Virus Burden in Lymph Nodes and Blood of Subjects with Primary Human Immunodeficiency Virus Type 1 Infection on Bitherapy

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At present, it is not known whether undetectable plasma viremia corresponds to an absence of human immunodeficiency virus type 1 (HIV-1) replication in lymphoid tissues. This issue has been explored in 11 subjects with primary HIV-1 infection treated with zidovudine plus didanosine by evaluating virologic markers in blood and lymphoid tissues 9–18 months after initiation of treatment. These markers include plasma viremia, measured with a sensitive assay with a detection limit of 20 HIV-1 RNA copies/mL, infectious virus titers and proviral DNA in lymph node mononuclear cells, and HIV-1 RNA in lymphoid tissue. Five subjects had plasma viremia <20 copies/mL and showed no evidence of viral replication in lymphoid tissue. Six subjects had both detectable plasma viremia and evidence of HIV-1 RNA in lymphoid tissue. The results indicate that absence of detectable HIV RNA in lymphoid tissue is associated with viremia levels of HIV-1 RNA <20 copies/mL.

The primary goal of combined antiviral therapy is to achieve complete suppression of viral replication and possibly eradication of human immunodeficiency virus type 1 (HIV-1). Recently, several studies showed a dramatic reduction of viremia to levels below the detection limit of currently used assays (i.e., 500–200 HIV-1 RNA copies/mL of plasma) in HIV-1–infected subjects receiving combined antiviral therapy [1–3]. However, the recent development of a sensitive assay for the determination of plasma viremia, with a lower detection limit of 20 copies/mL, demonstrated that 45% of plasma samples with viremia <200 copies/mL, had >20 HIV-1 RNA copies/mL of plasma [4].

To date, it is difficult to assess whether the absence of detectable HIV-1 RNA in plasma reflects the situation in other anatomic compartments, such as lymphoid tissue, which is the primary site for viral spreading and replication [5–7]. Therefore, it seems crucial to compare the levels of viral replication in blood and lymphoid tissue and to identify plasma viremia levels that correspond to undetectable viral replication in lymphoid tissue.

To address these issues, 11 subjects with primary HIV-1 infection receiving combination therapy [8] were enrolled in a prospective study. At 9–18 months after treatment initiation, lymphoid tissue biopsies were obtained from lymph nodes, tonsils, and adenoids, and viral replication was assessed in parallel in blood and lymphoid tissues.

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Patients and Methods

Study population. All subjects with primary HIV-1 infection referred to us between January 1995 and March 1996 were offered an open-label therapeutic regimen with zidovudine (250 mg twice a day) and didanosine (200 mg twice a day) [8]. All subjects experienced an acute retroviral syndrome at the time of or in the month preceding initiation of antiviral therapy, except 1 subject who was included 2 months after the onset of the acute retroviral syndrome. The subjects were Caucasians; 7 were male and 4 female, with a mean age at study entry of 37 years (range, 26–54). HIV-1 infection was acquired through homosexual contacts in 6 subjects and heterosexual contacts in 5. At 9–18 months after initiation of treatment, excisional lymph node biopsies were performed in the inguinal area in 11 subjects; tonsil and adenoid biopsies were collected in parallel in 3 of them.

HIV-1 RNA assay. Viremia levels were determined in batches using EDTA-treated plasma samples, stored in aliquots at −75°C, using the Amplicor HIV-1 Monitor test (Roche, Basel, Switzerland) according to the manufacturer's instructions, with a detection limit of ~200 HIV-1 RNA copies/mL. Samples with viremia <200 copies/mL were retested using a ''boosted'' modification of the Amplicor HIV Monitor test with a detection limit of 20 HIV-1 RNA copies/mL [4].

Quantification of proviral DNA. The proviral DNA concentration associated with lymph node mononuclear cells (LNMC) and peripheral blood mononuclear cells (PBMC) was measured in duplicate as previously reported [9]. The input of cellular DNA was 2.5 μg/assay, corresponding to 375,000 cells. The limit of detection of the assay was 1–3 HIV-1 DNA copies/2.5 μg of DNA. Samples with <3 copies were retested in triplicate using the same procedure.

