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19 ABSTRACT (Continue on reverse if necessary and identify by block number) Monocytes were maintained in tissue culture for - 3 mo in media supplemented with rCSF-1. These cultures provided susceptible target cells for isolation and propagation of virus from PBMC of HIV-infected patients. HIV isolated into monocytes readily infected other rCSF-1 treated monocytes but only inefficiently infected PHA-stimulated lymphoblasts. Similarly, laboratory HIV strains passaged in T cell lines or virus isolated from patients' leukocytes into PHA-stimulated lymphoblasts inefficiently infected rCSF-1 treated monocytes. Persistent, low-level virion production was detected in macrophage culture fluids by reverse transcriptase activity or HIV antigen capture through 6 to 7 wk. Marked changes in cell morphology with cell death, syncytia, and giant cell formation were observed in monocyte cultures 2 wk after infection, but at 4 to 6 wk, all cells appeared morphologically normal. However, the frequency of infected cells in these culture 6 wk was 60 to 90% as quantified by in situ hybridization with HIV RNA probes or by immunofluorescence with AIDS patients' sera. Ultrastructural analysis by EM also showed a high frequency of infected cells; vir-			
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19. ABSTRACT (Continuation)

→ tually all HIV budded into and accumulated within cytoplasmic vacuoles, and virus particles were only infrequently associated with the plasma membrane. Retention of virus within macrophages and the macrophage tropism of HIV variants may explain mechanisms of both virus persistence and dissemination during disease. *print*

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EFFICIENT ISOLATION AND PROPAGATION OF HUMAN IMMUNODEFICIENCY VIRUS ON RECOMBINANT COLONY-STIMULATING FACTOR 1-TREATED MONOCYTES

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Infection with the human immunodeficiency virus (HIV) (1-3) often results in clinically apparent disease only after intervals of months to years. During this latent or subclinical phase of infection, HIV continues to replicate at low levels despite an often vigorous but apparently ineffective host immune response (4). Mechanisms that contribute to this persistent, low-level infection, as well as the cellular reservoirs for HIV during this latent period, are not fully understood. Several lines of evidence now document cells of the monocyte/macrophage lineage as major targets for persistent HIV in vivo (5-12). In this respect, HIV is similar to several ruminant lentiviruses that show strong tropism for macrophages during both viral latency and active replication (13-15). If macrophages also serve as a viral reservoir during HIV infection, then analysis of these infected cells may explain mechanisms of viral persistence, dissemination, and ultimately clinical disease.

In this report, we describe an in vitro system that allows replication of HIV in blood-derived monocyte/macrophages from normal donors. Purified monocytes were cultured for intervals > 3 mo in medium supplemented with human rCSF-1 (16, 17). These cultures provided susceptible target cells for HIV infection. Cocultivation of PBMC from patients with AIDS or AIDS-related complex (ARC)¹ and rCSF-1-treated monocytes from normal donors resulted in isolation of progeny HIV virions in the majority of patients tested.

Materials and Methods

Isolation and Culture of Peripheral Blood Monocytes. Populations of monocytes were isolated by countercurrent centrifugal elutriation of mononuclear leukocyte-rich fractions

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¹Abbreviations used in this paper: ARC, AIDS-related complex; UA, uranyl acetate.

of blood cells from normal donors undergoing leukopheresis (18). Cell suspensions were $> 96\%$ monocytes by the criteria of cell morphology on Wright-stained cytosmears ($96 \pm 2\%$, mean $\pm$ SEM for six determinations), by granular peroxidase ($95 \pm 3\%$), and by nonspecific esterase ($98 \pm 2\%$). Elutriated monocytes were cultured as adherent cell monolayers in DMEM (formula 780176AJ, Gibco, Grand Island, NY) supplemented with 10% freshly obtained, heat-inactivated, normal human serum, 50 $\mu\text{g}/\text{ml}$ gentamicin, and 1,000 U/ml rCSF-1 (Cetus Corp., Emeryville, CA) (16, 17).

Isolation and Culture of PHA-stimulated PBMC (Lymphoblasts). PBMC isolated from whole blood by Ficoll-diatrizoate density gradient centrifugation were cryopreserved and stored in liquid nitrogen. 3 d before use for virus isolation, cells were quickly thawed and stimulated with the T cell mitogen, PHA (1–3).

Virus Isolation by Monocyte or Lymphoblast Cocultivation. Monocytes treated with rCSF-1 and maintained in culture for at least 7 d were used for cocultivation experiments with freshly isolated PBMC from seropositive HIV-infected individuals. Aliquots of Ficoll-diatrizoate-separated PBMC (5×10^5 cells/culture well) were admixed with equal numbers of adherent rCSF-1-treated monocytes in 16-mm-diameter culture wells (Cluster²⁴; Costar Data Packaging Corp., Cambridge, MA) or with suspensions of PHA-stimulated lymphoblasts (1–3). Fluids from all cultures were sampled daily and assayed by ELISA (Cellular Products, Inc., Buffalo, NY) for presence of HIV-specific antigens and/or reverse transcriptase activity for at least 40 d. Reverse transcriptase assays were performed with [³²P] deoxythymidinetriphosphate in a protocol modified from that described by Goff et al. (19, 20).

Immunofluorescence Analysis by Flow Cytometry. Uninfected (10 d) and HIV-infected (40 d) rCSF-1-treated monocytes were cultured and recovered from Teflon-coated tissue culture flasks (Cole-Parmer Instrument Co., Chicago, IL). For all experiments, 10^6 cells were incubated with 1:100 dilution of mAb anti-HLc-1 (CD45, Becton Dickinson & Co., Mountain View, CA), Leu-M3 (CD14, Becton Dickinson & Co.) B4 (CD19), J5 (CD10), T4 (CD4), T6 (CD1), T8 (CD8), and T11 (CD2; all from Coulter Immunology, Hialeah, FL) or 1:50 dilution of pooled AIDS patients' sera from HIV-1- and HIV-2-infected individuals. After the initial antibody incubation, cells were washed after centrifugation and resuspended in 1:100 dilution of fluorescein-conjugated horse anti-mouse or goat anti-human IgG. Immunofluorescence of individual cells previously fixed in 1% paraformaldehyde were analyzed by FACS flow cytometry.

