

Mononuclear phagocytes of blood and bone marrow: Comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections

M. J. McELRATH*, J. E. PRUETT, AND Z. A. COHN

The Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY 10021

Contributed by Z. A. Cohn, October 10, 1988

ABSTRACT We examined human immunodeficiency virus (HIV) production in cultured mononuclear cells from 36 seropositive homosexual males. Production was detected by using an HIV p24 antigen ELISA assay in blood mononuclear cells in 54% of asymptomatic, 71% of acquired immunodeficiency syndrome (AIDS)-related complex, and 100% of AIDS patients. When the peripheral blood mononuclear cells were separated into monocytes and CD4⁺ T cells, we found that the CD4⁺ T-cell fraction was preferentially infected in the three clinical stages. The ability to isolate HIV from blood monocyte-derived macrophages was similar in the three stages (24-33%) and required coculture with phytohemagglutinin-stimulated lymphoblasts. Bone marrow and blood mononuclear cells cultured simultaneously yielded virus from both sources in 13 individuals. Again the prime source of virus was the nonadherent bone marrow mononuclear cells, which contained CD4⁺ T cells, and not the adherent monocyte-enriched fraction. We conclude that blood mononuclear cells yield productive virus more readily as disease progresses and that infection is detected in association with CD4⁺ T-cell-enriched fractions. In our large sample of patients, monocyte infection was detected in only a small fraction, suggesting that this cell type is neither a primary nor an exclusive reservoir of HIV infection.

Human immunodeficiency virus (HIV) infection can remain clinically silent in man for months to years, but with time it can inexplicably lead to profound CD4⁺ T cell deficiency accompanied by opportunistic infections, neurologic and neoplastic disease, and eventually death. Throughout the various clinical stages, HIV can be isolated from culture supernatants of peripheral blood mononuclear cells (PBMC) in some but not all seropositive individuals (1, 2). HIV infects the human CD4⁺ T cell and in some cases has been reported to cause extensive lysis of these cells in culture (3, 4). In contrast, infection of blood monocyte-derived macrophages with exogenous virus appears to be noncytolytic (5) and persistent (6). In view of these findings and consistent with reports concerning lentivirus infection in animals, the human mononuclear phagocyte system has been implicated as a major reservoir for HIV infection, particularly during the latent disease period (7-9). However, a large clinical sample of patients has not been studied to determine the extent to which monocytes yield productive virus.

We have separated PBMC into fractions enriched in monocytes and CD4⁺ T cells from a group of 36 homosexual HIV-seropositive individuals in various stages of clinical infection. In 13 individuals, we have compared HIV production in peripheral blood with that in bone marrow mononuclear cells. Our results indicate that the ability to detect HIV infection in the CD4⁺ T cell fraction correlates with the severity of disease. Virus production by monocytes is not

detectable in the majority of patients, does not correlate with disease severity, and requires amplification with lymphoblast coculture.

MATERIALS AND METHODS

Patient Population. Thirty-six male homosexuals from the New York City area who previously tested HIV-1 antibody-positive by ELISA and Western blot analyses were recruited for study at the Rockefeller University Hospital. Informed consent was obtained for every procedure performed. Individuals using 3'-azido-3'-deoxythymidine (AZT) or experimental antiviral agents were not excluded from the study. Complete blood cell counts, prothrombin and partial thromboplastin times, and peripheral blood CD4⁺ and CD8⁺ T cell counts were performed on all participants upon entry in the study. Patients were classified into one of three clinical stages of HIV infection: (i) acquired immunodeficiency syndrome (AIDS) according to the revised case definition reported by the Centers for Disease Control (10); (ii) AIDS-related complex (ARC), with CD4⁺ T cell counts <250; (iii) asymptomatic, with CD4⁺ T cell counts >250. Many patients have been studied longitudinally and disease has progressed in some, but results are reported only once per patient.

Mononuclear Cell Isolation. *Peripheral blood.* Peripheral blood was collected by venipuncture into heparinized syringes, diluted 3:1 with sterile RPMI 1640 (GIBCO), layered over Ficoll-Paque (Pharmacia), and centrifuged at 1000 × g for 20 min at room temperature. The interface (containing PBMC) was washed twice in RPMI at 4°C by centrifugation at 650 × g for 10 min and cultured as described below. If cell counts were adequate, a portion of the PBMC was then layered over prespun continuous Percoll (Pharmacia) density gradients (11) and centrifuged at 1800 × g for 25 min at 4°C. The high- and low-density interfaces were collected separately, washed three times, and kept at 4°C for further enrichment.

