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High Viral Load in Lymph Nodes and Latent Human Immunodeficiency Virus (HIV) in Peripheral Blood Cells of HIV-1-Infected Chimpanzees

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We have examined human immunodeficiency virus type 1 (HIV-1) infection in chimpanzees by analyzing HIV-1 DNA and RNA in lymph nodes and peripheral mononuclear cells (PBMCs). Like certain asymptomatic HIV-infected persons, these chimpanzees had no detectable viral replication in their PBMCs. However, viral replication and a high viral load were observed in the lymphatic tissue. Despite the absence of viral replication in PBMCs, 1/1,000 to 1/10,000 of the PBMCs contained HIV-1 proviral DNA, and HIV transcription could be rapidly induced in these cells in vitro. These results provide direct evidence of cellular latency of HIV in vivo and suggest that HIV infection in chimpanzees may be a useful model for clinical latency of HIV infection in humans.

Chimpanzees can be infected with relatively low doses of human immunodeficiency virus type 1 (HIV-1), resulting in an infection that is in many ways similar to that of humans (1, 13, 19). As in humans (29), HIV-1 infection in chimpanzees results in a transient viremia followed by an extended period with low or undetectable levels of cell-free virus in plasma and few infected cells in the peripheral blood (14–16). Chimpanzees also develop HIV-specific neutralizing antibodies (15, 25, 26) and cytotoxic T cells (4, 7, 39). Despite these immunological responses, persistent viral replication occurs, and infectious virus can typically be isolated from chimpanzee peripheral blood mononuclear cells (PBMCs) at any time of the infection (2, 14, 15, 26).

Although episodes of lymphadenopathy (1, 22) and cases of decreased numbers of CD4-positive lymphocytes accompanied by thrombocytopenia (18) have been reported, for reasons that are currently not well understood, none of the more than 100 chimpanzees infected with HIV-1 have so far have developed AIDS. Nevertheless, it is apparent that many features in the pathobiology of HIV infection are shared between humans and chimpanzees. The persistent infection in chimpanzees despite a specific immune response and the absence of overt disease suggests a virus-host interaction that might be similar to that in HIV-positive asymptomatic humans.

A lower level of viral gene expression (HIV-1 mRNA) and fewer HIV-infected cells (proviral DNA) are typically observed in the peripheral blood of HIV-infected persons during the asymptomatic phase of the disease than in later stages of the infection (3, 9, 23, 28, 34). We have recently extended these observations by analyzing serial PBMC samples collected during a long-term prospective study. We found that the expression of HIV mRNA differs dramatically among HIV-infected individuals with very similar clinical indices, and this variation strongly correlates with the future course of their disease (32). Significantly, we found no evidence of viral replication in the blood of long-term asymptomatic HIV carriers who would continue to have normal numbers of $CD4^+$ cells for more than 5 years.

Although HIV replication in PBMCs is variable over the course of the disease, it has been suggested that lymphoid germinal centers are an important reservoir of HIV and support active viral replication in all stages of HIV infection (8, 12, 27, 28). Notably, high viral load has been demonstrated in the lymphatic tissues of asymptomatic individuals with little or no detectable viral replication in their peripheral blood (28).

In all stages of the disease, the number of cells harboring HIV provirus appears to be larger than the number of cells expressing HIV-1 mRNA (8, 21, 35). It is somewhat controversial whether this ratio significantly changes during the progression of the disease (3, 8, 23); if it does, a possible explanation could be an increase in the number of new productively infected cells rather than transcriptional activation of quiescent proviruses in latently infected cells. Therefore, it is not known whether latently infected cells which can be productively activated exist in vivo.

In this study, we have used HIV-infected chimpanzees as a model system with which to study HIV growth in vivo. We have compared the viral nucleic acid load in the lymph nodes (LN) and peripheral blood of these animals by quantitative polymerase chain reaction (PCR) assays specific for HIV-1 DNA and RNA. These studies have revealed a dramatically higher amount of HIV-1 RNA in chimpanzee LN than in their PBMCs. However, a short stimulation of PBMCs in vitro was sufficient to induce readily detectable expression of HIV-1 mRNA, providing direct evidence that cells with a transcriptionally competent but yet quiescent HIV exist in vivo.