Detection of HIV-specific Polypeptides by Radioimmunoprecipitation. Adherent monolayers of rCSF-1-treated monocytes chronically infected (30 d) with HIV-1 (patient Ada, second passage) were washed twice and cultured in methionine-free DMEM with 2% dialyzed FCS for 2 h. Cells were labeled with [³⁵S]methionine (100 $\mu\text{Ci}/\text{ml}$) for 8 h. Radiolabeled cell lysates were mixed with AIDS patients' sera for 12 h at 4°C and the immune complexes were recovered on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Eluted immune complexes were subjected to SDS-PAGE as described (21).

Detection of HIV-specific DNA by Southern Blot Hybridization. DNA was prepared from HIV-infected (patient 120 isolate, second passage at 30 d) rCSF-1-treated monocytes and analyzed for presence of virus-related sequences by Southern blot hybridization of Hind III-digested cellular DNA with the pBenn6 *gag-pol-env* probe (22).

In Situ Hybridization with HIV RNA Probes. Subgenomic viral DNA fragments present in pBI (23), pBenn6 (22), pB11 (23), and a recombinant plasmid (pRG-B) that contains a 1.35-kb Hind III fragment mapping between 8.25 and 9.6 kb on the proviral DNA were subcloned into SP6/T7 vectors (Promega Biotec, Madison, WI), and the pooled DNAs were transcribed using ³⁵S-UTP (Amersham Corp., Arlington Heights, IL). The labeled RNAs were incubated with 40 mM NaHCO₃/60 μM Na₂CO₃, pH 10.2, before hybridization to facilitate their entry into cells. Cytosmears of cultured monocytes were prepared onto polylysine-coated glass slides, fixed in periodate/lysine paraformaldehyde-glutaraldehyde, and pretreated with proteinase K, triethanolamine, and HCl. Specimens were prehybridized in 10 mM Tris (pH 7.4), 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 1X Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA), and 200 $\mu\text{g}/\text{ml}$ yeast tRNA at 45°C for 2 h, and hybridized in this

solution with 10% dextran sulfate, 5 μ M dithiothreitol and 10⁶ cpm ³⁵S-labeled HIV RNA. Slides were serially washed in solutions with RNase to reduce binding of nonhybridized probe. Autoradiography was performed in absolute darkness (6).

To control for the specificity of in situ hybridization, probes synthesized in the sense orientation (same polarity as viral mRNA) were incubated with replicate cell preparations. Additionally, uninfected cells were hybridized with antisense probes (i.e., complementary to viral mRNA).

EM Examination of Monocyte Cultures. HIV-infected or uninfected rCSF-1-treated monocytes were grown on plastic dishes or recovered from Teflon flasks. Cells were harvested at 10 and 40 d, washed in PBS, and immediately fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C. Fixed cells were gently transferred to 1.5-ml microfuge tubes using a large-bore Pasteur pipette and were pelleted after centrifugation. The cell pellet was further processed through 1% OsO₄, blocked in uranyl acetate (UA), stained for 1 h in saturated UA in 50% ethanol, dehydrated in graded ethanol and propylene oxide, and embedded in epon. Thin sections were stained with UA and lead citrate and examined in a Zeiss EM 10A^R EM operating at 60 kV.

Results

Culture of rCSF-1-treated Blood Monocytes. Relatively pure populations of monocytes were obtained by countercurrent centrifugal elutriation of mononuclear leukocyte-rich fractions of blood cells from normal donors undergoing leukopheresis (18). Such cell suspensions were > 96% monocytes by criteria of cell morphology on Wright-stained cytosmears, by granular peroxidase, and by non-specific esterase. Purified monocytes were cultured in medium supplemented with 1,000 U/ml rCSF-1. After 5–7 d of culture, clusters of rounded, loosely adherent, proliferating monocytes were observed scattered throughout a monolayer of adherent fusiform cells (Fig. 1). Low levels of cell division were confirmed by [³H]thymidine incorporation and the presence of mitotic figures in 1–5% of the cells. In coincident experiments, monocytes in aliquots of the same cell suspension cultured without rCSF-1 for 7 d appeared spread, vacuolated, and granular. No proliferating cell clusters were observed and the absolute cell number was < 20% of the initial inoculum. In contrast, the number of cells in rCSF-1-treated monocyte cultures at 7–10 d ranged from 90–150% of the initial inoculum. EM examination of 100 individual cells after 10 d in culture showed that all cells had ultrastructural characteristics typical of macrophages: irregular outlines, abundant lysosomes, prominent perinuclear Golgi, and eccentric nuclei. Cell surface antigens in these monocyte cultures were also characterized at 10 d by mAbs and analyzed by FACS flow cytometry. More than 98% of cells were positive for HLe-1 (CD 45) and Leu-M3 (CD 14); binding of anti-B4 (CD 19), J5 (CD10), T4 (CD 4), T6 (CD1), T8 (CD 8) or T11 (CD2) were each below levels of detection. Thus by antigenic, histochemical, morphologic, and ultrastructural analysis, virtually all of the cells in these 10-d suspensions were identified as monocytes/macrophages.

