Productive Human Immunodeficiency Virus Type 1 (HIV-1) Infection of Nonproliferating Human Monocytes

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Summary

Human immunodeficiency virus type 1 (HIV-1) infection of T lymphocytes requires cellular proliferation and DNA synthesis. Human monocytes were shown to have low DNA synthesis rates, yet the monocytotropic BaL isolate of HIV-1 was able to infect these cells efficiently. Monocytes that were irradiated to assure no DNA synthesis could also be readily infected with HIV-1_{BaL}. Such infections were associated with the integration of HIV-1_{BaL} DNA into the high molecular weight, chromosomal DNA of monocytes. Thus, normal, nonproliferating monocytes differ from T lymphocytes in that a productive HIV-1 infection can occur independently of cellular DNA synthesis. These results suggest that normal nonproliferating mononuclear phagocytes, which are relatively resistant to the destructive effects of this virus, may serve as persistent and productive reservoirs for HIV-1 in vivo.

Productive infection of cells by retroviruses generally requires cellular proliferation and DNA synthesis for integration of viral nucleic acid, and synthesis of new virions (1–3). Productive infection of T lymphocytes with HIV-1, the pathogenic agent of AIDS, likewise requires cellular proliferation and DNA synthesis (4–7). HIV-1 was demonstrated initially to infect and destroy CD4-bearing T lymphocytes (8, 9). Subsequently, however, it has been demonstrated that several strains (isolates) of HIV-1 can also infect mononuclear phagocytes (monocytes and macrophages) in vitro with high efficiency (10–12). Unlike the bulk of infected T lymphocytes that are rapidly killed by HIV-1, infected mononuclear phagocytes survive for weeks in in vitro culture (11, 13, 14) and persist in tissues in vivo (11, 12). Because tissue macrophages and blood monocytes are generally nonproliferating cells and monocytes synthesize little or no DNA in in vitro culture (15, 16), we set out to determine the requirement for monocyte proliferation/DNA synthesis for productive HIV-1 infection in vitro.

Materials and Methods

Cell Isolation. Blood was removed from normal, anti-HIV-1 antibody-negative individuals after appropriate informed consent, and normal monocytes (of ~98% purity) were prepared as noted earlier (16). PBL were isolated from normal donors by gradients using Ficoll/sodium diatriazoate and then cultured for 3 d in PHA (5 μg/ml). 3 d after isolation and culture, the lymphocytes were washed three times and placed into chambers of microtiter plates (10^5/chamber) with rIL-2 (20 U/ml; Cetus Corp., Emeryville, CA). All cultures were in DME of low endotoxin content (17) with autologous, unheated 10% serum.

Viruses and Virus-related Assays. The following HIV-1 strains were used: BaL, IIIB, D.U.7887-8, and D.U.6587-7 (12, 18). BaL, D.U.7887-8, and D.U.6587-7 are monocytotropic strains, and IIIB is a lymphocytotropic strain. The cells were inoculated with multiplicity of infections (MOI)[1] of ~0.1–0.01. The micro-reverse transcriptase (RT) assay and the p24 ELISA (DuPont Co., Wilmington, DE) were done as described before (18).

Thymidine Incorporation, Autoradiography, and Immunofluorescence. After 3 d in culture, methyl-[^{3}H]thymidine (2 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to the cultures (1 μCi/chamber in microtiter plates, and 3.5 μCi/chamber on LabTek slides [Miles Scientific, Naperville, IL]). After a further 18 h, microtiter plates were harvested using an multiple automated sample harvester, depositing radioactive thymidine insoluble in 10% TCA onto glass fiber paper. Cells on LabTek slides were processed as described before for autoradiography (16). In some experiments, monocytes were irradiated with 3,000 cGy from a Shepherd ^{137}Cs irradiator, before they were plated. In immunofluorescence experiments, monocytes in LabTek slides were inoculated with HIV-1_{BaL} (MOI

[1] *Abbreviations used in this paper:* GM-CSF, granulocyte/macrophage colony-stimulating factor; LTR, long terminal repeat; MOI, multiplicity of infection; RT, reverse transcriptase.

