TR cells were stimulated with phytohemagglutinin (PHA) and rIL-2. In some cases, fixed adherent cells (accessory cells [AC]) were added to the culture for maximum activation (3). After 3 days of stimulation, activated TR cells were designated TA cells.

LR and TR cells, shown to be 99.9% $CD4^+$ (as measured by Leu3a monoclonal antibody), were maintained in RPMI 1640 supplemented with 5% heat-inactivated autologous serum and the other supplements noted above. In some studies, AIM V serum-free medium (Gibco BRL, Gaithersburg, Md.) replaced RPMI 1640 for long-term culture.

Measurement of DNA synthesis and flow cytometry. Cellular DNA synthesis was monitored by incorporation of [^{3}H]thymidine by standard techniques (5). Flow cytometry for cell subset analyses was conducted with fluorescein-conjugated antibodies to CD3, CD4, CD14, CD16, CD25, CD56, and HLA-DR (provided by Becton Dickinson, San Jose, Calif.) (14). Cell cycle progression was also monitored by flow cytometry by using the propidine iodide staining technique for detergent-isolated nuclei (9). The results were determined with a FACscan (Becton Dickinson). When the results indicated arrest in the cell cycle at the G_0/G_1 phase, a distinction between these two phases could not be made because of the requirement for detecting all levels of DNA synthesis. Thus, although these cells acted biologically as if they were in the G_0 phase, we refer to this stage as measured by flow cytometry as the G_0/G_1 phase.

TABLE 1. Cell surface markers of LR and TR cells and cell cycle analysis[a]

Cell population	% Cells with cell surface markers			% Cells in cell cycle phase			
	$CD4^+/CD3^+$	$CD4^+/DR^+$	$CD4^+/CD14^+$	G_0/G_1	G_0/G_1, S	G_2	M
LR	98.0	16.0	14.0	100	0	0	0
TR	99.8	0.06[b]	0	100	0	0	0

[a] Freshly isolated LR and TR cells were double stained with monoclonal antibodies directed at CD3, CD4, HLA-DR, and CD14 for determinations of cell surface markers (14). Cells were stained with propidium iodide for nuclear DNA staining to determine cell cycle progression (9). Cell analysis was performed with a FACscan. Cells from more than five healthy seronegative donors were analyzed in these studies and showed similar results.

[b] DR^{Dim}.

RESULTS

Identification of resting lymphocyte populations. To identify the physiological state of lymphocytes, we analyzed the LR and TR cell populations which had been isolated from fresh peripheral blood. As described in Materials and Methods, to avoid any possible contamination and stimulation, all cells were maintained in RPMI 1640 containing only 5% heated autologous serum. Processing for cell isolation and purification was performed without any mitogen and exogenous cytokines. The resting states of both LR and TR cells were ascertained by analyses of the cell cycle and by identification of cell surface markers CD3, CD4, HLA-DR, and CD14. The purity of T cells and contamination with activated T cells, monocytes, and dendritic cells were also assessed by flow cytometry.

Table 1 shows the characteristics of LR cells isolated by a conventional panning procedure. In this $CD4^+$ cell population about 14 to 16% of the cells expressed HLA-DR and CD14; these cells were part of the adherent cells harvested from Leu3a-coated plastic dishes after 24 h. The LR cell population contained primarily $CD4^+$ T cells, including a small portion of activated T cells (or memory cells). After further elimination of monocytes/macrophages and $HLA\text{-}DR^+$ cells from the LR population (see Materials and Methods; Fig. 1), the residual TR cells contained only very small numbers of $CD4^+/DR^{Dim}$ cells and cells with no CD14 expression, indicating that monocytes were not present (Table 1). However, the existence of a very small portion of $CD4^+$ cells with weak major histocom-

TABLE 2. Induction of progeny HIV-1 production in LR cells with PHA stimulation on day 15 postinfection[a]

Treatment	RT (10^3 cpm/ml)	p25 (pg/ml)	Infectious cell center (%)	% Cells with surface marker(s)				Mean cell proliferation ± SD (cpm)
				CD4	CD14	CD25	HLA-DR	
HIV (−)/PHA (−)	1.0	0	0	96	14	42	9	348 ± 72
HIV (−)/PHA (+)	1.6	0	0	100	ND[b]	90	62	14,616 ± 515
HIV (+)/PHA (−)	1.0	30	0	98	ND	36	18	4,603 ± 1,057
HIV (+)/PHA (+)	298.5	>500	1.55	88	ND	98	65	5,712 ± 980