Isolation of HIV from PBMC of Seropositive Individuals onto rCSF-1-treated Monocytes of Normal Donors. Repeated attempts to propagate established laboratory strains of HIV in monocytes were uniformly negative over a time interval of >6 mo (data not shown). These attempts were repeated with the rCSF-1-treated monocyte culture technique described above. Monocytes treated with rCSF-1 for at least 7–10 d were used for cocultivation experiments with freshly

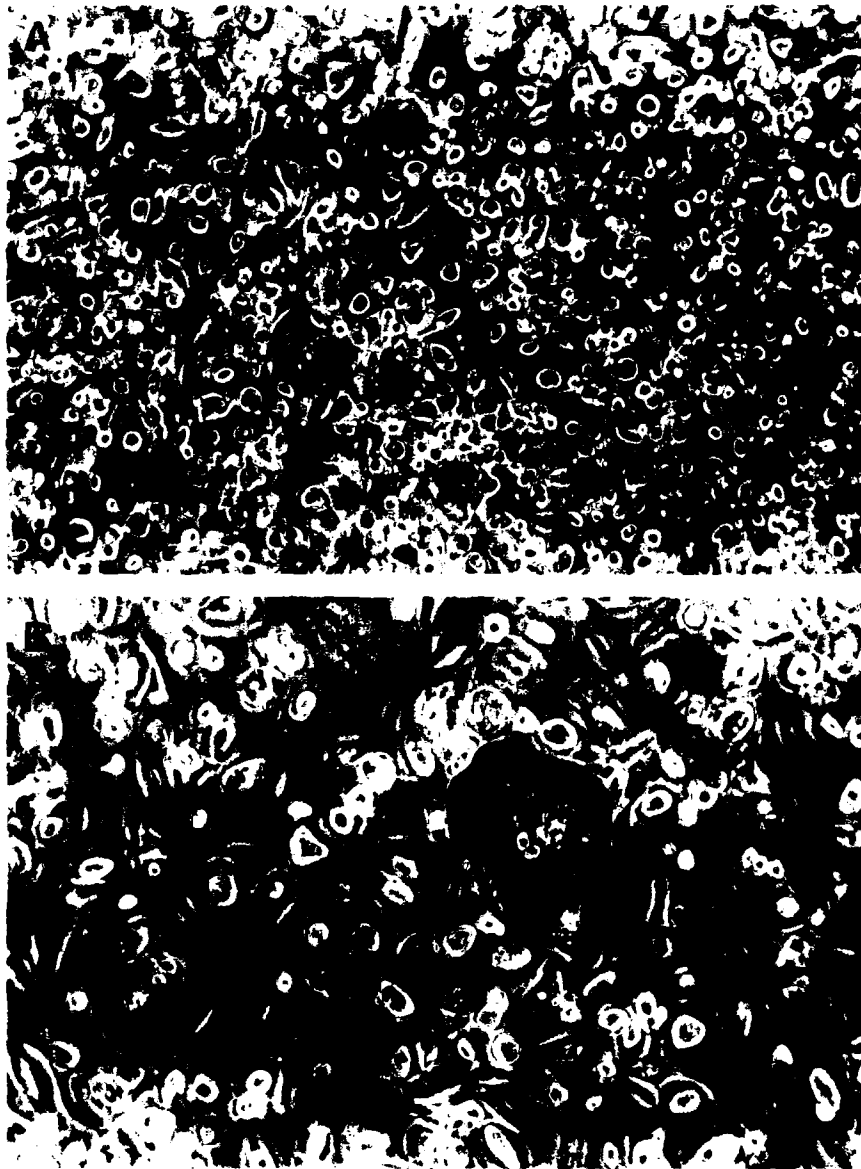


FIGURE 1. Light microscopic characteristics of blood-derived monocytes cultured with rCSF-1 for (A) 10 and (B) 40 d. Note heterogeneous cell populations: small cells with dense nuclear chromatin and moderate cytoplasm; larger cells with extensive and diffuse cytoplasm and eccentric nucleus; and individual fusiform cells with fibroblast-like morphology; islands of aggregated and proliferating cells. Original magnification, $\times 80$.

isolated PBMC from seropositive HIV-infected individuals (Table I). Aliquots of PBMC from each of five patients were cocultivated with rCSF-1-treated adherent monocyte monolayers and suspensions of PHA-stimulated PBMC (lymphoblasts) from normal donors. Culture fluids were sampled daily and assayed for HIV-specific antigens and/or reverse transcriptase. Isolation of HIV was suc-

TABLE I
Virus Isolation from PBMC of HIV-infected Patients by Monocyte or Lymphoblast
Cocultivation

Patient	HIV reverse transcriptase activity in culture fluids of patient PBMC cocultivated with:		
	CD4 ⁺ T cells/mm ³	PHA-induced lymphoblasts	rCSF-1-treated monocytes
		<i>cpm/ml</i> $\times 10^{-3}$	
Ada: 36-yr-old male homosexual with Kaposi's sarcoma for 4 yr	252	1,000	70 (35 d)
Ree: 44-yr-old male homosexual with Kaposi's sarcoma for 1 yr	208	900	100 (22 d)
120: 27-yr-old male i.v. drug abuser with ARC for 1 yr	360	200	40 (25 d)
121: 32-yr-old male i.v. drug abuser with ARC for 9 mo	582	500	None
167: 24-yr-old male homosexual with ARC for 1 yr	433	None	160 (36 d)

Aliquots of Ficoll-diatrizoate-separated PBMC were admixed with equal numbers of adherent rCSF-1-treated monocytes or with PHA-stimulated lymphoblasts. Fluids from all cultures were sampled daily and assayed for HIV-specific reverse transcriptase activity for 40 d. Reverse transcriptase levels represent peak activity. Day at which virus was first detected in monocyte cultures is shown in parentheses.

cessful in all five patients by cocultivation with rCSF-1-treated monocytes or lymphoblasts. Antigen-capture assays confirmed isolation of HIV in each instance. One patient had a lymphoblast isolate and not a monocyte isolate; another patient had a monocyte but not a lymphoblast isolate. With three patients, both monocyte and lymphoblast isolates were obtained; the peak reverse transcriptase activity and HIV viral antigen level in lymphoblast cultures were 10-fold higher than those in monocyte cultures.