~0.1) and cultured for 14 d. Cells were then fixed with cold acetone, and stained with a mouse mAb against HIV-1 p24 antigen using indirect fluorescent techniques.

DNA Analyses. Cells were washed with PBS, and total DNA was isolated by lysis of the culture with 0.5% SDS, digestion of the lysate overnight at 37°C with proteinase K, repeated (at least six) phenol/chloroform extractions, RNase A treatment, and ethanol precipitation. Total DNAs were subjected to electrophoresis through a 0.8% agarose gel and examined by Southern blot analysis. The filter was hybridized initially with a viral-specific probe derived from *nef* and U3/R regions of the 3' long terminal repeat (LTR) (HXB-3 isolate of HIV-1 [19]) and subsequently with a γ actin probe. In other experiments, high molecular weight DNA was purified after extended electrophoresis through a 1% low melting point agarose gel. These preparations, as well as unfractionated total DNAs, were digested extensively with EcoRI, subjected to electrophoresis through a 1.1% agarose gel, transferred to a filter, and hybridized first with *nef*/LTR probe and second with an internal probe that recognizes the 3' end of *pol*, *vif*, and the 5' end of *vpr*.

Results and Discussion

As previously reported, HIV-1_{BaL} efficiently infected monocytes from normal individuals (Fig. 1). The level of HIV-1 infection and expression in monocytes was as high (or higher) than that seen in PBL cultured with PHA and IL-2. In general, viral RT appeared in supernatants of lymphocyte cultures more rapidly than it did in those of monocytes, while RT in monocyte culture supernatants appeared later and stayed higher for a more prolonged period. All of the tested strains efficiently infected lymphocytes with approximately the same kinetics. The monocytotropic strain HIV-1_{BaL} produced the highest levels of supernatant RT in monocyte cultures. In addition to strain BaL, monocytes reproducibly could be infected with the field isolates D.U. 7887-8 (Fig. 1) and D.U. 6587-7 (data not shown). The levels of monocyte infection with strain IIIB were variable; in some experiments, as shown in Fig. 1, IIIB produced low or moderate RT levels, but in others inoculation of monocytes with IIIB produced no evidence of infection.

While some investigators have shown that incubation of the monocytes with granulocyte/macrophage CSF (GM-CSF) or M-CSF (two growth factors that can cause proliferation of immature monocytic cells [20]) will enhance infection with HIV-1 of monocytes (13, 21), others have noted that human monocytes can be infected with HIV-1 without the need for added growth factors (10, 22, 23). In our experiments, inclusion of GM-CSF (Amgen, Thousand Oaks, CA) or M-CSF (Cetus Corp.) (10–1,000 U/ml) throughout the cultures did not appreciably modify HIV-1_{BaL} infection of monocytes. For example, in one typical experiment, supernatant medium from control infected monocytes on day 21 had a p24 level of 875 ng/ml above background, whereas that of infected monocytes with either 1,000 U/ml M-CSF or 1,000 U/ml GM-CSF had 485 and 710 ng/ml, respectively. The monocytes, however, were clearly responsive to cytokines; both GM-CSF and M-CSF caused increased spreading of the monocytes and an increase in the ability of the cells to produce hydrogen peroxide in response to PMA (data not shown).