MATERIALS AND METHODS

Chimpanzees. Chimpanzee C-487 (male) was infected intravenously with a chimpanzee-passaged lymphadenopathy-associated virus 1 (LAV-1) strain, LAV-1b (37), 4.5 years before the analyzed samples were obtained and has been described in detail previously (14). C-435 (male) was infected 3 years before sampling by transfusion of 7×10^5 PBMCs from another

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HIV-1_{Lai}-infected chimpanzee (C-087 in reference 20). A more detailed description of the infection and follow-up of C-435 is reported in reference 17. C-120 (female) was infected 10 months before sample collection via the endocervical-vaginal route, using an inoculum of 10^7 PBMCs from C-435. C-120 became virus positive in PBMCs at week 4 postinfection and seroconverted quickly thereafter. All chimpanzees were maintained in biosafety level 2 and 3 facilities at the Laboratory for Experimental Medicine and Surgery in Primates, and the experimental procedures were performed according to accepted practices for the care and use of primates in biomedical research.

Sample collection and preparation. Peripheral blood and inguinal LN biopsies were obtained simultaneously under ketamine anesthesia. The LN biopsies were frozen on dry ice and stored at −70°C until analyzed. PBMCs were purified immediately after the blood was drawn by using Ficoll-Hypaque (Pharmacia) centrifugation, washed twice with RPMI 1640, and either stored as a frozen pellet until analyzed or resuspended in RPMI 1640 with 10% fetal calf serum plus antibiotics when used for in vitro activation studies. These cells were kept at room temperature until split into aliquots and put into culture 1 h later. The unstimulated control sample was harvested at this point. Phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) were purchased from Sigma and used as specified in the text.

Nucleic acid extractions and PCR assays. Total RNA was extracted by the acid phenol-guanidium isothiocyanate method (5). The RNA preparations were then treated for 1 h with 100 U of RNase-free DNase (Boehringer Mannheim) in the presence of 50 U of placental RNase inhibitor (Boehringer Mannheim), extracted with phenol-chloroform, and precipitated with ethanol. High-molecular-weight DNA was extracted by standard methods and incubated for 1 h with 100 U of boiled RNase A (Boehringer Mannheim) before addition of proteinase K to the lysates. The reverse transcription-PCR (RT-PCR) analysis of the RNA samples was performed as follows. One microgram of DNase-treated RNA in 20 μl of water was heated for 5 min at 80°C, transferred onto ice, and combined with a mixture containing 6 μl of 5× reaction buffer (250 mM Tris [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 1.5 μl of deoxynucleoside triphosphates (dNTPs; 10 mM each), 0.6 μl of RNasin, 1 μl of random hexamers (1 mM; Pharmacia), and 2 μl of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) or 2 μl of water in the control lacking enzyme. After a 1-h incubation at 42°C, 2 μl samples of these reverse transcription reactions were added to each 50-μl PCR mixture containing 10 mM Tris (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.02% gelatin, 50 μM each unlabeled dNTP, 100 ng of each 23-mer oligonucleotide primer, 0.25 μl of [α-^{32}P]dCTP (3,000 Ci/mmol; NEN), and 0.25 μl of *Taq* polymerase (5 U/μl; Boehringer). These reactions were cycled in tightly fitting tubes under mineral oil in a Perkin-Elmer Cetus DNA Thermal Cycler 480 as follows: one cycle of 94°C for 40 s, 65°C for 1 min, and 72°C for 30 s, followed by 32 cycles of 94°C for 20 s, 65°C for 30 s, and 72°C for 20 s. β-Actin PCRs were cycled only 22 times after the first cycle, using the conditions specified above, and 10% dimethyl sulfoxide was added to inhibit primer-dimer formation. The sequences (sense and then antisense) of the oligonucleotides used were as follows: unspliced (US) HIV-1, 5′-TCT CTA GCA GTG GCG CCC GAA CA-3′ and 5′-TCT CCT TCT AGC CTC CGC TAG TC-3′; multiply spliced (MS) HIV-1, 5′-CTT AGG CAT CTC CTA TGG CAG GAA-3′ and 5′-TTC CTT CGG GCC TGT CGG GTC CC-3′; and β-actin, 5′-CGA GCA CAG AGC CTC GCC TTT GC-3′ and 5′-CAT AGG AAT CCT TCT GAC CCA TG-3′. To confirm the lack of contaminating proviral HIV-1 DNA in the PBMC cDNA preparations, a duplicate cDNA synthesis of each sample was also performed without adding reverse transcriptase and analyzed in a US HIV-1 PCR assay. In addition, in each set of RNA extractions, a negative control with 20 μg of yeast tRNA and no cells added to the lysis buffer was processed together with the PBMC preparations and analyzed in MS and US HIV-1 RT-PCR to exclude any contamination of the samples during sample processing or RT-PCR analysis. Quantitations of the HIV-1-specific RNA were based on comparisons of the intensities of the autoradiographic PCR signals from the chimpanzee samples with those derived from parallel analyses of in vitro-transcribed HIV-1 RNAs serially diluted into a constant amount (1 μg) of HIV-negative human PBMC RNA (32). For amplification of HIV-1 proviral sequences, 1 μg of DNA in 2 μl was added to 50-μl PCR mixtures otherwise identical to those used to amplify cDNA specific for US HIV-1 RNA.