Progeny virions released in supernatant fluids of infected monocyte and lymphoblast cultures were used to serially infect other rCSF-1-treated monocyte and lymphoblast cultures. In all cases, viral inoculum was adjusted to 5×10^4 cpm/ml reverse transcriptase activity in 0.5-ml filtered (0.22- μ m filter units; Millipore Continental Water Systems, Bedford, MA) culture fluid. HIV initially isolated on monocyte or lymphoblast cultures serially infected normal homologous cells with all isolates tested. Serial passage of HIV (patient 120 isolate, see Table I) initially isolated on monocytes into other rCSF-1-treated monocyte cultures resulted in a progressive increase in viral titer (Fig. 2). In the second passage, distinctive changes in cell morphology and some cell death were noted in monocyte cultures 8–14 d after HIV infection. Appearance of cell debris and changes in cell morphology in the rCSF-1-treated monocyte monolayer were coincident with peaks of reverse transcriptase activity in culture fluids. Multi-nucleated giant cells with > 20 nuclei/cell were noted in 10–20% of monocytes. Replicate rCSF-1-treated monocytes cultured alone as adherent monolayers or cocultivated with PBMC from HIV seronegative control donors did not show these changes. HIV-specific reverse transcriptase activity was detected in culture

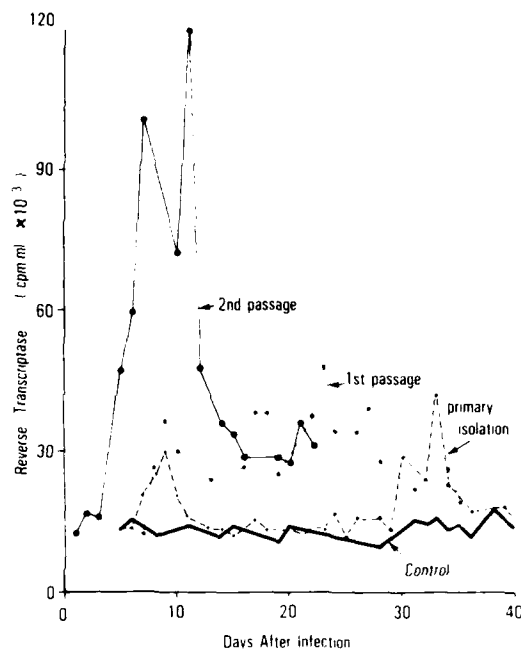


FIGURE 2. Serial passage of virus isolated from PBMC of patient 120 into rCSF-1-treated monocytes. Aliquots of Ficoll-diatrizoate-separated PBMC suspensions from patient 120 were admixed with equal numbers of adherent rCSF-1-treated monocytes (primary isolation). Culture fluids were sampled daily and assayed for HIV-specific reverse transcriptase activity. For each subsequent passage into rCSF-1-treated monocytes, viral inoculum was adjusted to 5×10^4 cpm/ml reverse transcriptase activity in filtered culture fluid (0.5 ml).

fluids through 40 d. Significantly, at these later time points, most of the cells in the HIV-infected monocyte monolayer appeared morphologically normal.

After three passages on homologous cells, monocyte and lymphoblast HIV isolates were each added to heterologous cells; in each instance, sustained, productive viral infection was not demonstrated. Similarly, several different strains of HIV-1, such as lymphadenopathy associated virus or LAV that were each maintained for long intervals in normal lymphoblasts or continuous T cell lines, all failed to infect rCSF-1-treated monocyte/macrophage cultures even at viral inocula 20-fold higher than that needed to infect lymphoblasts. One macrophage-tropic HIV patient isolate (Ada) infected PHA-stimulated lymphoblasts after five serial passages in rCSF-1-treated monocytes. In marked contrast to the preceding observations, a well-characterized HIV-2 isolate (ROD) that had been serially passaged in lymphoblasts and continuous T cell lines (24) infected rCSF-1-treated monocytes; peak reverse transcriptase activity, 2×10^5 cpm/ml, was detected 8 d after infection.

Characterization of the Macrophage Variant HIV. HIV-specific proviral DNA was detected by Southern blot hybridization of Hind III-digested DNA prepared from HIV-infected (patient 120 isolate, second passage at 30 d) rCSF-1-treated monocytes (Fig. 3 A). Two cleavage products (4.5 and 2.0 kb) reacted with the pBenn6 DNA probe. Radioimmunoprecipitation of HIV-associated proteins from [³⁵S]methionine-labeled, HIV-infected rCSF-1-treated monocytes (patient Ada isolate, second passage at 30 d) showed detectable levels of synthesis for envelope (gp 160 and gp 120) and gag (p55 and p39) proteins (Fig. 3 B). Levels of HIV gene synthesis in chronically infected monocyte cultures were further estimated by assay of virus-specific RNA and proteins. In situ hybridization

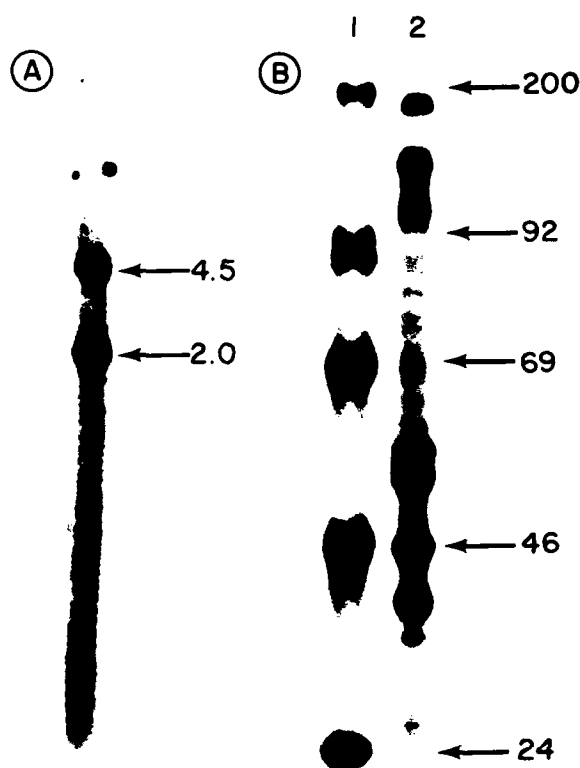


FIGURE 3. (A) Detection of HIV-specific DNA by Southern blot hybridization. DNA was prepared from HIV-infected (patient 120 isolate, second passage at 30 d) rCSF-1-treated monocytes and analyzed for presence of virus-related sequences by Southern blot hybridization of Hind III-digested DNA with the pBem6 probe. (B) Detection of HIV-specific polypeptides by radioimmunoprecipitation of cell lysates from adherent monolayers of rCSF-1-treated monocytes chronically infected (30 d) with HIV-1 (patient Ada, second passage). Supernatant fluids from [35 S]methionine-labeled cell lysates were mixed with pooled AIDS patients' sera and the immune complexes were subjected to SDS-PAGE. Immunoprecipitate bands representing envelope (gp 160 and gp 120) and *gag* (p55 and p39) proteins are clearly identified in lane 2. Molecular weight markers were applied to lane 1.