In situ hybridization. In situ hybridization for the detection of HIV-1 RNA was performed on paraffin-embedded tissue sections using RNA probes that comprised 90% of the HIV-1 genome, as previously described [5]. The number of individual cells expressing HIV-1 RNA were quantified by first determining the area of the biopsy section, using computerized planimetry, followed by counting the number of HIV-1 RNA–expressing cells in the biopsy section, using a darkfield microscope at ×100 magnification. The sensitivity of the in situ hybridization procedure was determined by constructing a standard containing a known number of HIV-1 virions and was found to correspond to 13 virus particles (5 silver grains). HIV-1 RNA–expressing cells were reported as the number of cells per 10 mm^2.

Infectious HIV-1 titers. Suspensions of LNMC were obtained after mincing lymph node tissue with a scalpel, teasing out the cells, and isolating them by ficoll-hypaque gradient centrifugation. Aliquots of 5×10^6 cells in 20% fetal calf serum and 10% dimethyl sulfoxide were stored in liquid nitrogen. Cellular infectious titers were determined using quantitative cell microculture [10]. Six dilutions of LNMC, depleted of CD8 cells, starting at a concentration of 10^6 LNMC, were cocultivated in duplicate with 10^6 phytohemagglutinin (PHA)-stimulated CD8 cell–depleted PBMC from HIV-negative donors. Half of the culture medium was replaced with fresh medium twice a week. Fresh uninfected PHA-stimulated PBMC were added to the culture once a week. On days 14 and 21, culture fluid from each well was tested for HIV-1 p24 antigen level using a commercial ELISA (Abbott Laboratories, North Chicago). Results are expressed as infectious units per million cells.

Immunologic parameters. CD3, CD4, and CD8 lymphocyte counts were determined on fresh material in lymph nodes and blood by flow cytometry (Coulter EPICS IV; Instrumente Gesellschaft, Basel, Switzerland) using fluoresceinated DAKO-CD3, DAKO-CD8, and R-Phycoerythrin DAKO-CD4 (Dako, Glostrup, Denmark). To minimize variations linked to fluctuations in total lymphocyte counts, results were expressed as the CD4/CD8 ratio.

Results

HIV-1 replication in blood. Viral replication in blood was evaluated, at the time of lymph node collection, via the determination of plasma viremia. Five of 11 subjects (subjects 1–5; table 1) had levels of plasma viremia <20 HIV-1 RNA copies/mL. Subjects 6–8 had levels of plasma viremia just above the detection limit, ranging between 22 and 25 copies/mL, whereas plasma viremia was >200 copies/mL in subjects 9–11 (table 1). The sensitive assay allowed the detection of viremia in subjects 6–10, in whom viremia levels were below the detection limit (500 to 200 HIV-1 RNA copies/mL) of the commercial assay. Figure 1A shows the kinetics of plasma viremia in the 5 subjects with <20 HIV-1 RNA copies/mL at the time of lymph node collection. All 5 had plasma viremia of <20 copies/mL at 6 months after initiation of combination therapy.

HIV-1 replication in lymph nodes. Viral replication in lymph nodes was evaluated by in situ hybridization of cell-associated HIV-1 RNA and by determination of infectious virus titers in suspensions of LNMC (table 1). Subjects 1–6 showed no evidence of cells expressing HIV-1 RNA. Cells expressing HIV-1 RNA were, however, detected in subjects 7–11 (figure 2). A second lymph node biopsy was performed in subjects 1 and 2 at 7 months after the first biopsy. Like the first lymph node analyzed, the second had no detectable cells expressing HIV-1 RNA. Infectious virus was recovered from subjects 6, 7, and 9–11. The titers of infectious virus were very low in subjects 6 and 7 and higher in subjects 9–11 (table 1). No evidence of active viral replication as assessed by coculture was found in subjects 1–5, whereas virus was isolated in subjects 6–11 (table 1). Failure to detect HIV-1 replication in lymph nodes was confined to subjects 1–5, who had plasma viremia of <20 copies/mL.