RESULTS

PBMCs and parts of LN were obtained from three HIV-1-infected chimpanzees, C-487, C-435, and C-120, and used for nucleic acid extractions (see Materials and Methods). HIV-specific DNA and RNA were measured by PCR and RT-PCR with two different sets of amplification primers that match precisely the HIV-1_{Lai} sequence of the viral strains used to infect the animals. One set of primers was located on each side of the major splice donor site in the 5′ region of the virus and was used to amplify a 160-bp fragment specific for HIV proviral DNA and cDNA derived from US HIV RNA. US HIV RNA could represent genomic RNA in viral particles or intracellular full-length HIV mRNA. Another set of primers separated by the intron between the two coding exons of *tat* and *rev* was used to amplify a 131-bp fragment from cDNA representing MS HIV mRNA. MS HIV mRNA is obligately intracellular and therefore reflects ongoing viral replication. To control for the quantity and quality of the RNA preparations and the efficiency of subsequent cDNA synthesis, RT-PCR specific for β-actin mRNA was also performed.

As shown in Fig. 1A, no MS HIV-1 RNA was detected in the PBMC samples from any of the three chimpanzees (Fig. 1A, lanes P), indicating less than 50,000 molecules of MS HIV-1 mRNA per microgram of total PBMC RNA. Similarly, little or no US HIV RNA was observed in the PBMCs. In longer exposures of the autoradiogram, a weak signal specific for US HIV RNA was seen in PBMCs from chimpanzee 435 (not shown), whereas samples from the others (chimpanzees 487 and 120) were still negative. In the absence of MS HIV mRNA, however, the low levels of US HIV mRNA signal in the PBMC sample of chimpanzee 435 is unlikely to reflect HIV replication and probably was caused by virus particles produced elsewhere in the animal. The intensities of β-actin mRNA signals from all PBMC samples were comparable and similar to what we typically observe when using cDNA prepared from 1 to 2 μg of spectrophotometrically quantitated human PBMC RNA, indicating that the chimpanzee PBMC RNAs were intact and the cDNA synthesis had been successful. Thus, no evidence of viral replication in the PBMCs of these animals was observed.

In contrast, intense autoradiographic signals specific for US HIV RNA were detected in LN samples from all three animals (Fig. 1). This was even more striking considering that two of these LN RNA preparations (435 and 120) had much less intact RNA, as indicated by the weak β-actin signal obtained from these cDNAs (Fig. 1). From results for our in vitro-transcribed HIV RNA controls and the β-actin signals ob-