Blood monocytes were found primarily in the low-density fraction and were further purified by a 1- to 2-hr adherence in R20 [RPMI containing 20% (vol/vol) HIV-seronegative A⁺ human serum, penicillin (100 units/ml), and streptomycin (0.01 mg/ml) (Sigma)] onto plastic 25-cm² flasks (Costar) or 12-mm glass coverslips (Propper Manufacturing, Long Island City, NY) in 16-mm 24-well plates (Costar), depending upon the adequacy of cell counts. Nonadherent cells were removed by aspiration with several washes immediately after adherence and the day after isolation. The adherent cells were carefully washed until nonadherent cells were no longer visualized by inverted phase microscopy.

High-density mononuclear cells containing primarily lymphocytes and dendritic cells were enriched for CD4⁺ T cells

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

*To whom reprint requests should be addressed.

by negative selection through panning. The cells were incubated with OKT8 (CD8; Ortho Diagnostics) and 3C10 (CD14; American Type Culture Collection) hybridoma supernatants on ice for 1 hr, washed by centrifugation, and panned onto bacteriologic Petri plates coated with goat anti-mouse immunoglobulin Fc fragment (Cappel Laboratories). The panning procedure was repeated at least twice or until cells adhering to the plate were no longer visualized by inverted microscopy. The cells negatively selected by this procedure were enriched CD4⁺ T cells. Further enrichment by incubating with 9.3F10 hybridoma supernatant (anti-major histocompatibility complex class II; American Type Culture Collection) along with OKT8 and 3C10 was performed in some samples to remove contaminating B and dendritic cells.

Purity of enriched monocyte and CD4⁺ T cell fractions was analyzed by immunofluorescence with anti-human leukocyte mouse monoclonal antibodies by using a FACScan (Becton Dickinson) flow cytometer. Cells (10⁶) suspended in 50 μ l of phosphate-buffered saline at pH 7.4 were reacted at 4°C for 30 min with 10 μ l of the following Becton Dickinson reagents: anti-Leu-3a (CD4) fluorescein isothiocyanate (FITC) conjugate, anti-Leu-2a (CD8) phycoerythrin (PE) conjugate, anti-Leu-M3 (CD14) PE conjugate, anti-leukocyte (CD45) FITC conjugate, mouse IgG1 FITC conjugate (control), and IgG2a PE conjugate (control). Cells were washed twice, fixed in 10% formalin for 20 min, and washed twice. Two-color contour plots were obtained. Anti-Leu-16 monoclonal antibody (CD20; Becton Dickinson) followed by goat anti-mouse FITC was used to determine B cell content in the mononuclear cell fractions.

Bone marrow. Ten milliliters of bone marrow fluid was aspirated from the iliac crest of consenting patients into a heparinized syringe. The cell suspension was diluted in RPMI, washed by centrifugation at 650 \times g for 10 min, and resuspended to a final volume of 20 ml with RPMI. Ten milliliters was layered over 4 ml of Ficoll-Paque (twice) and centrifuged at 1000 \times g for 20 min. The two interfaces were collected, combined, washed by centrifugation three times, and resuspended in R20. Depending upon cell yield, total bone marrow mononuclear cells only or in combination with adherent and nonadherent cell populations were cultured. Nonadherent cells were removed from adherent cells immediately after a 2-hr adherence and the day after isolation, and the two fractions were combined. In one experiment, T cells were separated by positive selection from other nonadherent cells by incubation with OKT8 and OKT3 (Ortho Diagnostics) hybridoma supernatants followed by panning as described above. In addition, the pellet from the Ficoll gradient was recovered, the erythrocytes were lysed with distilled water, and the remaining multinucleated cells were cocultured with lymphoblasts.