of infected monocytes with HIV RNA probes and analysis of the hematoxylin-stained cytosmears by autoradiography documented a large subpopulation of cells (60–90%) that expressed viral RNA (Fig. 4). Similarly, analysis of chronically infected monocyte populations (patient 120 isolate, second passage at 40 d) by binding of antibodies in AIDS patients' sera as quantified by FACS flow cytometry also documented a large subpopulation of cells (60–88%) that expressed viral protein. These results, however, stand in sharp contrast to the relatively low levels of reverse transcriptase activity or HIV antigens found in culture fluids. Independent estimates of viral RNA and protein produced by HIV-infected monocytes suggested a massive infection, yet the amount of virus released into culture fluids was exceedingly small.

EM analysis of HIV-infected monocyte cultures resolved this apparent paradox. Monocytes chronically infected (40 d) with HIV-1 (patient 120, second passage) initially isolated in rCSF-1-treated monocyte cultures were fixed in 2% glutaraldehyde and were prepared for transmission EM. Virus particles were identified in ~15% of macrophages examined. Virions typical of lentiviruses (25) were numerous (100–300 particles/cell) and uniformly localized to intracytoplasmic vacuoles (Fig. 5). Viral particle size and nucleoid appearance was pleomorphic. Virions were commonly seen budding into cytoplasmic vacuoles but only rare viral particles were observed associated with the plasma membrane. Similar findings were evident in rCSF-1-treated monocytes chronically



FIGURE 4. In situ hybridization of HIV-infected (patient Ada isolate, third passage) rCSF-1-treated monocytes. Silver grains (HIV-specific RNA) overlie infected cells.

infected (40 d) with the HIV-2 (ROD) isolate (Fig. 6). With HIV-1, plasma membrane budding was observed at low levels during the acute phase of infection (10–14 d), but again intracellular accumulation of virus particles within cytoplasmic vacuoles was the predominant finding.

The pattern of HIV replication in monocytes and T cells is thus very different. HIV-infected monocytes accumulate large numbers of budded virus in intracytoplasmic vacuoles during both acute and chronic infections; release of virus from the plasma membrane is infrequent and at relatively low levels (0–10 particles/cell section). This pattern of viral replication in rCSF-1-treated monocytes was observed with three different patient isolates of HIV (patients Ada and 120), and HIV-2 (ROD); viral particles were identified in ~15% of monocytes examined at 4–6 wk. In contrast, the HIV-infected T cell releases large numbers of viral particles from the plasma membrane (often hundreds of virions/cell section); the number of virions that reside in cytoplasmic vacuoles in these cells is exceedingly small. The concept that HIV virions produced in macrophages accumulate intracellularly and are only inefficiently transported out of the cell was confirmed by comparison of fluid-phase reverse transcriptase levels in monocyte cultures before and after three successive freeze-thaw cycles. The amount of reverse transcriptase activity detected in monocyte cultures after the freeze-thaw cycles was 10–20 times higher (9×10^5 cpm/ml) than that of control levels. Virus released into culture fluids by freeze-thaw cycles was fully infectious for rCSF-1-treated monocytes.