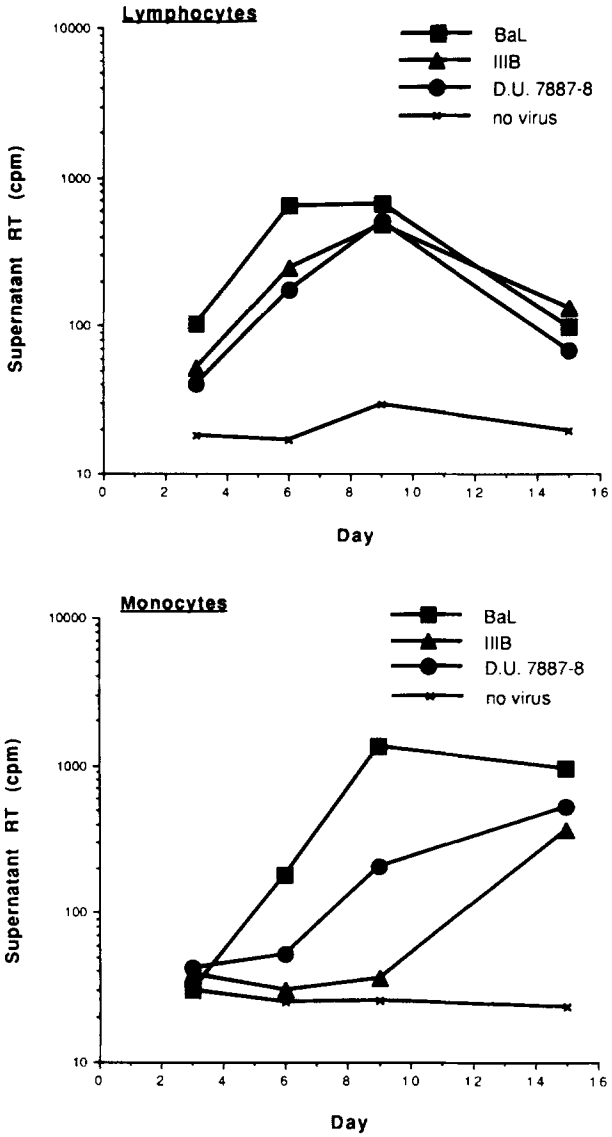

Figure 1. Infection of PBL and monocytes with different strains of HIV-1. PBL were cultured for 3 d in PHA (5 μg/ml), washed three times, and placed into chambers of microtiter plates (10^5/chamber) with rIL-2 (20 U/ml). These lymphocytes and monocytes were inoculated with HIV-1, strains BaL, IIIB, and the field isolate D.U. 7887-8 (MOI ~0.01). Supernatant samples were collected at the indicated times and assayed for RT activity. Each point represents the mean from triplicate samples. Comparable results were obtained in a total of three experiments.

Adherent monocytes synthesized very little DNA when compared to an actively dividing transformed cell line. To assure absolutely no monocyte DNA synthesis, some cells were irradiated. Thymidine incorporation quantitation showed 66.7 ± 8.5 × 10^3 cpm (mean ± SEM of triplicate samples) for mouse fibrosarcoma L929 cells (labeling index [Li] ~80% by autoradiography), 2.4 ± 0.4 × 10^3 cpm for nonirradiated monocytes (LI ~$1/10^3$), and 0.4 ± 1 × 10^3 cpm for irradiated monocytes (LI ~0). Despite the irradiation, the monocytes survived and appeared morphologically similar to nonirradiated cells for up to 21 d in culture. RT and p24