[a] LR cells were inoculated with HIV-1_{SF33}. Subsequently, cells were trypsinized to remove input virus (18) and maintained in RPMI 1640 containing 5% heated autologous serum. Cells were treated with PHA (3 μg/ml) on day 15 postinfection for 3 days. Then infectious cell center assays of MT-4 cells were performed with aliquots of these cells. HIV-1 replication in the original culture was monitored 6 days later by assays for RT activity and p25 antigen. Cell surface markers were analyzed with a FACscan. Cell proliferation was evaluated by [^{3}H]thymidine incorporation (see Materials and Methods). The results of a representative experiment (of at least four studies) are shown.

[b] ND, not done.

patibility complex class II expression is possible. In some studies, DR^+ cells were further eliminated by depletion twice with anti-DR antibody (see below). By cell cycle analysis, although they contained some different cell markers, both the LR and TR cell populations were in the G_0/G_1 phase; none of the cells showed detectable DNA synthesis (data not shown). In all our experiments, both LR and TR cells maintained a stable resting state during cell cultivation from several days to weeks.

HIV-1 infection of LR cells. The LR cell population derived from PBMC was in the G_0/G_1 phase of cell cycle, as determined by flow cytometry (Table 1). Cells in this resting state can maintain a viability of >90% without cell proliferation for up to 4 weeks in culture. Upon stimulation with mitogen, they progress in the cell cycle and have surface markers of T-cell activation (data not shown).

When HIV-1_{SF33} was inoculated onto LR cells, no RT activity was detected in the culture supernatant for up to 15 days postinfection. Residual input virus had been eliminated by standard trypsin treatment (18) (as described in Materials and Methods). However, very low but persistent amounts of p25 antigen (14 to 30 pg) were detected in the culture fluids of these cells after virus inoculation (Table 2). Subsequent treatment of infected LR cells with PHA alone 15 days postinfection led to high-level production of infectious HIV-1, as measured by RT activity and p25 core antigen in the culture fluid (Table 2). In addition, mixing PHA-treated LR cells with the MT-4 cell line led to high levels of virus replication, as detected by RT activity. In some studies, LR cells inoculated with virus and showing very little viral expression (5 pg of p25 antigen) could be induced to replicate infectious HIV up to 40 days postinfection (data not shown).

Treatment with mitogen also produced cytopathic effects in LR cells, as shown by balloon degeneration and syncytium formation. At that time, immunofluorescent antibody staining indicated high levels of HIV antigen expression in these cells (data not shown). The number of resting LR cells that maintained this persistent state of virus infection without HIV replication was estimated to be 1 to 2%, as measured by the infectious cell center assay with MT-4 cell monolayers (Table 2).

Role of macrophages in LR cell infection. Since LR cells contain up to 14% monocytes and FACscan analysis further showed that 96% of these $CD14^+$ cells can express both CD4 and HLA-DR (data not shown), the role of these latter cells in the early steps and maintenance of HIV infection was evaluated. A series of experiments was conducted. At different times before and after HIV-1 inoculation, LR cells were depleted of monocytes by using the lysosome-tropic agent, LME, that selectively kills this cell population (20). This drug had no observed effect on $CD4^+$ lymphocytes. In these studies, a role for monocytes/macrophages in permitting efficient virus infection was noted (Table 3). In cultures receiving LME, HIV-1 replication was substantially reduced (experiments 2, 4, 6, and 8 through 10). However, LME treatment was not sufficient to eliminate completely productive infection of resting $CD4^+$ LR cells; subsequent exposure of these cells to PHA after LME treatment induced some HIV-1 production (experiments 4, 8, and 10). This latter observation most likely reflected the presence of low numbers of $CD4^+$ lymphocytes activated by PHA treatment in the LR cell population. In support of these overall observations, the culture with the highest level of virus production was the one that had not been treated with LME and had been exposed to PHA twice (experiment 7).

