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Abstract: We have developed sensitive assays for viremia and cell-associated human immunodeficiency virus type 1 (HIV-1) RNA and DNA to assess the predictive value of virological parameters determined in blood for virus load in lymph nodes (LNs). Eighteen patients were included; 13 received stavudine/didanosine/hydroxyurea and 5 stavudine/didanosine, and all had viremia <500 copies/mL for >3 months. At the time of LN biopsy (median, 10 months), the median viremia was 2.09 log copies/mL (range, <0.70-3.34). Cell-associated HIV-1 RNA and DNA were detectable in blood and LNs of all patients. The median cell-associated RNA and DNA were 2.16 log copies/10⁶ cells and 2.60 log copies/10⁶ cells in blood versus 4.31 log RNA copies/10⁶ cells and 3.26 log DNA copies/10⁶ cells in LNs. Regression analysis shows that, in treated patients with sustained low viremia, cell-associated RNA and DNA in blood are better predictors of virus load in LNs than viremia

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CONCISE COMMUNICATIONS

Cell-Associated HIV-1 RNA in Blood as Indicator of Virus Load in Lymph Nodes

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We have developed sensitive assays for viremia and cell-associated human immunodeficiency virus type 1 (HIV-1) RNA and DNA to assess the predictive value of virological parameters determined in blood for virus load in lymph nodes (LNs). Eighteen patients were included; 13 received stavudine/didanosine/hydroxyurea and 5 stavudine/didanosine, and all had viremia <500 copies/mL for >3 months. At the time of LN biopsy (median, 10 months), the median viremia was 2.09 log copies/mL (range, <0.70–3.34). Cell-associated HIV-1 RNA and DNA were detectable in blood and LNs of all patients. The median cell-associated RNA and DNA were 2.16 log copies/10⁶ cells and 2.60 log copies/10⁶ cells in blood versus 4.31 log RNA copies/10⁶ cells and 3.26 log DNA copies/10⁶ cells in LNs. Regression analysis shows that, in treated patients with sustained low viremia, cell-associated RNA and DNA in blood are better predictors of virus load in LNs than viremia.

The virological response in human immunodeficiency virus type 1 (HIV-1)-infected patients on antiretroviral therapy is routinely assessed by the measurement of plasma HIV-1 RNA. Because lymphoid tissue is the main site of production and storage of the virus [1], lymph node biopsies have been used to evaluate residual virus load in patients with low or undetectable viremia [2–4]. In several studies, viremia below the limit of detection (LOD), that is, <500 or <50 copies/mL, was associated with a marked decrease of viral deposits in follicular dendritic cells (FDCs) and in cells producing HIV-1 RNA in lymph nodes [2–7]. However, in most instances, HIV-1 RNA was still detectable in lymph nodes of patients with viremia below detection limits, indicating that complete clearance of HIV-1-producing cells was not achieved [3, 5, 7].

Lymph node biopsy is a surgical procedure that cannot be often repeated, whereas alternative procedures (i.e., needle aspiration of lymph node cells) provide an insufficient number of cells for in-depth virological evaluation, in particular in patients

with low viremia [8]. In contrast, peripheral blood mononuclear cells (PBMC) are easily accessible. We have thus developed highly sensitive assays for viremia and cell-associated HIV-1 RNA and DNA to assess the predictive value of virological parameters determined in blood for virus load in lymph nodes. In the present investigation, this approach was evaluated in a cross-sectional study including treated patients with viremia <500 RNA copies/mL.

Methods

Patients. Patients were participants in a randomized, controlled clinical trial examining the effect of the adjunction of hydroxyurea, 500 mg every 12 h, to didanosine, 200 mg every 12 h, plus stavudine, 40 mg every 12 h [9]. Patients included in this virological substudy were selected according to the following criteria: at least 6 months of therapy and plasma HIV-1 RNA <500 copies/mL for at least 3 months. Lymph node biopsy was proposed to the 24 patients fulfilling these criteria; 18 signed the informed consent form approved by the local ethical committee.

Lymph node biopsies. Inguinal biopsy was performed under local anesthesia. Only 40% of the patients had palpable lymph nodes; the inguinal area was selected because local exploration there is easier than in other locations. Lymph node tissue was minced with a scalpel in tissue culture medium, and the cells were teased out by use of small tweezers. The lymph node mononuclear cells (LNMC) were isolated by gradient centrifugation (Ficoll Hypaque; Pharmacia, Dubendorf, Switzerland), and aliquots of 2 × 10⁶ cells were stored in liquid nitrogen.