Culture System for HIV Production. Total PBMC, monocytes, and CD4⁺ T cells were cultured in 25-cm² flasks (Costar) (5 \times 10⁶ cells in 6 ml of R20) or in 16-mm wells (Costar) (5 \times 10⁵ cells in 1 ml of R20) in a 37°C, 5% CO₂/95% air incubator. Virus production was stimulated by 5% phytohemagglutinin (PHA) treatment for 72 hr or by coculture with 48–72 hr PHA-treated PBMC from seronegative donors (5 \times 10⁶ per flask, 5 \times 10⁵ cells per well). Recombinant human interleukin 2 (400–800 units/ml, NEN) or 10% human T-cell-conditioned medium containing interleukin 2 was added to the R20. One-half of the culture medium was removed twice a week and replaced with fresh R20 containing interleukin 2 with or without PHA-stimulated lymphoblasts. Cultures were maintained until virus production was detected or for 28–30 days.

Detection of HIV Production. Viral concentration. To concentrate HIV from tissue culture supernatants, 2 ml of cell culture supernatants were incubated with 30% (wt/vol) polyethylene glycol 8000 (Sigma) on ice for 2 hr and centri-

Table 1. Production of HIV from PBMC cultures of 36 seropositive homosexual males

Clinical status	Positive cultures, no.	Patients tested	Positive cultures, %
AIDS	7	7	100
ARC	5	7	71
Asymptomatic	12	22	54

Cultures included total mononuclear cells, enriched CD4⁺ T cells, and monocyte-derived macrophages. Virus production was determined by the presence of HIV p24 antigen in the culture supernatant.

fuged at 600 \times g for 20 min. The supernatant was removed, and the hard pellet was resuspended by vortexing in R20 containing 0.5% Triton X-100. The resulting concentrate was used for the ELISA assay.

HIV p24 ELISA assay. The DuPont HIV p24 ELISA system (NEN) was used to detect viral production in culture supernatants. Concentrated culture supernatants (200 μ l) were run in duplicate along with known positive (HIV-infected H9 and CEMT4 cell lines) and negative (HIV-seronegative cultured mononuclear cells) control supernatants. Optical density, which correlates linearly with HIV p24 antigen captured, was measured at 490 nm against a reference filter of 630 nm with a Dynatech MR700 microplate reader and was compared to that of inactivated viral lysate standards. Values obtained that were <0.01 ng/ml are reported as negative.

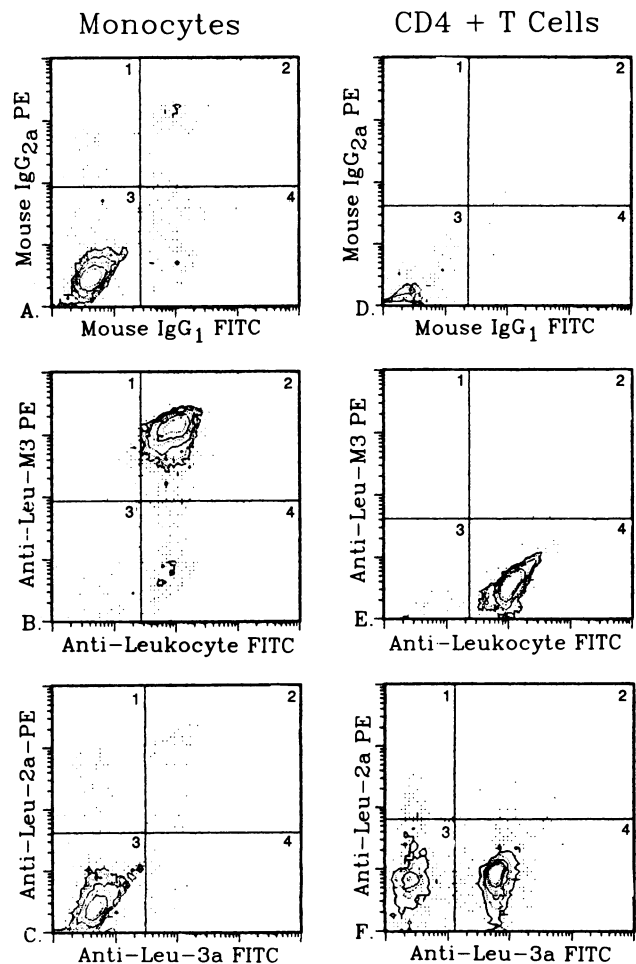


FIG. 1. Flow cytometry of enriched monocyte and CD4⁺ T cell populations. Monocyte (A–C)- and CD4⁺ T cell (D–F)-enriched populations were reacted with fluorescent-labeled mouse anti-human leukocyte monoclonal antibody.