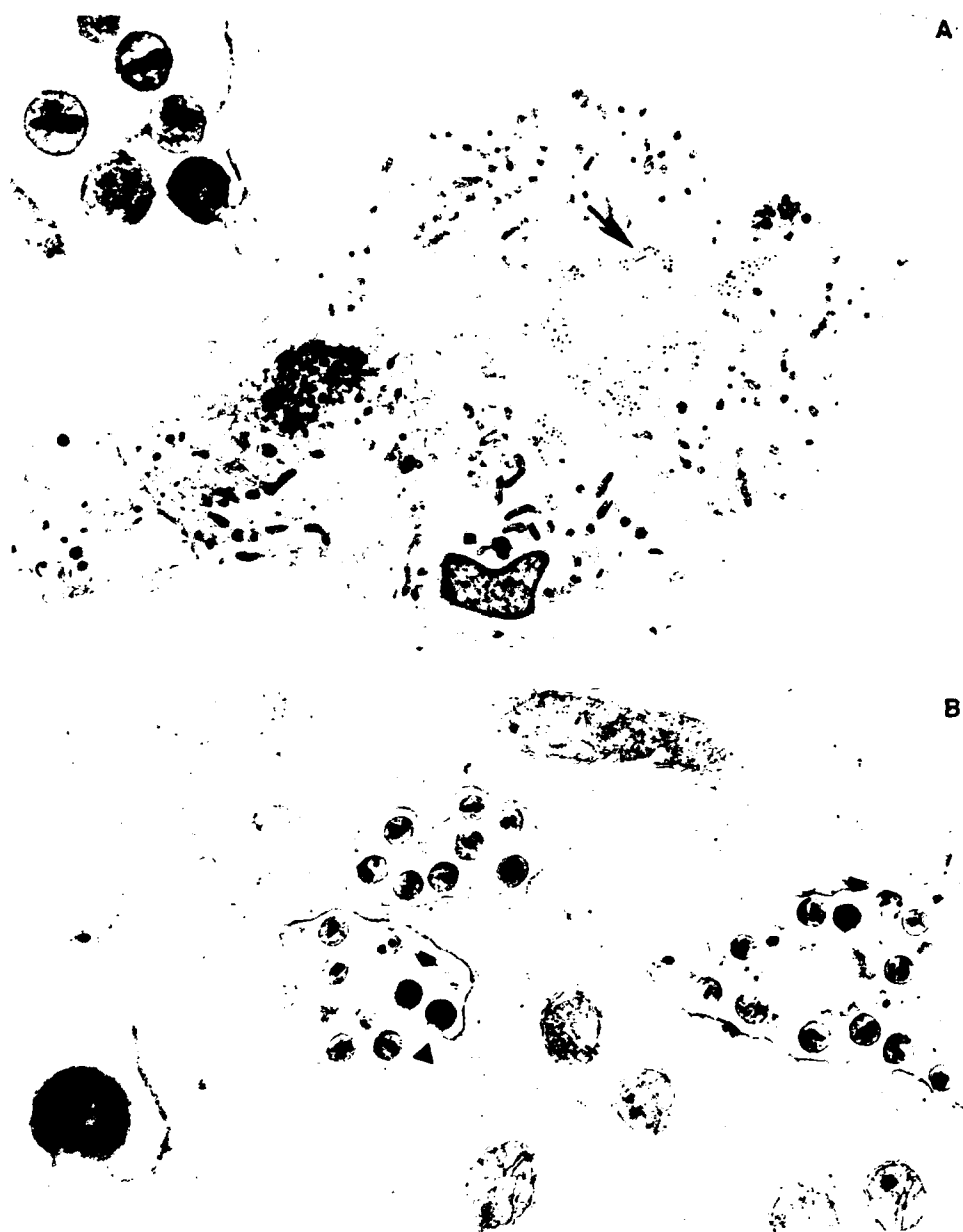


FIGURE 5. (A) Transmission EM of an HIV-1-infected (patient 120 isolate, second passage) rCSE-1-treated monocyte at 40 d of culture. Part of a relatively small eccentric nucleus, numerous electron-dense lysosomes and an irregular surface with pinocytic vesicles are seen. Several vacuoles with numerous viral particles are present in the central cytoplasm. The inset shows virus budding into a vacuole (*arrow*) that also contains several mature virions with dense conical nucleoids. Virus surface spikes are not apparent. Original magnification, $\times 6,500$ (*inset*: $\times 100,000$). (B) In the same preparation, three irregular cytoplasmic vacuoles contain many mature virions and several budding or incomplete (immature) viral particles (*arrows*). One of the budding virions (*arrowhead*) is enlarged in the inset. A mature virus particle is associated with the plasma membrane. Virus surface spikes are not apparent. Original magnification, $\times 58,000$ (*inset*: $\times 200,000$).