Viremia quantitation. Plasma HIV-1 RNA was quantified first by use of the standard Amplicor HIV Monitor, version 1.5 (Roche Diagnostics, Basel, Switzerland). The samples with RNA <500 copies/mL were retested by use of a modified version of Amplicor HIV

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All patients included in this study have signed the informed consent form approved by the local ethical committee.

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Table 1. Virological and immunological parameters in lymph node and peripheral blood.

Parameter	Peripheral blood	Lymph node	P ^a
Viremia (log RNA copies/mL)	2.09 (0.70–3.34)	—	—
Cell-associated RNA (log copies/10 ⁶ cells)	2.16 (0.70–3.14)	4.31 (1.23–5.27)	<.001
Cell-associated DNA (log copies/10 ⁶ cells)	2.60 (0.78–3.45)	3.26 (2.06–3.79)	<.001
CD4 cells (%)	26 (15–38)	48 (25–59)	<.001
CD8 cells (%)	50 (31–64)	26 (14–46)	<.001
Ratio CD4/CD8	0.55 (0.26–1.23)	1.98 (0.78–3.36)	<.001

NOTE. Values are median (range).

^a Paired Wilcoxon test.

Monitor assay [10]. Briefly, 1 mL of plasma was centrifuged at 50,000 *g* for 80 min at 4°C. After removal of plasma, the next steps were performed according to the manufacturer's instructions, with the exception of a reduction (7.3×) in the input of internal quantitative standard (IQS) and in the volume of specimen diluent (55 vs. 400 μ L) and a prolongation to 15 min of the substrate incubation step. The LOD is 5 RNA copies/mL, and the mean coefficient of variation is 40% (range, 23%–52%). A value of 5 HIV-1 RNA copies/mL (0.70 log) was attributed to patients with HIV-1 RNA below the LOD.

Cell-associated HIV-1 RNA and DNA quantitation. PBMC were isolated from 7 mL of EDTA blood by gradient centrifugation (Ficoll Hypaque; Pharmacia). Cell-associated HIV-1 RNA and DNA were measured on the same cell aliquot by use of the reagents of the Amplicor HIV-1 Monitor assay (Roche Diagnostics). Pellets of 2×10^6 PBMC or LNMC were lysed in 600 μ L of lysis containing RNA IQS at 50% of the concentration recommended. After adding 600 μ L of 100% isopropanol, half of the lysate was transferred into a new tube for RNA quantitation, and the 2 tubes were centrifuged at 16,000 *g* for 15 min. After ethanol washing, nucleic acids were resuspended in 20 μ L of buffer containing 10 mM Tris-HCl at pH 7.4, 5 mM MgCl₂, 50 mM KCl, and 0.5 mM CaCl₂.

For cell-associated HIV-1 RNA quantitation, the nucleic acid preparation was incubated for 1 h at 37°C with 20 units of DNase I RNase-free (Boehringer, Mannheim, Germany), then for 5 min at 95°C, and diluted in 80 μ L of specimen diluent. Then 50 μ L of RNA preparation, corresponding to 6.25×10^5 cells, was amplified according to manufacturer's instructions by use of primers located in the *gag* gene.

For cell-associated HIV-1 DNA quantitation, the nucleic acid preparation was incubated 1 h at 37°C with 10 μ g of RNase A DNase-free (Sigma, Buchs, Switzerland) and diluted in 80 μ L of specimen diluent. Then 50 μ L of DNA preparation was added to mastermix buffer containing 25 copies of DNA IQS (Roche Diagnostics) and directly amplified, bypassing the reverse transcriptase incubation step. Negative controls included PBMC from normal blood donors. Results are expressed in log₁₀ HIV-1 RNA or DNA copies/10⁶ PBMC or LNMC.