Table 2. Summary of isolation of HIV from enriched mononuclear cell populations in peripheral blood of seropositive patients

Clinical status	Positive cultures/patients tested (% positive)		
	PBMC	Monocytes	CD4 ⁺ T cells
AIDS	5/5 (100)	2/6 (33)	6/6 (100)
ARC	5/6 (83)	1/5 (20)	3/4 (75)
Asymptomatic	5/19 (26)	5/21 (24)	10/20 (50)

RESULTS

Virus Production in PBMC at Different Stages of HIV-1 Infection. HIV production from mononuclear cells of peripheral blood (total, monocytes, and CD4⁺ T cells) was detected in all clinical groups, as shown in Table 1. Productive infection was more common as disease progressed; it varied from 54% in the asymptomatic, 71% in the ARC, and 100% in the AIDS group. Of those individuals using 3'-azido-3'-deoxythymidine, five of five with AIDS, one of two with ARC, and one of two asymptomatic seropositives had HIV-positive mononuclear cell cultures.

Separation and Purity of Peripheral Blood Mononuclear Cells. To establish the specific cell type(s) infected with HIV, we further purified the blood mononuclear cells into two enriched populations, monocytes and CD4⁺ T cells. The purity of each was evaluated by flow cytometry with fluorescent-labeled monoclonal antibodies against T cell subset and monocyte surface markers. Typical two-color channel plots are shown in Fig. 1 for monocyte and CD4⁺ T cell-enriched populations in an asymptomatic patient. Nonspecific staining with mouse immunoglobulin was negligible for both fractions (Fig. 1 A and D). The monocyte fraction was 95% Leu-M3-positive (Fig. 1B), and <1% stained with either anti-Leu-3a or anti-Leu-2a (Fig. 1C). The CD4⁺ T cell-enriched fraction failed to stain with anti-Leu-M3 (Fig. 1E), and <1% stained with anti-Leu-2a (Fig. 1F). Anti-Leu-3a (CD4) staining increased from 39% in the PBMC to 79% in the CD4⁺ T-cell-enriched fraction (Fig. 1F). The CD4⁺ T cells were primarily contaminated with anti-Leu-16⁺ B cells, which could be removed by incubation with 9.3F10 along with OKT8 and 3C10 hybridoma supernatants before panning (data not shown).

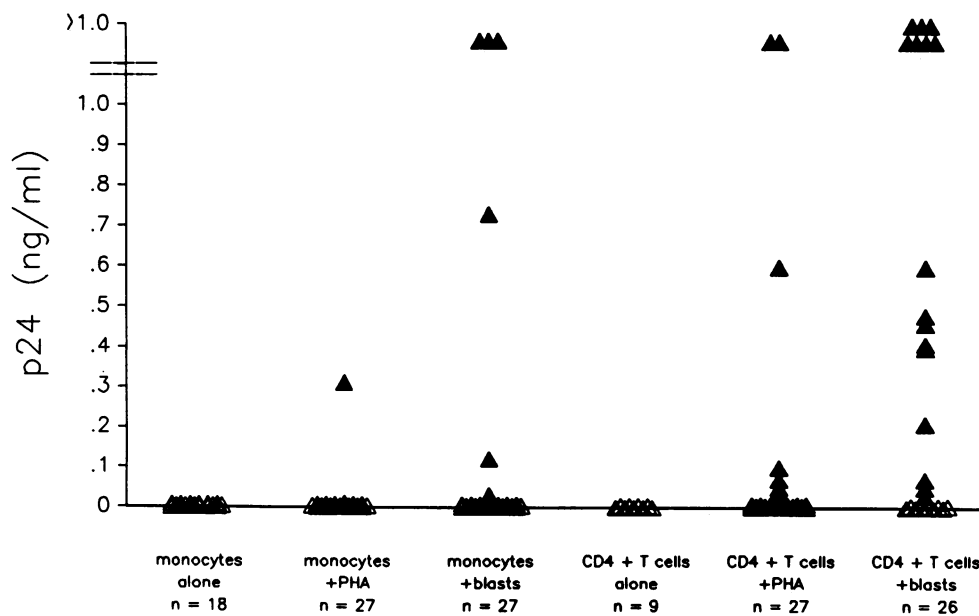


FIG. 2. Expression of p24 antigen in cultures of monocytes and CD4⁺ T cells. blasts, Lymphoblasts.