HIV-1 infection of TR cells. Since the results discussed above indicated that activated $CD4^+$ cells or differentiated macrophages could be the targets of HIV-1 infection in the resting $CD4^+$ cell population (i.e., LR cells), we evaluated further the susceptibility of highly purified quiescent $CD4^+$ lymphocytes to HIV-1 infection. For these studies, extensive elimination of contaminating monocytes and activated (HLA-DR-expressing) $CD4^+$ lymphocytes was conducted. By using sequential selection with monoclonal antibodies, the TR cell population was obtained (Fig. 1) (see Materials and Methods). These cells showed no evidence of contaminating macrophages and only

TABLE 3. Role of monocytes and cell activation in HIV-1_{SF33} infection of LR cells

Expt	Treatment of cells[a]				RT activity (10^3 cpm/ml)[b]
	Before HIV-1 inoculation		After HIV-1 inoculation		
	LME	PHA	LME	PHA	
1					1.7
2	+				3.5
3		+			17.1
4	+	+			6.9
5				+	23.1
6	+			+	2.4
7		+		+	249.6
8	+	+		+	43.1
9			+	+	3.0
10		+	+	+	12.5

[a] LR cells were treated with LME (5 mM) for 45 min at room temperature before and/or after HIV-1_{SF33} inoculation. LR cells were also left alone or stimulated with PHA (3 μg/ml) for 3 days before and/or after infection. +, the indicated procedure was performed in this experiment.

[b] Viral replication was monitored by RT activity on day 8 after virus inoculation. An RT value of <5.0 is considered negative. The results of a representative experiment (of three studies) are presented.

TABLE 4. HIV-1 infection of TR cells and subsequent attempts at induction of virus replication[a]

Treatment	RT (10^3 cpm/ml)	p25 (pg/ml)	Infectious cell center (%)	% Positive				Mean cell proliferation ± SD (cpm)
				CD4	CD14	CD25	HLA-DR	
None	1.1	0	0	99.8	0	6.6	0.01	60 ± 3
PHA	1.2	0	0	99.9	ND[b]	23.1	0.6	39 ± 15
rIL-2	2.5	0	0	99.7	ND	59.8	12.5	6,093 ± 309
PHA + rIL-2	1.5	0	0	99.7	ND	50.4	25.0	7,995 ± 1,593
PHA + rIL-2 + AC	2.2	18.5	0	99.8	ND	49.4	68.1	10,587 ± 2,121

[a] TR cells were prepared as described in Materials and Methods. HLA-DR$^+$ cells were removed by one or several steps. After HIV-1_{SF33} inoculation, infected TR cells were treated for 3 days with PHA, rIL-2, or paraformaldehyde-fixed AC (macrophages) alone or with a combination thereof at 1 h post virus inoculation. Then cells were washed twice and maintained in medium for FACscan analysis and [^{3}H]thymidine incorporation. Virus replication was determined on day 10 postinoculation by p25 core antigen, RT, and infectious cell center assays.

[b] ND, not done.

very weak levels of HLA-DR^{Dim}-expressing CD4$^+$ cells (Table 1). By flow cytometry, these cells were in the G_0/G_1 phase of the cell cycle, but they could be induced by PHA and exposure to macrophages into the S phase with augmentation of DNA synthesis and expression of HLA-DR and CD25 (IL-2 receptor). TR cells do not make IL-2; thus, for mitogen activation, exogenous IL-2 was added. Maximal stimulation of these TR cells with induction of high-level DR expression required the addition of 5 to 10% AC consisting of either live or paraformaldehyde-fixed macrophages (Table 4).

With these observations using TR cells, this CD4$^+$ cell population was exposed to HIV-1_{SF33} and the susceptibility to virus infection was measured. Stimulation factors in the culture medium were avoided by using sucrose gradient-purified HIV-1_{SF33}. Virus was inoculated onto purified TR cells at a multiplicity of infection of 3 (virus input was measured by the MT-4 plaque assay). Incubation was for 1 h in RPMI 1640 with 2 μg of Polybrene per ml. After infection, TR cells were trypsinized (25 μg) to remove residual virus and then maintained in medium containing 5% autologous serum.