The mean coefficient of variation, evaluated by use of dilutions of the 8E5 LAV cell line, was 12% (range, 3%–26%) for cell-associated RNA and 18% (range, 2%–29%) for cell-associated DNA. The LOD of cell-associated RNA was approximately 3 RNA copies/10⁶ cells (9/9 replicates containing 10 and 3.3 RNA copies/10⁶ PBMC and 4/6 replicates containing 1.1 RNA copies/10⁶ PBMC were found to be positive). The LOD of cell-associated DNA was approximately 5 DNA copies/10⁶ cells (10/10, 8/8, and 4/8 replicates

containing 10, 5, and 2.5 DNA copies/10⁶ PBMC, respectively, were found to be positive).

Immunological parameters. CD3, CD4, and CD8 lymphocyte cell counts were determined by flow cytometry (EPICS IV; Coulter, Nyon, Switzerland) by use of fluoresceinated DAKO-T3, DAKO-T8, and R-Phycoerythrin DAKO-CD4 (Dako, Glostrup, Denmark).

Statistical analysis. Associations between parameters were assessed by use of the Spearman rank correlation analysis. Comparisons between parameters were done by use of the paired Wilcoxon-test. Assessment of parameters associated with cell-associated RNA in lymph node was performed by use of univariate and multivariate linear regression models. To allow for comparisons between variables, we computed the relative predictive ability (RPA) of each predictor, that is, the relative percentage of variance explained, compared with the best predictor. The RPA is the F-statistic of a predictor divided by the F-statistic of the best predictor [11].

Results

Patients. Eighteen patients were included; 13 patients received didanosine/stavudine/hydroxyurea, and 5 patients received didanosine/stavudine/placebo. The median baseline CD4 cell count was 347/mm³ (range, 220–482), and the median plasma HIV-1 RNA was 4.50 log copies/mL (range, 3.20–5.08). Lymph node biopsies were performed 6–12 months after treatment initiation (median, 10 months). At the time of lymph node biopsy, the median CD4 cell count was 512/mm³ (range, 261–648) in blood. The median CD4 cell count change from before therapy was +121/mm³ (range, –54 to +360). The percentage of CD4 cells was approximately 2-fold higher in lymph nodes than in blood (table 1).

Quantitation of cell-associated HIV-1 RNA and DNA. The median decrease of viremia from before therapy to the time of lymph node biopsy was –2.08 log RNA copies/mL (range, –3.60 to –1.16). The median time to first viremia <500 RNA copies/mL was 1 month (range, 1–3), and the median duration with viremia <500 RNA copies/mL was 5 months (range, 3–11).

Virological parameters measured in blood and lymph nodes at the time of lymph node biopsy are reported in table 1. The median plasma RNA was 2.09 log copies/mL. Eight patients had plasma RNA <50 copies/mL, and 3 of them had <5 copies/

mL; all were on hydroxyurea. Cell-associated RNA and DNA were detectable in all patients in both blood and lymph nodes. Plasma RNA/mL was, on average, 44-fold lower (95% confidence interval [CI], 11- to 174-fold) than LNMC-associated RNA/ 10^6 cells, and PBMC-associated RNA was, on average, 34-fold lower (95% CI, 11- to 107-fold) than LNMC-associated RNA.

A strong correlation was observed between cell-associated RNA and DNA in both PBMC ($r = .80, P < .001$) and LNMC ($r = .82, P < .001$). There was only a weak correlation between plasma RNA and PBMC-associated RNA ($r = .42, P = .08$) or PBMC-associated DNA ($r = .40, P = .10$). Similarly, a weak correlation was observed between plasma RNA and both LNMC-associated RNA ($r = .46, P = .05$) and LNMC-associated DNA ($r = .58, P = .02$). In contrast, a strong correlation was found between PBMC-associated RNA and LNMC-associated RNA ($r = .67, P = .002$) or LNMC-associated DNA ($r = .78, P < .001$).

Parameters predictive of virus load in lymph nodes. In univariate linear regression analysis, plasma RNA, PBMC-associated RNA, and DNA determined at the time of lymph node biopsy significantly predict LNMC-associated RNA (table 2). Pretherapy plasma RNA, CD4 cell counts, and the CD4/CD8 ratio did not predict LNMC-associated RNA, but the duration of viremia <500 copies/mL did. To compare predictors, we calculated the relative predictive ability (RPA) for each parameter; the RPA values of PBMC-associated RNA and DNA were in the same range and were 3 times higher than that of plasma RNA.

