Quantification of HIV-1 Proviral DNA in Patients with Undetectable Plasma Viremia over Long-Term Highly Active Antiretroviral Therapy

Maurizia Debiaggi, PhD;* Francesca Zara, MD;† Angela Pistorio, MD;‡ Raffaele Bruno, MD, PhD;†
Paolo Sacchi, MD, PhD;† Savino F.A. Patruno, MD, PhD;† Giorgio Achilli, PhD;†
Egidio Romero, MD, PhD;* and Gaetano Filice, MD, PhD†

ABSTRACT

Objectives: To assess the prognostic role of proviral DNA in peripheral blood mononuclear cells (PBMC) of patients with undetectable viremia over long-term highly active antiretroviral therapy (HAART).

Methods: Eighty-two human immunodeficiency virus (HIV)-1-infected patients, free of acquired immunodeficiency syndrome (AIDS), received zidovudine plus lamivudine plus indinavir. Levels of plasma HIV-RNA, and PBMC proviral DNA and RNA unspliced (US) transcripts were evaluated by using competitive polymerase chain reaction (cPCR) assays, every 3 months over 1 year.

Results: Among patients with undetectable viremia at baseline, 13 of 18 with CD4 cell count 350/mm^3 or less and 12 of 16 with CD4 between 351 and 700/mm^3, constantly maintained undetectable RNA levels; in these patients, a mean proviral DNA decrease of 0.67 ± 0.7 and 1.03 ± 0.53 log ($P < 0.001$), respectively, a significant decrease of RNA-US transcripts ($P < 0.001$), and significant correlations between decreases of proviral DNA and RNA-US transcripts ($P = 0.008$ and $P < 0.001$, respectively) were observed.

Conclusions: Proviral DNA quantitation permits the continued monitoring of HAART in patients with undetectable viremia.

Key Words: *HIV-1, HIV-1 RNA, PCR, proviral DNA*

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Several controlled clinical trials have provided evidence that plasma human immunodeficiency virus (HIV)-RNA levels are strongly correlated with disease progression, CD4 T-cell count, and response to antiretroviral therapy.[1-4] In a recent follow-up report, the Multicenter AIDS Cohort Study (MACS) group demonstrated improved predictability of outcome when viral load and CD4 count were used together.[5] The viral load monitoring, using several plasma HIV-RNA assays is incorporated into routine clinical practice and provides clinicians with the tools to start therapy as well as to monitor and adjust antiretroviral regimens on the basis of changes in viral load over time.

Emerging data from ongoing clinical trials indicate that in the majority of patients treated with highly active antiretroviral therapy (HAART), HIV-RNA in plasma reached undetectable levels in 24 weeks,[6,7] and the virus cannot be recovered from peripheral blood mononuclear cells (PBMC) or lymphoid tissue samples by standard co-culture techniques.[8,9]

However, several groups have reported that viral DNA remains detectable in PBMCs as well as in lymphoid tissues, and the virus can be isolated from PBMCs by using enhanced co-culture conditions even after more than one year of HAART.[10,11] These studies suggest that the virus could be derived from long-living cells that have been infected before the initiation of therapy and, despite prolonged suppression of plasma viremia, they represent an important viral reservoir that seems not to be modified during HAART.

Although these cells are present in low amounts (about 1 × 10^5 to 1 × 10^6 cells in the entire body), some of them harbor replication-competent provirus.[12] Given this, residual viral burden in PBMCs should be evaluated, as an indicator for monitoring of HAART, and further diagnostic tools are required for continued monitoring of patients whose plasma viremia has been reduced to undetectable levels by aggressive antiretroviral therapies. In

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*Department of Microbiology, and of †Infectious and Tropical Diseases, University of Pavia, IRCCS Policlinico San Matteo, Pavia, and ‡Clinical Epidemiology and Biometry Unit, Scientific Direction, IRCCS Policlinico San Matteo, Pavia, Italy.

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Address correspondence to Dr. Maurizia Debiaggi, Department of Microbiology, University of Pavia, via Brambilla, 74-27100, Pavia, Italy. E-mail: inftrop@unipv.it.

the past few years, much effort has been placed in the development of polymerase chain reaction (PCR)-based methods for the clinical applicability of proviral DNA quantitation in PBMCs.[13-16] In addition, the quantitative analysis of HIV-1 viremia, viral gag transcripts, and proviral DNA sequences in PBMCs by competitive PCR (cPCR) and reverse transcription PCR (cRT-PCR) has been extensively used to examine the level of HIV-1 activity in infected patients at different stages of the disease and during antiretroviral therapies, thus providing a more complete evaluation of the molecular profile of HIV-1 infection.[17-20]

In this study, sequential blood samples were obtained from 82 infected patients during 1 year of HAART; changes in proviral DNA levels were compared with those of unspliced (US) RNA transcripts in PBMCs and RNA in plasma, according to immunologic status.

The goal of this study was to evaluate the prognostic role of proviral DNA levels in PBMCs in aggressively treated patients with undetectable plasma viremia.