In contrast to the results with LR cells (Tables 2 and 3), after virus inoculation, TR cells showed no evidence of HIV infection, as measured by p25 core antigen production and RT activity in the culture supernatant (Table 4). Furthermore, the addition of inoculated TR cells to MT-4 cells did not show evidence of virus infection by infectious cell center assay. Similar results were obtained with HIV-1_{SF2} and HIV-1_{SF162} isolates; after virus inoculation, resting T cells could not be activated to produce virus. In one experimental group, HIV-1_{SF33} expression (low-level p25 antigen production) was noted when infected TR cells were stimulated with PHA in the presence of rIL-2 and paraformaldehyde-fixed AC. On these occasions, only 10 to 20 pg of p25 was detected from 10^6 infected TR cells that had been fully activated for virus induction (Table 4). These results indicate that a rare DR$^+$ T cell was present in the TR cell population at the time of initial infection. This possibility was supported by the inability to detect p25 antigen expression after mitogen stimulation unless 10^6 TR cells were used in these studies (data not shown). Moreover, the treatment of TR cells twice to eliminate DR$^+$ cells by the panning procedure and with magnetic beads coated with anti-DR antibodies removed all biologic evidence of HIV-1 infection of these cells. At that time, only 0.06% of CD4$^+$ cells were HLA-DR^{Dim} (Table 5).

Susceptibility of TR cells to HIV-1 infection after activation. To determine what factors induce TR cells to be susceptible to HIV-1 infection, portions of this cell population were pretreated with PHA alone, with IL-2 alone, with both substances, and with both substances in combination with fixed adherent cells (i.e., macrophages [AC]). Unless supplied with exogenous IL-2 and AC, TR cells failed to be activated by PHA alone (Table 4). After 3 days, cell cycle progression and the expression of HLA-DR were monitored and cells were infected with HIV-1_{SF33}. Infection and subsequent trypsinization were performed as described in Materials and Methods. The results indicated that any level of T-cell activation (measured by cell cycle or DNA expression) induced sufficient TR activation and proliferation to permit susceptibility to HIV-1 (Table 6). The combined use of all three preinoculation procedures gave optimal conditions for HIV production. In these studies, both autologous and allogeneic AC provided sufficient activation signals after stimulation. Following each procedure, virus infection was most efficient in the TR cells with the highest expression of HLA-DR antigen. It is noteworthy that monoclonal antibodies to HLA-DR did not prevent infection of these cells with HIV-1_{SF33} (data not shown).

PCR analyses. Despite the inability to recover virus from virus-inoculated TR cells after activation, PCR analyses indicated that virus entry had occurred. At zero time postinoculation, no evidence of an early DNA transcript (U3-R) of the viral LTR was detected (Fig. 2A, lane 2). By 1 h and later, a short DNA transcript (~400 bp) was found; this transcribed product remained present in cells up to 7 days postinfection (Table 7) (Fig. 2A, lanes 3 through 6). No evidence of a late transcript (U3-*gag*) was found in these cells (Fig. 2B, lanes 2 through 6). In contrast, activated CD4$^+$ lymphocytes (TA cells) showed some early transcripts and low levels of late transcripts even at zero time, which represents the 1 h of virus incubation with cells (Fig. 2, lanes 7). By 24 h and up to 7 days, fully transcribed products were detected concomitantly with progeny virus replication (Table 7; Fig. 2, lanes 8 through 12).

TABLE 5. Comparison of virus production in TR cells depleted of HLA-DR-expressing cells by two methods[a]

Method of elimination	DR expression (%)	p25 production (pg/ml)	
		Without cell activation	With cell activation
DR-antibody-coated IM beads alone	1.2	22.1	ND[b]
DR-antibody-coated IM beads and panning procedure	0.06[c]	0	0

[a] To deplete HLA-DR$^+$-expressing cells, TR cells were treated with (i) anti-DR-coated immunomagnetic (IM) beads alone or (ii) anti-DR-coated IM beads plus anti-DR-coated petri dishes used in a panning procedure. p25 antigen production was measured with or without virus induction, which was performed by the addition of PHA (3 μg/ml), rIL-2 (100 IU/ml), and paraformaldehyde-fixed AC (10%) to inoculated TR cells.

[b] ND, not done.

[c] DR^{Dim}.

TABLE 6. HIV-1_{SF33} inoculation of TR cells after different methods of T-cell activation[a]

Treatment before virus inoculation	Cell cycle phase(s)	DR expression (%)	RT activity (10^3 cpm/ml)
None	G_0/G_1	0.3	1.1
PHA	G_0/G_1	0.5	1.2
rIL-2	G_0/G_1, S	8.6	25.4
PHA + rIL-2	G_0/G_1, S, G_2, M	29.6	34.5
PHA + rIL-2 + AC	G_0/G_1, S, G_2, M	34.8	82.1

[a] TR cells were pretreated with different inducers of cell activation, PHA (3 μg/ml), rIL-2 (50 IU/ml), and 10% fixed macrophage (or AC), for 3 days. Then stimulated and unstimulated TR cells were inoculated with HIV-1_{SF33}. After 1 h, cells were trypsinized to remove residual virus (18). All infected cells were maintained in RPMI 1640 containing 5% autologous serum for 10 days, and then RT activities in culture fluids were determined. The levels of T-cell activation were monitored after 3 days of stimulation by fluorescent anti-DR antibody and propidium iodide staining (for analyses of cell cycle phases) (see Materials and Methods).