HIV-1 RNA associated with follicular dendritic cells (FDC). Previous studies showed that the diffuse HIV-1 RNA hybrid-

Table 1. Virus burden and CD4/CD8 cell ratio in blood and lymph nodes after >9 months of antiviral therapy.

		Blood			Lymph nodes				
					In situ hybridization				
Patients	Time, months	HIV RNA, copies/mL	DNA, copies/10^6	CD4/CD8 cell ratio	FDC-associated HIV RNA	HIV RNA–positive cells/10 mm^2	DNA, copies/10^6	Viral culture, IU/10^6 LNC	CD4/CD8 cell ratio
1	11	<20	<3	1.57	Absent	ND	61	0	4.00
2	13	<20	16	1.03	Present	ND	317	0	5.60
3	14	<20	<3	1.32	Absent	ND	37	0	2.59
4	12	<20	<3	0.87	Absent	ND	<3	0	4.92
5	10	<20	<3	1.03	Present	ND	24	0	4.64
6	10	22	<3	2.22	Present	ND	63	5	3.53
7	11	25	<3	0.97	Present	0.66	42	5	2.63
8	11	25	<3	1.32	Absent	0.66	<3	0	5.00
9	10	185	16	0.67	Present	0.66	79	100	2.32
10	18	440	50	0.73	Present	3.4	4433	625	2.38
11	9	32,790	290	0.68	Present	3.4	11,336	3125	1.44

NOTE. ND, not detected; FDC, follicular dendritic cells; IU, infectious units; LNC, lymph node cells.