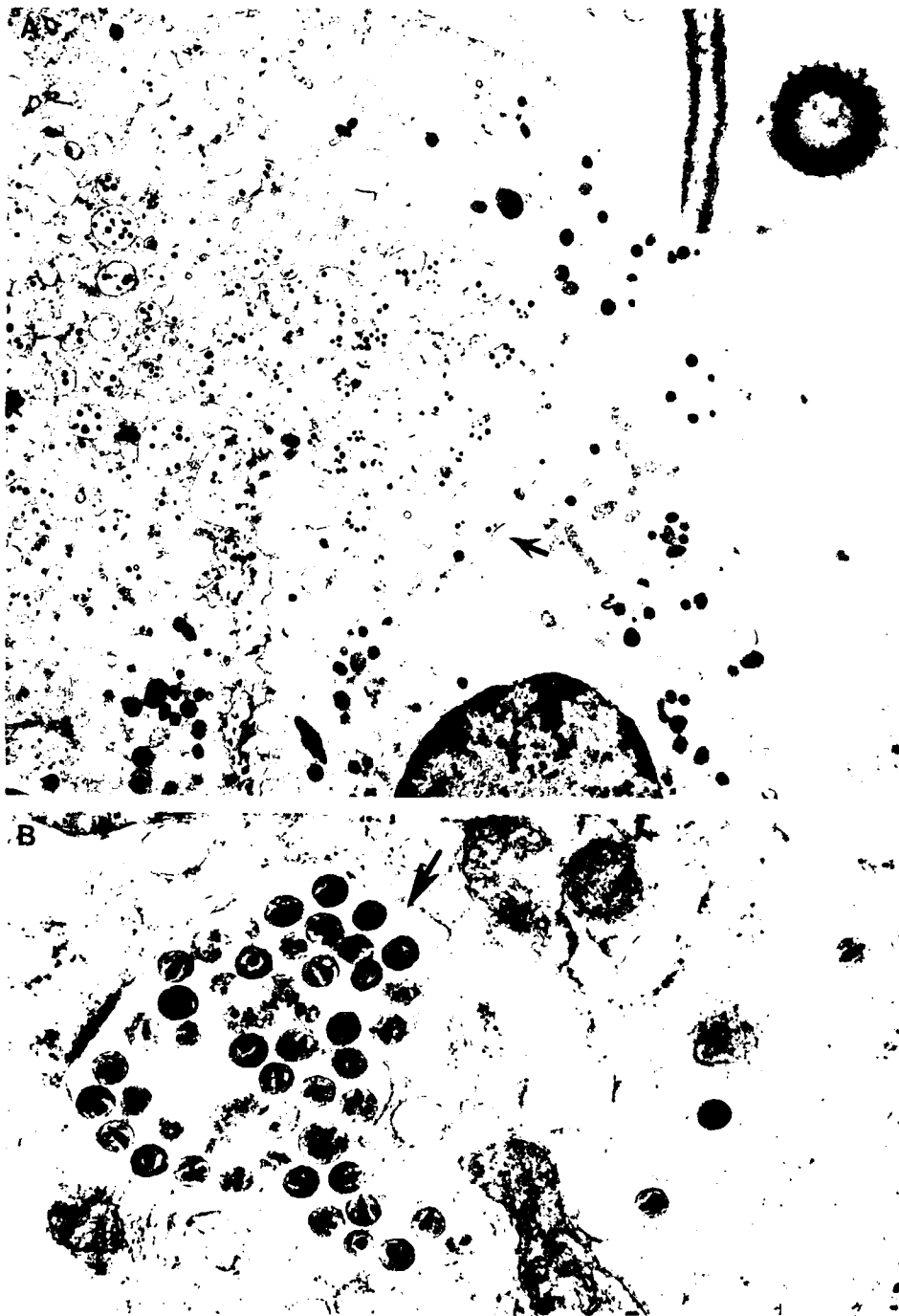


FIGURE 6. (A) Transmission EM of an HIV-2 (ROD)-infected rCSE-1-treated monocyte at 40 d of culture. Irregular surface processes, pinocytic vesicles, lysosomes, and lipid vacuoles are observed. Innumerable virus-bearing vacuoles of varying sizes fill the central cytoplasm. One of several budding virions (*arrow*) covered by prominent surface spikes is enlarged in the inset. Original magnification, $\times 9,400$ (inset: $\times 200,000$). (B) In the same preparation, one large cytoplasmic vacuole contains numerous pleomorphic mature and a cluster of three immature virus particles (*arrow*). Three small vacuoles each contain a single virion. Original magnification, $\times 58,000$.

Discussion

The preceding observations document recovery of HIV tropic for macrophages in a majority of patients tested. It is not clear at this point whether the efficient isolation of HIV from patients' leukocytes into rCSF-1-treated monocytes represents a change in target cell susceptibility to virus or to increased monocyte viability in culture over extended time intervals. The role of CSF-1, a macrophage growth factor, in HIV infection of macrophages may not be analogous to that of the T cell growth factor, IL-2, for T cells (1-3). We were able to document only a small subpopulation of proliferating monocytes (~1-5%) during culture with rCSF-1, yet the percentage of HIV-infected cells detected by in situ hybridization with HIV RNA probes or by immunofluorescence with AIDS patients' sera exceeded 60-90%. By whatever mechanism, the findings presented in these studies, as well as those in previous reports that document biologically distinct HIV in brain and lung tissue, implicate variant HIV as major participants in disease pathogenesis (5). The evidence in toto strongly suggests the macrophage variant HIV as a major virus reservoir in early and late disease. This concept must now be included in future drug testing and vaccine development strategies. Moreover, the intracellular sequestration of virions in chronically infected macrophages suggests new models for viral persistence and the dissemination of disease. Indeed, accumulation of HIV within cytoplasmic vacuoles of macrophage-derived, multinucleated giant cells in the brain has been recently described (26). These observations in brain tissue from AIDS patients closely parallel the ultrastructural findings in HIV-infected macrophages reported here. *Retention of virus within macrophages is not novel for retroviruses.* Other lentiviruses, such as caprine arthritis encephalitis and ovine progressive pneumonia virus also bud into and accumulate in cytoplasmic vacuoles (27, 28). These viruses have strong tropism for blood monocytes or tissue macrophages, yet viral replication is restricted and entirely dependent upon host cell (macrophage) differentiation. The visna virus-infected macrophages act as true "Trojan horses" (13). Infected, immature blood monocytes restrict virus replication to minimal levels. After these cells enter tissue and differentiate into mature macrophages, however, visna virus replication increases more than several-thousand-fold (15). Whether these same mechanisms apply to HIV-infected human macrophages remains to be determined.

Although patient-derived HIV efficiently infects rCSF-1-treated monocyte target cells, low numbers of progeny virus are released into culture fluids. During chronic infection, these cells appear morphologically unaffected by the infection, yet EM analysis documents large factories of virions in cytoplasmic vacuoles. In tissues of AIDS patients, HIV-infected mononuclear phagocytes are detected at high frequency in the brain, lymph node, and skin (5-7, 10-12). Do these hidden virus factories explain how HIV escapes a competent host immune surveillance response? It is interesting to further speculate that macrophage variant HIV are the forms responsible for virus latency and dissemination. At some time during disease, the macrophage variant HIV acquires T cell tropism. Perhaps viral envelope glycoprotein undergoes successive mutations to acquire affinity for the CD4 determinant and T cell tropism. This acquired change in structure, and thus function, of HIV would represent a second stage of virus

infection heralded by T4 helper cell depletion and followed by the inevitable development of opportunistic infection and death. The full biologic consequence of distinct T cell and macrophage tropic viruses in AIDS awaits further inquiries. The system for in vitro maintenance of viable, HIV-susceptible monocyte/macrophages described in this report can facilitate this search.

Summary

Monocytes were maintained in tissue culture for > 3 mo in media supplemented with rCSF-1. These cultures provided susceptible target cells for isolation and propagation of virus from PBMC of HIV-infected patients. HIV isolated into monocytes readily infected other rCSF-1-treated monocytes but only inefficiently infected PHA-stimulated lymphoblasts. Similarly, laboratory HIV strains passaged in T cell lines or virus isolated from patients' leukocytes into PHA-stimulated lymphoblasts inefficiently infected rCSF-1-treated monocytes. Persistent, low-level virion production was detected in macrophage culture fluids by reverse transcriptase activity or HIV antigen capture through 6–7 wk. Marked changes in cell morphology with cell death, syncytia, and giant cell formation were observed in monocyte cultures 2 wk after infection, but at 4–6 wk, all cells appeared morphologically normal. However, the frequency of infected cells in these cultures at 6 wk was 60–90% as quantified by in situ hybridization with HIV RNA probes or by immunofluorescence with AIDS patients' sera. Ultrastructural analysis by EM also showed a high frequency of infected cells; virtually all HIV budded into and accumulated within cytoplasmic vacuoles and virus particles were only infrequently associated with the plasma membrane. Retention of virus within macrophages and the macrophage tropism of HIV variants may explain mechanisms of both virus persistence and dissemination during disease.