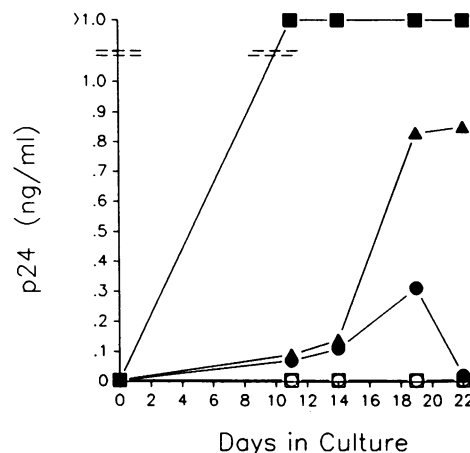


FIG. 3. Temporal expression of p24 antigen in cultures of blood monocyte-derived macrophages and CD4⁺ T cells from an asymptomatic individual. Virus was not detected in unstimulated cultures (○, □). HIV p24 levels were greater in CD4⁺ T cell cultures but were detectable in both macrophages plus lymphoblasts (▲) and CD4⁺ plus PHA (■) within 11 days. ●, Macrophages plus PHA.

Virus Production in Monocytes and CD4⁺ T Cells from Blood. As shown in Table 2, HIV could be isolated from monocytes and CD4⁺ T cells in some individuals from all three clinical groups. The CD4⁺ T cell-enriched cultures were most likely to contain active viral production of the three populations tested (total PBMC, monocytes, and CD4⁺ T cells). Virus was produced in the CD4⁺ T cell fraction preferentially over the total PBMC in the asymptomatic group (Table 2) and over the monocytes in all three groups (Fig. 2). We were unable to detect virus production in resting CD4⁺ T cell cultures from nine individuals tested, but upon stimulation with PHA or coculture with PHA-stimulated lymphoblasts, four of nine CD4⁺ T cell cultures produced HIV.

In contrast, one-third or less of the blood monocyte-derived macrophage cultures demonstrated HIV productive infection in the three clinical groups tested (Table 2). HIV production in blood monocyte-derived macrophages could only be detected by coculture with stimulated lymphoblasts or in one case by treatment with PHA alone. Monocytes were

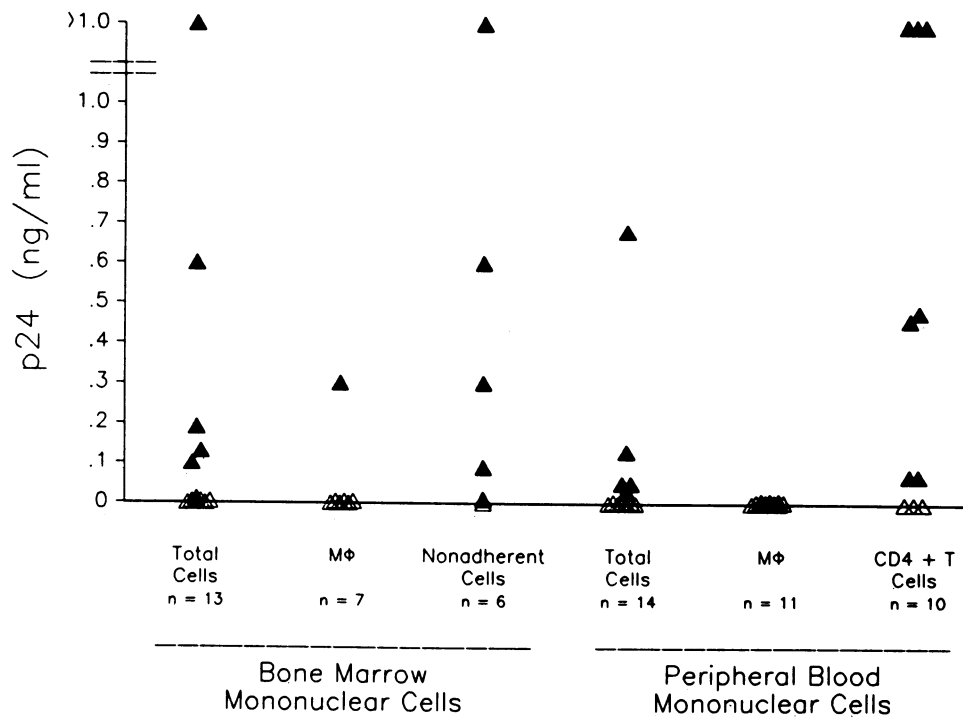


FIG. 4. Comparison of HIV infection in bone marrow and PBMC of seropositive individuals cocultured with PHA-stimulated lymphoblasts. M ϕ , Macrophages.

exclusively infected in only one individual, and upon repeat examination both monocytes and CD4⁺ T cell fractions were infected. In eight patients we found virus production in both monocyte and CD4⁺ T cell populations, an example of which is shown in Fig. 3. The levels of viral production in the CD4⁺ T cell-enriched cultures were consistently higher than in the monocyte-enriched cultures.