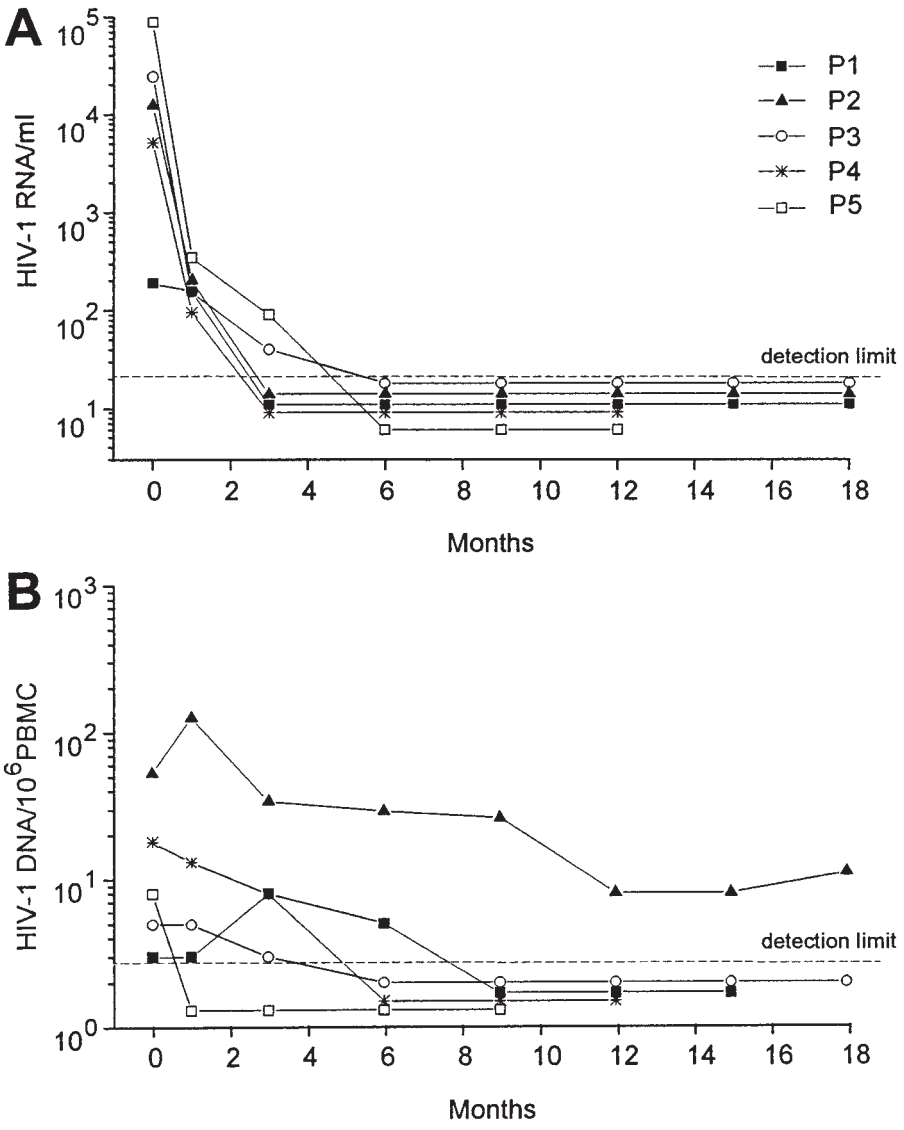

Figure 1. Viremia (**A**) and proviral DNA (**B**) levels in 5 subjects treated with zidovudine plus didanosine with no detectable viral replication in lymph nodes. Patient nos. (P1–P5) indicated in **A** correspond to those reported in table 1. Detection limit in dashed line corresponds to 20 HIV-1 RNA copies/mL of plasma (**A**) and 3 proviral DNA copies/10^6 peripheral blood mononuclear cells (PBMC) (**B**).

ization signal associated with germinal centers corresponded to HIV-1 virions trapped in the FDC network [5–7]. In our study, trapping of HIV-1 in the FDC network was either absent (subjects 1, 3, and 4) or present in traces (subjects 2 and 5) in subjects with plasma viremia of <20 copies/mL (table 1, figure 2). In subject 2, trapping of HIV-1 was no longer detected in the second lymph node biopsy, performed 7 months later. Trapping of HIV-1 in the FDC network was present in all but 1 of the subjects with plasma viremia of >20 copies/mL (table 1).

HIV-1 distribution in lymph nodes, tonsils, and adenoids. Biopsies of tonsils and adenoids were performed in subjects 4 and 9 at the same time as the lymph node biopsy and in subject P2 at the time of the second lymph node biopsy. No differences in HIV-1 distribution, including trapping of virions and cell expression of HIV-1 RNA, were observed in subjects 2 or 4 in the various lymphoid tissues analyzed. In subject P9, however, only traces of HIV-1 trapped in the FDC network were observed in tonsil tissue (data not shown) compared with the presence of both trapped virions and individual cells expressing HIV-1 RNA in lymph node tissue (table 1).

Proviral HIV-1 DNA. Proviral HIV-1 DNA concentrations in blood were <3 copies/10^6 PBMC in 4 of 5 subjects with plasma viremia of <20 HIV-1 RNA copies/mL. Among the subjects with viremia of >20 copies/mL, proviral DNA in blood was below the detection limit in subjects 6–8, with viremia of 20–30 copies/mL, whereas the highest levels were observed in subjects 9–11, with viremia of >30 copies/mL (table 1). Proviral DNA was detected in lymph nodes of all subjects except 4 and 8 (table 1). Proviral DNA was significantly higher (6- to 39-fold) in LNMC than in PBMC (table 1). Kinetics of proviral DNA decay in PBMC from subjects 1–5 are shown in figure 1B. Four of 5 subjects had proviral DNA levels of <3 copies/10^6 PBMC within 9 months. The