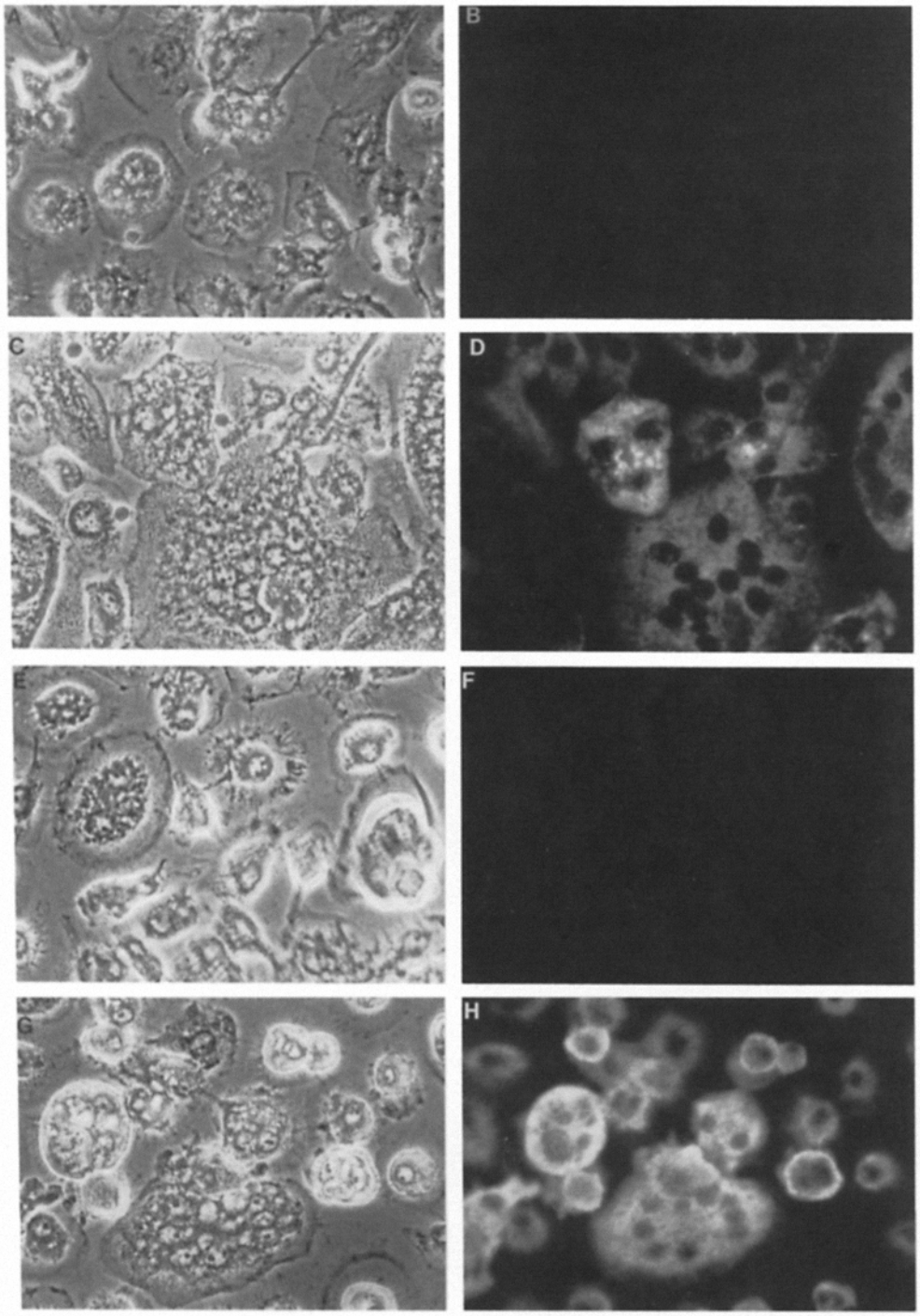

Figure 2. Demonstration of HIV-1_{BaL} p24 in infected nonirradiated and irradiated monocytes by indirect immunofluorescence. (*A*, *C*, *E*, and *G*) Phase microscopy; (*B*, *D*, *F*, and *H*) fluorescent microscopy of the same fields. (*A*, *B*, *E*, and *F*). Noninoculated monocytes; (*C*, *D*, *G*, and *H*) inoculated with HIV-1_{BaL}. (*A–D*) Nonirradiated monocytes; (*E–H*) irradiated monocytes. By phase microscopy, both nonirradiated and irradiated cells inoculated with HIV-1_{BaL} have multinucleated cells present (*C* and *G*), and 80–100% of both mononuclear and multinucleated cells in the inoculated, nonirradiated, and irradiated cultures contain cytoplasmic (but not nuclear) p24 antigen (*D* and *H*). Cultures not inoculated with HIV-1_{BaL} are negative for the p24 antigen (*B* and *F*). The original magnification of the photographs was 400.