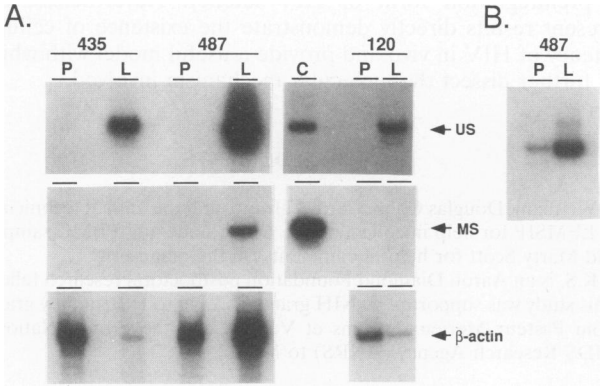

FIG. 1. Quantitation of HIV RNA and DNA in PBMCs and LN of HIV-1-infected chimpanzees. (A) Total RNA was extracted from PBMCs (P) or LN (L) of chimpanzees 435, 487, and 120 and subjected to RT-PCR specific for US HIV-1 RNA (US) or MS HIV-1 mRNA (MS). Cellular β-actin mRNA in these samples was also measured. The three PBMC RNAs and the LN RNA from chimpanzee 487 show similar β-actin signals, allowing comparison of their HIV expression. In contrast, despite similar spectrophotometrically determined RNA concentrations, the LN RNAs from chimpanzees 435 and 120 used for RT-PCR show only weak β-actin signals, indicating degradation of these samples. Lane C shows an analysis of a control sample containing 0.5×10^6 copies of both US and MS HIV-1 RNA per microgram of total cellular RNA. (B) DNA was isolated from the PBMCs (P) and LN (L) from chimpanzee 487, and 1 μg of each was analyzed for HIV-1 content by PCR, using the same primers as in US RT-PCR. Thus, the targeted PCR fragment is derived from the 5′ region of the virus just 3′ of the primer binding site and can be amplified only from more mature products of reverse transcription.

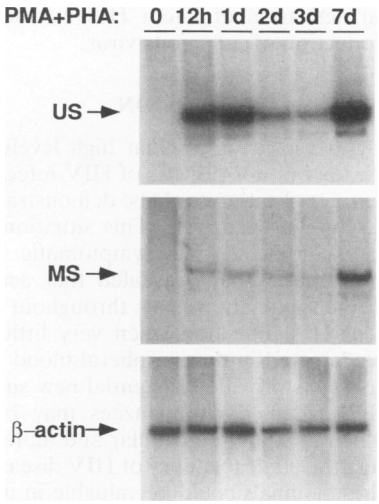

FIG. 2. In vitro activation of latent HIV in PBMCs from chimpanzee 487. The Ficoll-purified PBMCs from 75 ml of blood from chimpanzee 487 were diluted into culture medium and split into six identical aliquots. One was harvested immediately (0), the rest were first cultured for the indicated times (days [d]) in the presence of 100 nM PMA and 2 μg of PHA per ml. Two micrograms of total RNA from each sample was analyzed by RT-PCR as in Fig. 1A.

tained, we estimated that the LN RNA from animal 487 contained approximately 20 million copies of unspliced HIV RNA per microgram of total RNA. Although HIV RNA and cellular β-actin mRNA may be degraded at different rates, such calculations suggested that the viral load in the LN samples from animals 120 and 435 was even higher than that in the sample from animal 487.

The quality of the LN RNA from animal 487 allowed a reliable quantitative analysis of the different HIV RNA species. This RNA sample showed a readily detectable MS RNA signal under the linear and quantitative conditions of our assay (Fig. 1A) and was estimated to contain approximately 200,000 copies of MS HIV-1 mRNA per microgram of total RNA. Whether similar levels of MS HIV-1 mRNA were present in the LN samples of animals 435 and 120 could not be assessed because of the poor quality of these RNA preparations. Nevertheless, the result clearly demonstrates that active viral replication can occur in the lymphatic tissues of HIV-infected chimpanzees even when it is not detectable in the peripheral blood.

The amount of MS HIV-1 RNA in the LN of chimpanzee 487 was, however, less than 1% of that of US HIV-1 RNA. Such a dramatic difference in the ratio of these differentially spliced HIV RNAs is probably due to the fact that most of the US HIV RNA signal is derived from viral particles trapped in the LN and not from intracellular full-length HIV mRNA. This interpretation is in agreement with published in situ hybridization studies showing that most of the abundant HIV RNA present in the LN of HIV-infected humans is located extracellularly associated with the dendritic follicular cells (8, 28).

To examine the relative numbers of HIV-infected cells, genomic DNA was prepared from the PBMCs of chimpanzee 487 and from another part of the same LN of this animal that had been analyzed for its HIV RNA content. One microgram of each DNA was then used as the template for PCR amplification employing the primers and reaction conditions established for the US RT-PCR assay. The number of HIV-1 proviruses per microgram of genomic DNA was found to be approximately 10-fold higher in the lymphatic tissue than in the PBMCs (Fig. 1B). A similar ratio was observed between the LN and PBMCs of another chimpanzee (animal 411; data not shown) for which no LN RNA was available for RT-PCR analysis. Thus, the increased expression of HIV-1 RNA in the lymph nodes of HIV-infected chimpanzees is accompanied by a higher number of proviruses than in their peripheral blood.