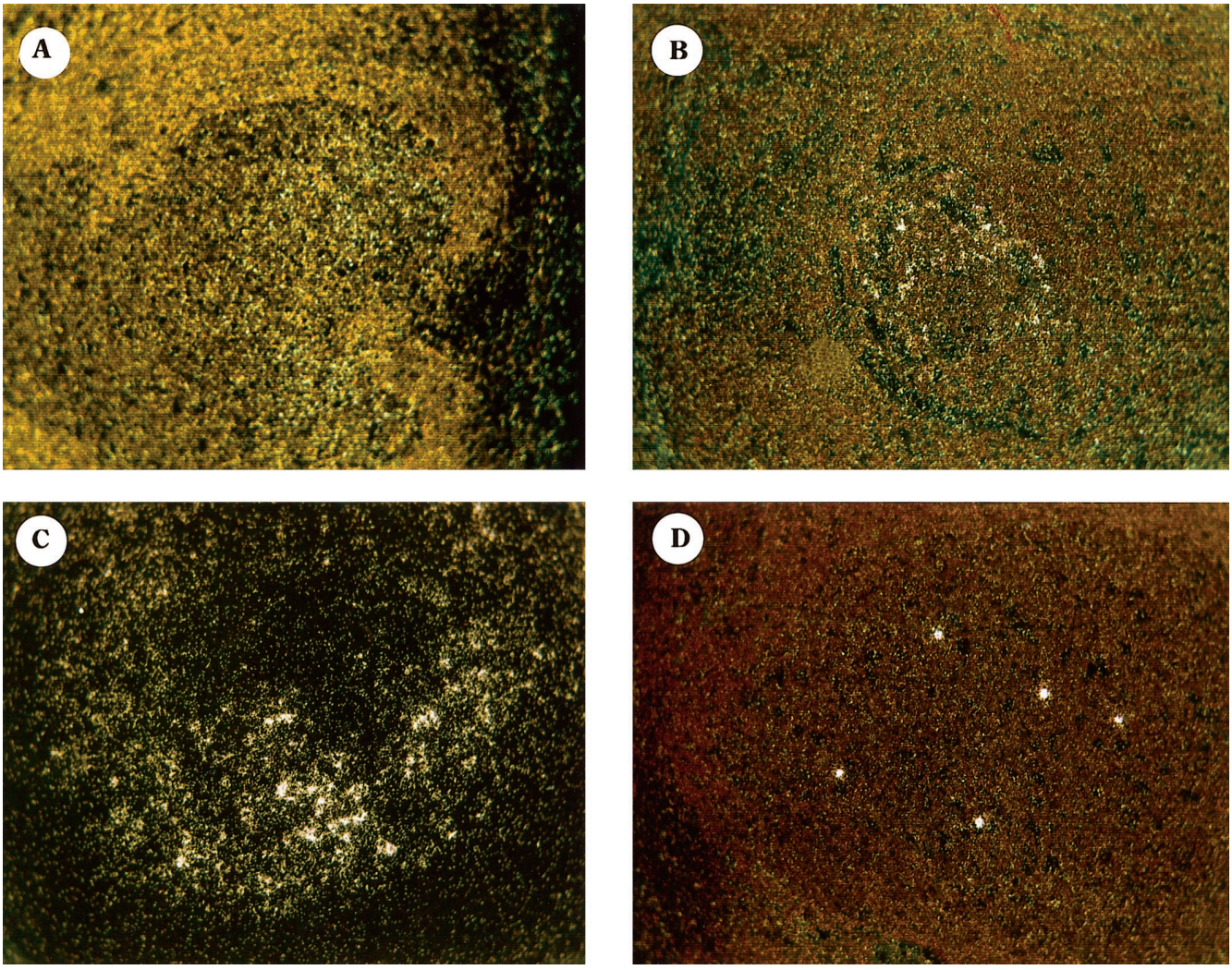

Figure 2. Sections of lymph node tissue from 4 subjects who received antiviral therapy with zidovudine plus didanosine at time of primary HIV infection and who had evidence of persistent viral replication and/or follicular dendritic cell (FDC)–associated deposits. Darkfield images of in situ hybridization for HIV RNA are shown. Location of HIV RNA is indicated by silver grains, which appear as white dots. **A–C**, Hybridization signal is localized over germinal centers; typical examples of different degrees of virus trapping in FDC are represented in **A** (very low), **B** (low), and **C** (intermediate). **D**, Typical example of hybridization signal localized in 5 isolated cells expressing HIV-1 RNA.

decrease of proviral DNA was slower than that of viremia (figure 1).