TABLE 7. PCR analysis of HIV-1 infection of TR and TA cells[a]

Transcript	Type of cells	PCR result at time postinfection				
		0 h	1 h	24 h	3 days	7 days
Early (U3-R)	TR	−	+	±	+	±
	TA	+	+	++	++	++
Late (U3-*gag*)	TR	−	−	−	−	−
	TA	±	±	++	+	++

[a] HIV-1_{SF33}-infected TR and TA cells (10^5 cells per sample) were harvested at the indicated time points after virus inoculation and were analyzed by PCR as described in Materials and Methods. Uninfected TR and TA cells and Jurkat cells chronically infected with HIV-1_{SF33} were used as negative and positive controls, respectively (Fig. 2). Experiments were performed in duplicate. The extent of viral DNA produced was measured by densitometry and ranged from a strong reaction (++) to no reaction (−). TR cells showed no evidence of virus production by p25 and RT assays (see text). TA cells showed ≥30,000 cpm of RT activity per ml after 5 days of infection.

As a control, Jurkat cells chronically infected with HIV-1_{SF33} showed both early and late transcripts (Fig. 2, lanes 13).

Similarly, when virus-inoculated TR cells were stimulated with PHA and rIL-2 after 7 days and then were cultured for 3 days, only early transcripts were detected 15 days later (Fig. 2, lanes 14). As expected, no virus production was noted (Table 8). However, when these infected TR cells were activated by PHA after 7 days and then infected again with HIV-1_{SF33}, both early and late transcripts of the viral genome were noted in association with virus production (Table 8; Fig. 2, lanes 16). Moreover, increased levels of p25 antigen and RT activity were observed in this reinfection experiment. Finally, when uninfected TR cells that had been maintained in a resting state for 7 days were stimulated with PHA for 3 days and then inoculated with HIV-1_{SF33}, the virus production that took place was associated with the expression of both early and late viral DNA transcripts (Fig. 2, lanes 17). This latter experiment indicated that after 1 week, activated TR cells were still susceptible to HIV-1 infection.