Discussion

In this investigation, we show that in treated HIV-1 patients with sustained low viremia, cell-associated RNA and DNA in

blood are better predictors of virus load in lymph nodes than viremia.

Patients selected for this substudy were included in a clinical trial assessing the effect of the addition of hydroxyurea to combined therapy with didanosine and stavudine [9]. As reported previously, the adjunction of hydroxyurea to a didanosine-containing regimen is associated with an additional decrease of viremia [9, 12]. As the main inclusion criterion for this study was a viremia level <500 copies/mL for at least 3 months, we included more patients receiving didanosine/stavudine plus hydroxyurea than receiving didanosine/stavudine plus placebo, precluding comparative analysis.

The 3 parameters evaluated in blood (plasma RNA, cell-associated RNA, and DNA) had similar levels of absolute sensitivity per unit volume, since approximately $1-3 \times 10^6$ PBMC are contained in 1 mL of blood. Comparison of plasma RNA with PBMC-associated RNA indicates a higher sensitivity of cell-associated RNA, since the 3 patients with plasma RNA <5 copies/mL had detectable PBMC-associated RNA. This suggests that, even in patients with plasma RNA <5 copies/mL, circulating cells containing HIV-1 RNA persist. These data are in agreement with previous data showing the persistence of replication-competent, infected CD4 cells in the blood of patients on highly active antiretroviral therapy with viremia <50 copies/mL [13].

We found, as previously reported, higher concentrations of cell-associated RNA in lymph nodes than in blood [2-5]. Several factors might explain this observation, including a release into blood restricted to cells with low levels of replication, rapid killing in the blood of cells engaged in active viral replication, impaired T cell functions in lymphoid tissues [14], or impaired penetration of antiretroviral drugs into lymph nodes. Interestingly, in treated patients with low viremia, only a weak correlation was observed between plasma RNA and LNMC-associated RNA, whereas a stronger correlation was observed

Table 2. Predictors of cell-associated RNA in lymph node (regression analysis).

Parameter	LNMC RNA change ^a (95% CI)	P	RPA (%) ^b
Before therapy			
Viremia (per 1 log higher)	0.75 (-0.05-1.94)	.21	8.6
CD4 PBMC (per 100/mm ³ higher)	-0.40 (-1.30-0.40)	.33	5.0
CD4/CD8 PBMC ratio (per 0.1 higher)	-0.35 (-0.89-0.20)	.20	8.9
Therapy			
Time to first viremia <500 copies/mL (per 1 more month)	-0.25 (-0.97-0.47)	.47	2.6
Duration with viremia <500 copies/mL (per 1 more month)	-0.24 (-0.51-0.03)	.07	18.0
Viremia (per 1 log higher)	0.89 (0.16-1.62)	.02	32.3
Change in viremia (per 1 log higher)	0.80 (-0.18-1.78)	.10	14.5
CD4 PBMC (per 100/mm ³ higher)	-0.05 (-0.60-0.50)	.85	0.2
CD4/CD8 PBMC ratio (per 0.1 higher)	-0.12 (-0.42-0.18)	.42	3.4
RNA PBMC (per 1 log higher)	1.41 (0.72-2.09)	$<.001$	92.8
DNA PBMC (per 1 log higher)	1.54 (0.82-2.26)	$<.001$	100

NOTE. LNMC, lymph node mononuclear cell; CI, confidence interval; PBMC, peripheral blood mononuclear cells.

^a Log RNA copies/ 10^6 LNMC.

^b RPA, relative predictive ability. The RPA is the F-statistic of a predictor divided by the F-statistic of the best predictor. The best predictor was DNA PBMC (RPA value of 100%).

between PBMC- and LNMC-associated RNA. Our preliminary data indicate that in treated patients, PBMC-associated RNA levels are better indicators of virus load in lymph nodes than plasma RNA, as confirmed by linear regression analysis. One likely explanation is that free viruses are rapidly removed from the blood through trapping in the FDC network and/or macrophages, whereas both acutely and latently infected cells in blood have a longer half-life [5, 15].

In conclusion, the main finding of this preliminary study is that the quantitation of cell-associated RNA and DNA in PBMC provides an excellent estimate of HIV-1 virus load in lymphoid tissues. These assays may represent in the future new standards for the assessment of treatment efficacy in patients with undetectable or low viremia.

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