Lymphocyte markers. CD4/CD8 ratios in lymph nodes and blood are reported in table 1. All subjects, regardless of plasma viremia levels, had a higher CD4/CD8 ratio in lymph nodes than in blood. However, no significant correlation was observed between the CD4/CD8 ratios in lymph nodes and blood. Subjects with no evidence of viral replication in lymph nodes tended to have a higher CD4/CD8 ratio in lymphoid tissue (median, 4.64 vs. 2.51; $P = .07$).

Discussion

Viremia levels in blood are routinely measured by commercial assays with a lower detection limit of 500 to 200 HIV-1 RNA copies/mL of plasma [1–3]. However, HIV-1 replication in lymphoid tissue was detected in subjects with plasma viremia below 500 to 200 copies/mL [11, 12]. The results of the present study, performed by using a sensitive assay with a detection limit of 20 HIV-1 RNA copies/mL, indicate that the plasma viremia levels corresponding to undetectable viral replication in lymphoid tissue, including lymph nodes, adenoids, and tonsils, are generally <20 copies/mL. When detectable, viral replication in lymphoid tissue appears to be associated with plasma viremia >20 copies/mL. It is important to underscore, however, that levels of plasma viremia <20 copies/mL and undetectable viral replication in lymphoid tissue do not necessarily reflect complete suppression of viral replication, since the techniques used are limited by their sensitivity and the size of the sample

analyzed [13]. Moreover, the use of frozen LNMC might decrease the recovery of infectious virus.

In situ hybridization analysis using lymph node sections presents advantages in terms of the surface area analyzed compared with tonsil biopsies, but the screening of a limited number of tissue sections for the presence of virus-expressing cells does not exclude the possibility of low viral replication in other lymph nodes or lymphoid tissues [12]. Furthermore, although virus distribution (trapping of virions by FDC) and viral replication levels appear to be quite homogeneous in different lymphoid tissues in the absence of antiviral therapy, heterogeneity was observed in 1 subject in comparing lymph nodes, tonsils, and adenoid biopsies.

Virions trapped within the FDC network might be a source of viral spreading and replication [7, 2]. Similarly, proviral HIV-1 DNA may allow reactivation of the latent viral genome leading to viral replication. Although most of the proviral DNA corresponds to defective virus, recombination of defective virus and generation of replication-competent virus in vivo may potentially contribute to maintain HIV-1 infection [14, 15]. Eradication of HIV-1 in infected individuals may require that viral replication, viral trapping, and proviral DNA are completely eliminated from all body tissues. In the present study, in only subject P4 were those virologic measures below the limit of detection of the assays used.

The slower decay of proviral DNA in PBMC compared with plasma viremia in our study is consistent with the recent mathematical model indicating a half-life of 1–3 weeks for chronically infected cells [16]. In the present study, proviral DNA in PBMC was not detectable in most subjects with undetectable viremia after 9–18 months of treatment. In contrast, proviral DNA in PBMC is consistently detected in subjects with chronic HIV infection treated with combination therapy and in whom viremia was undetectable [13] (unpublished data). Treatment at the time of primary infection might thus reduce spreading of the infection and the size of the pool of latently HIV-1–infected cells.

In conclusion, in this investigation there was a good correlation between viremia and HIV-1 replication in lymphoid tissues when viremia levels were assessed by a sensitive assay in subjects with primary HIV infection treated for >9 months.

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