Virus Isolation from Bone Marrow Mononuclear Cells. We obtained simultaneous blood and bone marrow aspirates from 13 individuals, and a comparison of the ability of the mononuclear cells from each source to produce HIV in culture is shown in Fig. 4. With one exception (patient 33), HIV production, when found in peripheral blood mononuclear cells, was also found in bone marrow mononuclear cells, but the levels of HIV p24 antigen in the culture supernatants were generally not comparable. We found HIV production in the nonadherent rather than the adherent fraction when tested in four of five cases (Fig. 4). We repeated the bone marrow aspirate in the exceptional case and again found infection in both nonadherent and adherent cells, as shown in Fig. 5. Upon separation of the nonadherent population into OKT3⁺, OKT8⁺ T cells and the OKT3⁻, OKT8⁻ population, which likely represent monocyte precursors, we found that the T cells alone (OKT3⁺, OKT8⁺) produced HIV within 15 days of culture (Fig. 5). The CD4⁺/CD8⁺ T cell ratio in both blood and bone marrow of this patient was 0.15 as determined by FACScan analysis. We were not able to detect virus in the multinucleated cell pellet, which likely contained polymorphonuclear leukocytes and megakaryocytes.

DISCUSSION

To determine early cellular events that may contribute to disease progression, we have examined the cellular tropism of HIV in a large group of patients in various stages of infection. We found that blood mononuclear cells from seropositive individuals are more likely to produce virus as disease progresses (Table 1) and that the primary source of virus is from a CD4⁺ T cell-enriched fraction (Table 2). Our

data do not implicate the monocyte as a primary or exclusive source of productive HIV infection in any stage of infection because we were only able to demonstrate blood monocyte infection in less than one-third of the patients tested (Table 2), nor were we able to demonstrate the bone marrow macrophage as a rich source of HIV productive infection (Fig. 4).

Recent studies (7-9), in which HIV has been found in mononuclear phagocytes from blood, brain, lung washings, and bone marrow aspirates, have suggested that macrophages may serve as a reservoir for virus and as a vehicle for viral dissemination. These studies were based upon relatively small clinical samples, and extensive efforts to remove all contaminating T cells were not reported. We suggest caution in attributing monocytes as an endogenous source of HIV infection unless every effort to remove other cellular contaminants has been made. Furthermore, the ability to infect blood monocytes *in vitro* does not necessarily correlate with

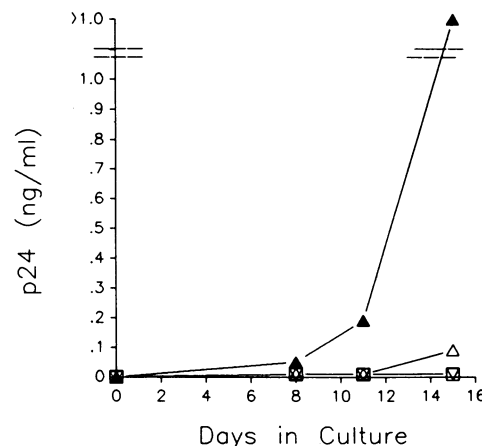


FIG. 5. Bone marrow cells from an AIDS patient were cultured with PHA-stimulated lymphoblasts and assessed for productive viral infection. ▲, OKT3⁺, OKT8⁺ nonadherent cells; △, macrophages; ▽, OKT3⁻, OKT8⁻ nonadherent cells; □, multinucleated cells.

the likelihood that these cells are infected *in vivo* or that they represent a rich source of HIV infection in the human host.