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References

1. Barré-Sinoussi, F., J.-C. Chermann, F. Rey, M. T. Nugevre, S. Chamaret, J. Gruest, C. Dautet, C. Axler-Blin, F. Vézinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science (Wash. DC)*. 220:868.
2. Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Parker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLA-III) from patients with AIDS and at risk for AIDS. *Science (Wash. DC)*. 224:500.
3. Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Lanois, J. M. Shimbukuro, and L. S. Oskiro. 1984. Isolation of lymphocytotropic retroviruses from San Francisco patients with AIDS. *Science (Wash. DC)*. 225:840.
4. A. S. Fauci. 1986. Current issues in developing a strategy for dealing with the acquired immunodeficiency syndrome. *Proc. Natl. Acad. Sci. USA*. 83:9278.

5. Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic. 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science (Wash. DC)*. 223:215.
6. Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci. 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science (Wash. DC)*. 233:1089.
7. Wiley, C. A., R. D. Schrier, J. A. Nelson, P. W. Lampert, and M. B. A. Oldstone. 1986. Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency patients. *Proc. Natl. Acad. Sci. USA*. 83:7089.
8. Nicholson, J. K. A., G. D. Cross, C. S. Callaway, and J. S. McDougal. 1986. *In vitro* infection of human monocytes with human T lymphotropic virus type III lymphadenopathy-associated virus (HTLV-III/LAV). *J. Immunol.* 137:323.
9. Ho, D. D., T. R. Rota, and M. S. Hirsch. 1986. Infection of monocyte macrophages by human T lymphotropic virus type III. *J. Clin. Invest.* 77:1712.
10. Tschachler, E., V. Groh, M. Popovic, D. L. Mann, K. Konrad, B. Safai, L. Eron, F. diMarzo Veronese, K. Wolff, and G. Stingl. 1987. Epidermal Langerhans cells. A target for HTLV-III/LAV infection. *J. Invest. Dermatol.* 88:233.
11. Tenner-Racz, K., P. Racz, M. Dietrich, and P. Kern. 1985. Altered follicular dendritic cells and virus-like particles in AIDS and AIDS-related lymphadenopathy. *Lancet*. I:105.
12. Armstrong, J. A. and R. Horne. 1984. Follicular dendritic cells and virus-like particles in AIDS-related lymphadenopathy. *Lancet*. II:370.
13. Haase, A. T. 1986. Pathogenesis of lentivirus infections. *Nature (Lond.)*. 322:130.
14. Narayan, O., and L. C. Cork. 1985. Lentiviral disease of sheep and goats: chronic pneumonia leukoencephalomyelitis and arthritis. *Rev. Infect. Dis.* 7:89.
15. Gendelman, H. E., O. Narayan, S. Molineaux, J. E. Clements, and Z. Ghotbi. 1985. Slow persistent replication of lentiviruses: role of tissue macrophages and macrophage precursors in bone marrow. *Proc. Natl. Acad. Sci. USA*. 82:7086.
16. Clark, S. C., and R. Kamen. 1987. The human hematopoietic colony-stimulating factors. *Science (Wash. DC)*. 236:1229.
17. Kawasaki, E. S., M. B. Ladner, A. M. Wang, J. V. Arsdell, M. K. Warren, M. Y. Coyne, V. L. Schwenkart, M-T. Lee, K. J. Wilson, A. Boosman, E. R. Stanley, P. Ralph, and D. F. Mark. 1985. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). *Science (Wash. DC)*. 230:291.
18. Wahl, L. M., I. M. Katona, R. L. Wilder, C. C. Winter, B. Haraoui, I. Scher, and S. Wahl. 1984. Isolation of human mononuclear cell subsets by counter flow centrifugal elutriation (CCE). I. Characterization of B-lymphocyte, T-lymphocytes and monocyte-enriched fractions by flow cytometric analysis. *Cell. Immunol.* 85:373.
19. Goff, S., P. Traktman, and D. Baltimore. 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. *J. Virol.* 38:239.
20. Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. Capon, and M. A. Martin. 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J. Virol.* 66:139.
21. Lightfoote, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan. 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. *J. Virol.* 60:771.

22. Folks, T. M., S. Benn, A. Rabson, T. Theodore, M. D. Hoggan, M. A. Martin, M. Lightfoote, and K. W. Sell. 1985. Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. *Proc. Natl. Acad. Sci. USA.* 82:4539.
23. Benn, S., J. Rutledge, T. Folks, J. Gold, L. Baker, J. McCormick, P. Feorino, P. Piot, T. Quinn, and M. A. Martin. 1985. Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. *Science (Wash. DC).* 230:949.
24. Clavel, F., F. Guetard, F. Vézinet-Brun, S. Chamaret, M. A. Rey, M. O. Santos-Ferreira, A. G. Laurent, C. Dauge, C. Katlama, C. Rouzioux, D. Klatzmann, J. L. Champalimaud, and L. Montagnier. 1986. Isolation of a new human retrovirus from West African patients with AIDS. *Science (Wash. DC).* 233:343.
25. Munn, R. J., P. A. Marx, J. K. Yamamoto, and M. B. Gardner. 1985. Ultrastructural comparison of the retroviruses associated with human and simian acquired immunodeficiency syndromes. *Lab. Invest.* 53:194.
26. Meyenhofer, M. F., L. G. Epstein, E-C Cho, and L. R. Sharer. 1987. Ultrastructural morphology and intracellular production of human immunodeficiency virus (HIV) in brain. *J. Neuropath. Exp. Neurol.* 46:474.
27. Dahlberg, J. E., J. M. Gaskin, and K. Perk. 1981. Morphological and immunological comparison of caprine arthritis encephalitis and ovine progressive pneumonia viruses. *J. Virol.* 39:914.
28. Lairmore, M. D., G. Y. Akita, H. I. Russell, and J. C. DeMartini. 1987. Replication and cytopathic effects of ovine lentivirus strains in alveolar macrophages correlate with in vivo pathogenicity. *J. Virol.* 61:4038.