antigen were detectable in the supernatant medium by 4–7 d after inoculation with HIV-1_{BaL}, reaching a maximum at 8–21 d after inoculation. Using a mouse anti-HIV-1 p24 mAb, immunofluorescence studies of fixed monocytes 14 d after HIV-1 infection demonstrated that 80–100% of the nonirradiated and irradiated cells expressed HIV-1 p24 antigen (Fig. 2). Many of the irradiated monocytes (like nonirradiated monocytes) inoculated with HIV-1_{BaL} were multinucleated, indicating that the formation of multinucleated cells was also not dependent on cellular DNA synthesis. In general, at high levels of virus inoculation, comparably high levels of p24 Gag were released by both irradiated and nonirradiated cells: for example, in a typical experiment, 83 ng/ml (irradiated) and 77 ng/ml (nonirradiated) at 7 d after inoculation, and 247 ng/ml (irradiated) and 86 ng/ml (nonirradiated) at 14 d after inoculation. Likewise, comparably high viral RT levels were noted in supernatant media of irradiated and nonirradiated cells: for example, 3,163 cpm (irradiated) and 2,435 cpm (nonirradiated) above background at 7 d after inoculation, and 11,348 cpm (irradiated) and 6,558 cpm (nonirradiated) above back-

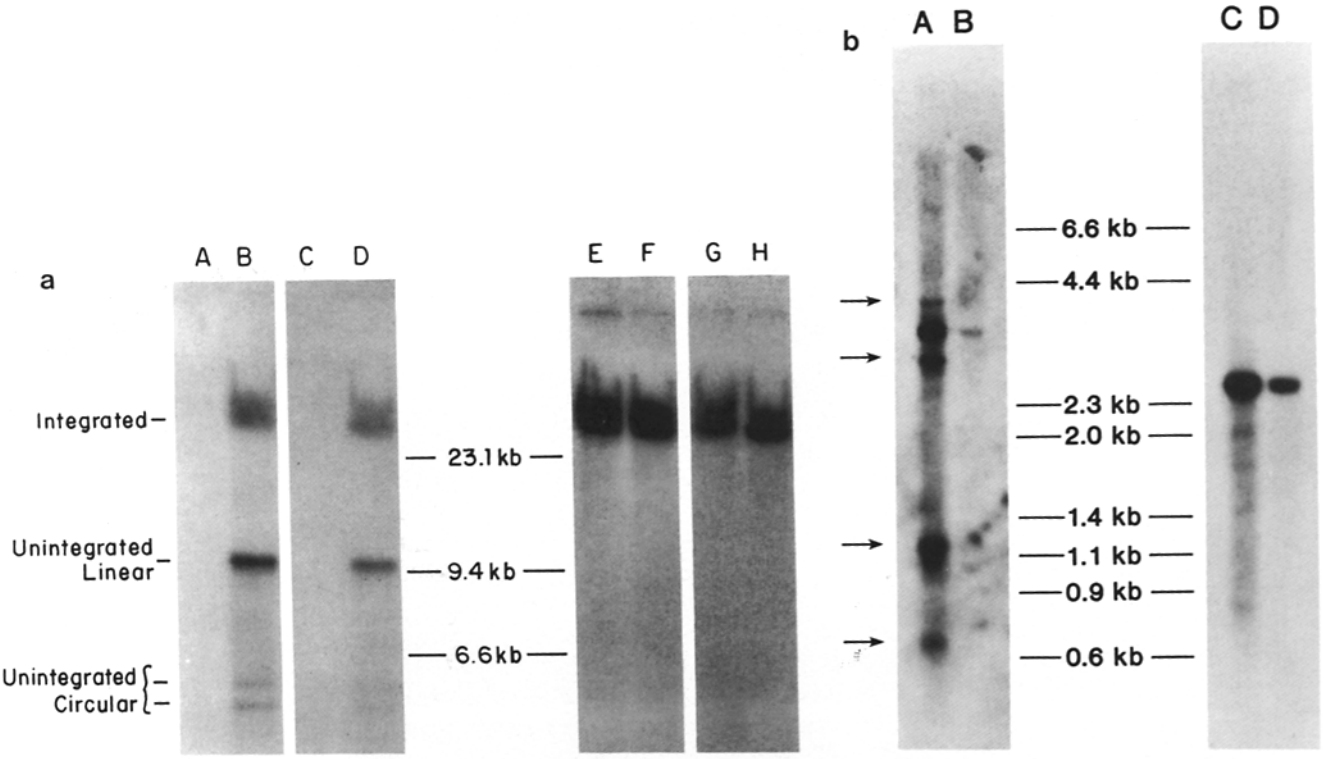

Figure 3. Southern blot analysis of DNA isolated from nonirradiated and irradiated monocytes after infection with HIV-1_{BaL}. Nonirradiated (lanes *A* and *B*) or irradiated (3,000 cGy; lanes *C* and *D*) monocytes were cultured adherent to 35-mm diameter culture dishes for 7 d with (lanes *B* and *D*) or without (lanes *A* and *C*) inoculation with HIV-1_{BaL} (MOI ~0.1) on day 0. (*a*) Southern blot analysis of total DNAs. The filter was hybridized initially with a viral-specific probe encompassing *nef* and U3/R regions of the 3' LTR (lanes *A–D*), and subsequently with a γ-actin probe (lanes *E–H*; these lanes correspond to lanes *A–D*, respectively). (*b*) Southern blot analysis of EcoRI-digested total (lanes *A* and *C*) and high molecular weight (lanes *B* and *D*) DNA. The filter was hybridized first with the *nef*/LTR probe (lanes *A* and *B*) and second with an internal probe specific for *pol*, *vif*, and *vpr* (lanes *C* and *D*). The four most intensely hybridizing HIV-1-specific bands (~4.3, 3.1, 1.2, and 0.7 kb) that are present in the unfractionated DNA (lane *A*), but completely absent from the high molecular weight DNA (lane *B*), are indicated by arrows. λ and ØX174 DNAs digested with HindIII and HaeIII, respectively, were used as molecular weight markers.