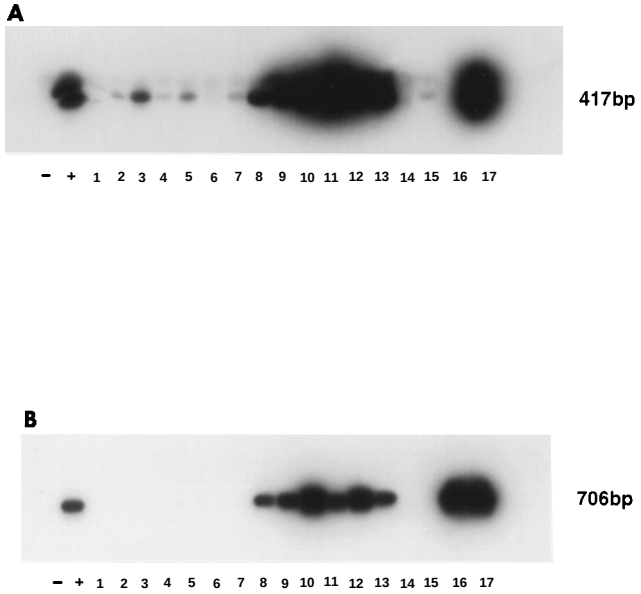

FIG. 2. PCR amplification of HIV-1 early (LTR U3-R) (A) and late (LTR U3-*gag*) (B) reverse transcripts, as described in Materials and Methods. Lanes: −, uninfected CEM cells; +, HIV-1-infected PBMC; 1, uninfected TR cells; 2, TR cells zero time postinfection; 3, TR cells 1 h postinfection; 4, TR cells 24 h postinfection; 5, TR cells 3 days postinfection; 6, TR cells 7 days postinfection; 7, TA cells zero time postinfection (in panel B, a light band at 706 bp can be detected on the original gel); 8, TA cells 1 h postinfection; 9, TA cells 24 h postinfection; 10, TA cells 3 days postinfection; 11, TA cells 7 days postinfection; 12, TA cells infected for 10 days; 13, HIV-1_{SF33}-infected Jurkat cells; 14, virus-inoculated TR cells after 7 days (lane 6) were stimulated with PHA for 3 days and 15 days later were harvested for PCR analyses; 15, TA cells surviving at day 20 postinfection (most infected cells died); 16, virus-inoculated TR cells after 7 days (lane 6) were stimulated with PHA for 3 days, inoculated with virus, and harvested 5 days later; 17, uninfected TR cells after 7 days in culture were stimulated with PHA for 3 days, infected with virus, and harvested 15 days later.

Effects of macrophages on the susceptibility of TR cells to HIV-1 infection. The inability of TR cells inoculated with HIV-1 to release virus on activation was further examined by a cell-to-cell contact system that involved normal and HIV-1-infected macrophages. In these studies, we found that TR cells mixed with autologous or allogeneic macrophages, whether infected or not, could not be made susceptible to HIV-1. After exposure for 5 to 6 days to normal or HIV-1_{SF33}-infected macrophages, the TR cells isolated were found to have no substantial modulation in CD25 (IL-2 receptor) or DR expression unless they had also been exposed to PHA (data not shown). These TR cells were not susceptible to productive infection by HIV-1 (data not shown). Thus, exposure to macrophages alone does not induce $CD4^+$ cell activation.

In experiments with infected macrophages, cells inoculated with HIV-1_{SF33} showed low levels of virus replication, as detected by the p25 antigen assay. However, cocultivation of these cells with activated PBMC led to efficient virus recovery in PBMC (data not shown). This macrophage infection could be maintained for at least 2 months without cytopathic effects. When cryopreserved autologous or allogeneic TR cells were cocultivated with these infected macrophages, no infection of TR cells took place, as detected by p25 and RT assays. Moreover, when these macrophages were treated with PHA for 3 days and then washed, the supernatant after 24 h did not induce HIV replication in these TR cells. Only when PHA was in the culture fluid along with TR cells and macrophages was HIV-1 transferred from the infected macrophages to the T cells (Table 9). These observations suggest that an infected macrophage is a reservoir for subsequent transfer of HIV-1 to lymphocytes through cell-cell contact, but only when the $CD4^+$ T lymphocyte is activated.

DISCUSSION

These studies were undertaken to determine if a highly purified quiescent $CD4^+$ T-lymphocyte population could be productively infected with HIV. By extensive purification of these cells, our results indicate that these $CD4^+$ cells are infectible by the virus, but after infection, these cells cannot be induced to release virus. PCR analyses indicated low and limited levels of early proviral forms; no late transcripts of the viral genome were detected. Only after some activation event and subsequent reinoculation of virus was progeny virion production detected. This release of virus was associated with the production of both early and late viral DNA transcripts (Table 7). Our experiments controlled for the possible contamination of short transcripts in virions attached to cell surfaces (15, 21). Trypsinization eliminates these viruses effectively (18). Moreover, these transcripts were not present at zero time.

These observations support the interpretation of previous findings (4) as to the inability to infect resting $CD4^+$ lymphocytes. In these early studies, however, PCR detection procedures were not available, so virus entry into cells could not be evaluated. Our data agree with the findings of Stevenson et al. (17) and Zack et al. (24), indicating that HIV can enter resting cells. However, they disagree with previous studies showing that virus replication can be induced after cell activation. The earlier reports demonstrated that quiescent $CD4^+$ lymphocytes can harbor HIV-1 in a linear unintegrated proviral DNA form for long periods without virus production. When they were activated, these cells released virus (17, 24). Our results with the LR cell population, derived in the same way as the cells used in these other studies (17, 24), mirror these findings (Table 2). We could induce virus from these cells up to 4 weeks after HIV-1 inoculation.

In previous studies of resting lymphocytes (24), both short and long viral DNA transcripts were detected by PCR. In our experiments with TR cells, only short transcripts were detected (Fig. 2). Conceivably, in the other investigations (17, 24), low-level contamination with DR^+ cells (0.4%) (either $CD4^+$ lymphocytes or macrophages) was responsible for these PCR results and the virus replication reported following activation. This factor appeared to be responsible for our recovery of virus from the LR cell population. When macrophages and DR^+ cells were eliminated, virus replication did not take place (Tables 3 and 5). Moreover, in our experiments with TR cells, only when small numbers of $CD4^+$ DR^+ T lymphocytes or macrophages were present were low levels of replicating virus detected. When all activated cells were removed, no virus replication was noted and virus production was not induced in these cells (Table 8). Finally, the detection of short reverse transcripts in resting cells agrees with previous observations (17, 24), except that in our studies, these transcripts were detected for a longer period (7 rather than 3 to 4 days). The reason for this increased stability is not known.