Although we found no evidence of viral replication in the peripheral blood, the intensity of HIV-specific DNA signal suggested that 1/1,000 to 1/10,000 of the PBMCs of chimpanzee 487 carried a provirus. To test whether viral replication could be induced in these cells by cellular activation, we collected a larger blood sample from this animal and analyzed HIV-1 mRNA expression in these PBMCs before and after stimulation in vitro with 100 nM PMA and 2 μg of PHA per ml. This second blood sample, collected more than 6 months after the first, confirmed the lack of HIV mRNA expression in the unstimulated PBMCs and indicated that this phenotype is stable over time. However, after only 12 h of stimulation in vitro, HIV gene expression was evident, and both MS and US HIV-1 mRNAs were readily detected by RT-PCR (Fig. 2). Since this time is too short to allow spread of virus in the culture, the induced HIV expression could not have derived from newly infected cells and must have been due to virus production by latently infected cells present in the original sample. This induction occurred rapidly and was already maximal at the 12-h time point. The expression of HIV mRNA relative to the total PBMC RNA thereafter decreased up to 3 days. However, in the sample collected after 1 week in culture, increased expression, even higher than that in the 12-h sample, was observed. This delayed increase in HIV expression was presumably due to spreading of the infection in the culture,

indicating that activation of latent HIV in these cells also resulted in production of infectious virus.

DISCUSSION

In this study, we have shown that high levels of HIV are associated with LN but not PBMCs of HIV-infected chimpanzees and that viral replication could be demonstrated in LN but not in PBMCs of these animals. This situation is strikingly similar to that seen in certain asymptomatic HIV-infected humans. Recent studies have revealed that active infection takes place in the lymphatic tissues throughout the clinically latent period of HIV infection when very little or no HIV mRNA can be detected in the peripheral blood (28).

Thus, these results provide substantial new support for the notion that HIV-infected chimpanzees may be a relevant model with which to study the cellular and molecular mechanisms regulating the clinical latency of HIV disease in humans. Specifically, these animals could be valuable in understanding the role of the lymphatic tissue in the establishment of a persistent HIV infection. Also, after the acute viremic phase of infection is over, HIV-infected chimpanzees probably represent a rather homogeneous population in regard to the replicative state of their virus, and this virus-host interaction could share significant pathobiological features with the subpopulation of human long-term survivors of HIV infection.

The sensitivity of our RT-PCR assay allowed us to determine the cellular basis of the lack of HIV expression in the peripheral blood of these chimpanzees. By directly monitoring HIV mRNA expression in the rare subpopulation of PBMCs carrying a provirus, we demonstrated rapid and efficient activation of HIV production in these cells upon mitogenic stimulation in vitro. Therefore, we conclude that cellular latency of HIV does indeed exist in vivo. HIV-1 DNA-positive cells which express no detectable HIV mRNA are also present in the peripheral blood (30) and lymphatic tissues (8) of HIV-infected humans. Given the present results, it is possible that the proviruses in many, if not most, of these cells can be activated to produce infectious virus. Interestingly, our preliminary studies to address this issue have shown that HIV mRNA expression can be similarly activated by PMA-PHA treatment of human PBMCs (33).

The PMA-PHA-induced activation of latent HIV in chimpanzee PBMCs was similar to the proviral activation observed in certain cell line models of HIV latency (10, 11). These latently infected cell lines have been derived from single cells which have survived the lytic effects of HIV after infection of human T-cell and monocytic leukemia cell cultures. Such cell lines support only very low levels of viral replication when cultured in an unstimulated state but can be induced to produce high amounts of virus when exposed to agents such as PMA and PHA or cytokines such as tumor necrosis factor alpha. The increased virus production after such stimulation of these cells is accompanied by a highly induced expression of HIV mRNA (31). Much of this induction is believed to be mediated by interaction of the transcription factor NF-κB with its two tandemly arranged cognate sites in the proviral long terminal repeat element (6, 24). Thus, it is likely that the increased activity of NF-κB, or a closely related factor, is involved in the activation of latent HIV in the in vitro-stimulated chimpanzee PBMCs. Transcriptional quiescence of integrated proviruses, however, may not be the only molecular mechanism of HIV cellular latency. It has been shown that unintegrated HIV can persist for extended times in infected cells and continue the viral life cycle once the host cell is activated (36, 38). Our findings do not exclude a role for such a preintegration form of HIV latency. Nevertheless, the present results directly demonstrate the existence of cellular latency of HIV in vivo and provide a useful model with which to further dissect the molecular mechanism involved.

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