ground at 14 d after inoculation. Cell-free supernatants from HIV-inoculated irradiated or nonirradiated monocytes both contained infectious virus, as indicated by their abilities to productively infect fresh monocytes (RT levels of 5,790 and 7,471 cpm above background, respectively, at 14 d after inoculation).

To determine if the genomic DNA of nonirradiated and irradiated monocytes contained integrated HIV-1_{BaL} DNA, total DNA prepared from both infected and mock-infected cells that had been cultured for 7 d were subjected to Southern blot analysis (Fig. 3 *a*). As expected, DNA isolated from mock-infected cells did not hybridize to the HIV-1-specific probe (lanes *A* and *C*). In contrast, at least three distinct classes of viral DNA were identified in the DNA from both irradiated and nonirradiated cells that had been infected with HIV-1_{BaL} (lanes *B* and *D*). These classes displayed the electrophoretic mobilities predicted for high molecular weight chromosomal DNA of monocytes containing integrated proviruses; unintegrated linear viral DNA (~9.7 kb); and a doublet of unintegrated circular viral DNAs, presumably representing one- and two-LTR circles. Rehybridization of the filter with a γ-actin probe (lanes *E–H*) suggested that the slowest migrating band was the high molecular weight DNA of monocytes. Others have noted that cytoplasmic forms of high molecular weight concatamers of HIV-1 DNA (defective "self-integrant" multimers) may exist in some chronically infected monocyte-like U937 cell line cells (24). However, in our analysis, which was performed after acute HIV-1 infection of a primary monocyte culture, no bands intermediate in size between the unintegrated linear HIV-1 DNA and high molecular weight DNA (corresponding to the macrophage γ-actin band) were noted. We therefore suggest that the high molecular weight HIV-1 DNA was unlikely to be composed of multimers of unintegrated viral DNA.

However, to further address the possibility that unintegrated HIV-1 DNA of any form might be noncovalently associated with (or trapped in) monocyte chromosomal DNA, the high molecular weight DNA of infected monocytes (Fig. 3 *a*, lane *B*) was separated from the faster-migrating DNA species by prolonged electrophoresis. The high molecular weight DNA, as well as an aliquot of the total DNA starting material (that used in Fig. 3 *a*, lane *B*), was then digested with the restriction enzyme EcoRI and examined by Southern blot analysis (Fig. 3 *b*). We predicted that DNA fragments derived from