TABLE 9. Viral transmission from macrophages latently infected with HIV-1_{SF33} to TR cells[a]

Coculture condition	RT activity (10^3 cpm/ml)		p25 antigen (pg/ml)	
	Isolated TR	Isolated macrophage	Isolated TR	Isolated macrophage
Without PHA	1.2	0.2	0	0
With PHA	34.2	0.8	492.8	10.9

[a] TR cells (5×10^6) were cocultivated for 6 days with HIV-1_{SF33}-infected adherent macrophages in the presence or absence of PHA (3 μg/ml). Then TR cells were removed from macrophages, and each cell subset was cultured individually for 8 days. The culture supernatants were harvested separately from TR cell and macrophage cultures to determine RT activities and p25 antigen production.

Our studies with highly purified quiescent $CD4^+$ cells do not explain why subsequent activation of infectible resting cells does not lead to the release of virus. Most likely, the lack of a long viral DNA transcript is involved. The intracellular mechanism for this arrest in reverse transcription (maintained for up to 7 days in quiescent cells) merits further evaluation. Importantly, infected resting $CD4^+$ cells once they have been activated and reinfected could be a source of recombinant viruses in vivo.

These observations may have relevance to a clinical situation in which virus entering a resting lymphocyte is not replicated even if the cell is subsequently activated. It is known that the number of unactivated $CD4^+$ HLA-DR^- T lymphocytes in the hematopoietic system is far greater than the number of fully activated cells (22). We show that these unactivated cells are in a state with limited susceptibility to HIV-1. By most FACscan analyses, very few normal circulating $CD4^+$ cells (5 to 7% in studies in our laboratory) show a phenotype sensitive to productive HIV-1 infection. Thus, the resting T lymphocytes in blood would not appear to be a major initial target for productive HIV-1 infection. In contrast, our results suggest that macrophages in an activated differentiated state, particularly in lymphoid tissues, can be readily infected by HIV (even relatively nonmacrophage-tropic strains such as HIV-1_{SF33}) and serve as an initial reservoir for HIV. Subsequently, these infected macrophages could transfer the virus to T lymphocytes, but only when they are activated.

In our macrophage-TR cocultivation system, the data showed that activation signals are needed during cell-to-cell

TABLE 8. Infection of TR cells under different culture conditions[a]

Procedure	Experimental conditions				Results			
	Day 1	Day 7	Day 10	Day 15	PCR[b] *Early*	PCR[b] *Late*	p25 (pg/ml)	RT (10^3 cpm/ml)
One-step infection	Inoculate HIV	Stimulate with PHA and rIL-2	Remove PHA	Harvest cells	+	−	115	0.7
Reinfection after activation	Inoculate HIV	Stimulate with PHA and rIL-2	Remove PHA; inoculate HIV	Harvest cells	++	++	>4,000	35.9
Pre-activated TR infection	No treatment	Stimulate with PHA and rIL-2	Remove PHA; inoculate HIV	Harvest cells	++	++	>4,000	1.9[c]

[a] HIV-1_{SF33} infection of TR cells was conducted as described in Materials and Methods. Cell pellets and supernatants were harvested on day 15 for PCR analysis and p25 and RT assays.

[b] Relative expression as determined by densitometry. ++, strong reaction; +, reaction; −, no reaction.

[c] This low value reflects cell death in culture.

contact to transfer infectious particles. This event could occur if an individual's immune system is activated against other invading organisms or through exposure to immunoactive proteins. Thus, our data suggest that in acute infection, HIV preferentially replicates in tissue or (blood) macrophages (or dendritic cells) where it can be maintained for long periods in a latent form or at low-level expression. These cells can serve as reservoirs for the spread of HIV in the host. During HIV transmission, infection would occur with much less frequency in the $CD4^+$ lymphocyte population since it is primarily in a quiescent state. However, further studies are needed to determine definitively which cell population serves as the major source of infection in the initial stages of HIV entry into the host.

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