We cannot exclude the possibility that the mononuclear phagocytes we have examined are latently but not productively infected. Conceivably the macrophage through its accessory cell function may transfer otherwise nonproductive HIV infection to CD4⁺ T cells through cell-to-cell contact. This situation is approximated by the coculture system with allogeneic PHA-stimulated lymphoblasts, and, as shown in Figs. 2 and 3, HIV replication (when found) was most likely to be detected by the coculture system. In addition, virus may be produced in such low levels that it is undetectable by our assay. To ensure that low levels of virus production could be detected, we chose an ELISA that is specific for HIV and in our hands more sensitive for the detection of HIV production than the standard reverse transcriptase assay. More sensitive methods to detect HIV infection in the macrophage that also circumvent prolonged *in vitro* culture, such as DNA amplification (12, 13), will be valuable in understanding the pathogenesis of HIV infection.

We found the CD4⁺ T cell-enriched population preferentially infected in our study population. In our culture system, the CD4⁺ T cell is stimulated to divide through either a mitogenic or mixed leukocyte response, which favors productive infection over resting CD4⁺ T cells and possibly in preference to other cell types, particularly monocyte-derived macrophages. The CD4⁺ T cell-enriched fraction may also contain B and dendritic cells, which may be additional potential sources of HIV infection.

We are indebted to Dr. Ralph Steinman for helpful discussions concerning the pathogenesis of HIV infection, to Judy Adams for preparation of the figures, and to Dr. John Jacobs at New York Hospital, Cornell University Medical Center, for patient referrals. This work was supported by the Aaron Diamond Foundation, National Institutes of Health Grant US PHS-AI-24775-01, and in part by National Institutes of Health General Clinical Research Grant

MO1-RR00102. M.J.M. is an Irvington House Medical Research Fellow.

1. Salahuddin, S. Z., Markham, P. D., Popovic, M., Sarngadharan, M. G., Orndorff, S., Fladagar, A., Patel, A., Gold, J. & Gallo, R. G. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5530–5534.
2. Levy, J. A. & Shimabukuro, J. (1985) *J. Infect. Dis.* **152**, 734–738.
3. Klatzmann, D., Barre-Sinoussi, F., Nugeyre, T., Dauget, C., Vilmer, E., Griscelli, C., Brun-Vezinet, F., Rouzioux, C., Gluckman, J. C., Chermann, J. C. & Montagnier, L. (1984) *Science* **225**, 59–63.
4. McDougal, S., Mawle, A., Cort, S. P., Nicholson, J. K. A., Cross, D., Scheppler-Campbell, J. A., Hicks, D. & Sligh, J. (1985) *J. Immunol.* **135**, 3151–3162.
5. Nicholson, J. K. A., Cross, G. D., Callaway, C. S. & McDougal, J. S. (1986) *J. Immunol.* **137**, 323–329.
6. Gendelman, H. E., Orenstein, J. M., Martin, M. A., Fernea, C., Mitra, R., Phipps, T., Wahl, L. A., Lane, C. L., Fauci, A. S., Burke, D. S., Skillman, D. & Meltzer, M. S. (1988) *J. Exp. Med.* **167**, 1428–1441.
7. Gartner, S., Markovits, P., Markovitz, D. M., Kaplan, M. H., Gallo, R. C. & Popovic, M. (1986) *Science* **233**, 215–219.
8. Ho, D. D., Rota, T. R. & Hirsch, M. S. (1986) *J. Clin. Invest.* **77**, 1712–1715.
9. Koenig, S., Gendelman, H. E., Orenstein, J. M., DalCanto, M. C., Pezeshkpour, G. H., Yungbluth, M., Janotta, F., Ak-samit, A., Martin, M. A. & Fauci, A. S. (1986) *Science* **233**, 1089–1093.
10. Centers for Disease Control (1987) *Morbid. Mortal. Wkly. Rep. Suppl.* **36**, 3–9.
11. Wright, S. D. & Silverstein, S. C. (1982) *J. Exp. Med.* **156**, 1149–1164.
12. Kwok, S., Mack, D. H., Mullis, K. B., Poiesz, B., Ehrlich, G., Blair, D., Freidman-Klein, A. & Sninsky, J. J. (1987) *J. Virol.* **61**, 1690–1694.
13. Ou, C.-Y., Kwok, S., Mitchell, S. W., Mack, D. H., Sninsky, J. J., Krebs, J. W., Feorino, P., Warfield, D. & Schochetman, G. (1988) *Science* **239**, 295–297.