the termini of unintegrated linear viral DNA species would be detected with an LTR-specific HIV-1 probe, whereas those from integrated proviruses would not. We reasoned this because the distances from the 5′ and 3′ termini of the unintegrated full-length linear viral DNA to the proximal internal viral EcoRI sites are essentially constant (and therefore would be detectable by this type of analysis), whereas the distances from these internal EcoRI sites to the EcoRI sites to the EcoRI sites in monocyte DNA adjacent to the integration sites are variable due to the heterogeneous nature of integration sites (and therefore would be undetectable by this type of analysis). Hybridization of the filter with a probe specific for the viral termini (the *nef*/LTR probe) revealed at least four bands (the more prominent of which are indicated by arrows) to be present in the total DNA (lane *A*) but absent from the high molecular weight DNA (lane *B*). We believe that these fragments are derived from linear and circular (one-LTR and two-LTR forms) unintegrated extrachromosomal viral DNA species. Because these experiments were performed with a nonclonal viral proof of HIV-1_{BaL}, the precise assignment of bands to regions of the virus is difficult. Nevertheless, we preliminarily consider the bands of 4.3 and 3.7 kb to represent various circular forms, the bands of 1.2 and 0.7 kb to represent the termini of linear DNA, and the band at 3.1 kb to contain species derived from both circular and linear forms. We speculate that the single band in lane *B* is derived from a restriction fragment contained entirely within the viral DNA that, by chance, comigrates with a DNA fragment from unintegrated species (lane *A*). The detection of the HIV-1 termini almost exclusively in the total DNA sample (material from cells containing both integrated and unintegrated HIV-1 DNA) indicates that the viral DNA present in the high molecular weight DNA of infected monocytes is covalently integrated into host cell genomic DNA. Rehybridization of this same filter with a probe derived from the *pol*, *vif*, and *vpr* genes (genes internal to the LTRs) revealed a relatively intense unique band in both samples, thus indicating the presence of significant amounts of HIV-1 DNA (either integrated or unintegrated) in each sample (lanes *C* and *D*). This signifies that the bands noted in lane *A* would have been detected in lane *B* if they had been present.

Cells of $CD4^+$ human T cell lines and normal human lymphocytes must be proliferating before they can be productively infected by HIV-1 (4–7). The virus enters nondividing T cells, and HIV-1 DNA synthesis is initiated. However, reverse transcription is not complete in quiescent cells and the infection is abortive unless the T cells are activated by mitogenic stimulation (5). Koyanagi et al. (21) demonstrated low viral expression by infected monocytes and low thymidine incorporation into monocytes not treated with GM-CSF, M-CSF, or IL-3; however, monocytes treated with these hematopoietic growth factors had increased thymidine incorporation and increased expression of viral p24 antigen (21), thus raising the possibility that efficient infection of mononuclear phagocytes is associated with DNA replication. In our system, productive infection of monocytes with HIV-1_{BaL} was found, however, to occur in the absence of significant DNA synthesis and was not enhanced by addition of GM-CSF or M-CSF. Other investigators have shown that visna virus, a related lentiviral pathogen of sheep, can infect populations of macrophages with low [^{3}H]thymidine labeling indices (25), and it has been suggested that this property may correlate with the proposed ability of visna virus to replicate without a requirement for viral DNA integration into cellular chromosomal DNA (26). However, we show here that HIV-1 replication takes place in nonproliferating monocytes and is associated with chromosomal DNA integration.

Mononuclear phagocytes are distributed widely throughout the body (e.g., in skin, brain, lung, lymph nodes, spleen, liver, kidneys, and gonads [15]). These phagocytes, despite being primarily nondividing cells, can serve as targets for HIV-1 infection, and then act as long-lived, nonproliferating reservoirs of the virus.

We thank T. S. Wortham, A. Faust, T. F. Greenwell, and R. J. Oakey for excellent technical assistance and advice.

This work was supported in part by the Veterans Affairs Medical Research Service and Research Center of AIDS and HIV, the James Swiger Hematology Research Fund, and National Institutes of Health grants CA-09307, AR-30162 AI-23308, and AI-30411.

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Received for publication 28 May 1991 and in revised form 5 August 1991.

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