MATERIALS AND METHODS

Study Subjects

Eighty-two HIV-1 seropositive patients, attending the Department of Infectious and Tropical Diseases of the University of Pavia were enrolled in the study. All patients were currently taking antiretroviral treatments, including zidovudine plus didanosine or zidovudine plus lamivudine from 3 months to 1 year and completed a screening visit within 1 month of the start of the study. Patients were selected who met each of the following criteria: (1) plasma HIV-RNA levels 5000 copies/mL or less, (2) CD4 cell count from 200 to 700/μm^3, and (3) strict adherence to current antiretroviral treatment.

Patients were excluded if they had received any HIV-protease inhibitor, if they required maintenance therapy for an opportunistic infection, or if they had received investigational or immunomodulatory drugs within 30 days before entry into the study. Exclusion criteria also included neutrophil count less than 1.0×10^9/L; hemoglobin levels more than three times the lower limit of the normal range; alkaline phosphatase and serum creatinine levels more than 2.5 or 1.5 times, respectively, of the upper limit of normal range, and Karnofsky score below 80 points. Patients were classified according to the Centers for Disease Control and Prevention (CDC) classification system.

Patients were given a combination of zidovudine (ZVD, 300 mg twice daily), lamivudine (3-TC, 150 mg twice daily), and indinavir (IDV, 800 mg three times daily). The patients were followed before starting the treatment and every 3 months for 1 year of triple drug therapy, with a clinical assessment and routine laboratory monitoring: CD4+ T-cell counts were determined twice at baseline and at each time point, and concentrations of HIV-1 RNA in plasma, proviral DNA, and US-RNA transcripts in PBMCs were determined twice at baseline and once at each time point.

Preparation of Clinical Samples and Nucleic Acid Purification

Ethylenediaminetetraacetic acid (EDTA)-treated peripheral blood was centrifuged at 600 g for 10 minutes. Plasma was recovered, and platelets and cell debris were removed at 1800 g for 10 minutes. Clarified plasma was aliquoted and stored at -80°C until RNA extraction. Blood cells were resuspended in Hank's balanced salt solution (HBSS; Gibco Life Tecnologies, Paisley, Scotland, UK) and centrifuged over a Ficoll density gradient (Lymphoprep, Nycomed Pharma AS, Oslo, Norway): PBMCs were recovered and washed three times with HBSS. For DNA preparation, PBMCs were resuspended in lysis buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCI, 0.5% Tween 20, 0.5% NP 40, 1.5 mmol/L $MgCl_2$) at the concentration of 10^7/mL and treated with proteinase K (120 μg/mL) for 4 hours at 56°C. The proteinase was inactivated by heating at 95°C for 10 minutes, and the lysate was stored at -20°C until PCR analysis. RNA was extracted from PBMCs and plasma samples using the guanidinium thiocyanate method as previously described.[21] RNA samples were then used immediately as RT-PCR template or stored at -80°C for subsequent analysis.

Quantitative Analysis of HIV-DNA and HIV-RNA

The following substrates were analyzed using quantitative cPCR and cRT-PCR: (1) genomic RNA from plasma, (2) US-RNA transcripts from PBMCs, and (3) proviral DNA from PBMCs. Quantitation of HIV-DNA and HIV-RNA, using cPCR and cRT-PCR, was performed as previously described in detail.[17]

Briefly, the same RNA (5 μL, equivalent to 100 μL of plasma or 10^5 PBMCs) or DNA (5 μL, equivalent to 10^5 PBMCs) sample was reverse transcribed or amplified in four reaction tubes containing 5 μL of competitor RNA or competitor DNA at increasing copy number (10-1250). Each RNA sample was reverse transcribed for 30 minutes at 42°C in 20-μL final volume containing $1 \times$ PCR reaction buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L $MgCl_2$), 100 units of Maloney murine leukemia virus reverse transcriptase (RT, Gibco Life Technologies), 50 pmol of the SK39 primer, 0.2 mM of each deoxynucleoside triphosphate (dNTPs), 2 units of RNasin and 5 μL of competitor RNA. The cDNA was then heated in the same mixture for 5 minutes at 95°C. Each cDNA sample was amplified in a 50-μL mixture containing, at a final concentration, $1 \times$ PCR reaction buffer, 0.2 mM of each dNTP, 50 pmol of each SK 38 and SK 39 primers and 1.5 units of Taq polymerase (Perkin-Elmer Cetus, Emeryville, California, USA). DNA samples were amplified in the same mixture containing 5 μL of competitor DNA. The PCR profile (94°C, 20 s; 60°C, 30 s; 72°C, 30 s) was repeated for 50 cycles. At the end of the last cycle the DNA was further extended at 72°C for 10 minutes. The amplification products were run on a 10% polyacrylamide gel and ethidium bromide stained. The peak areas of DNA band fluorescence emission were analyzed with a video densitometer (Gel Doc 1000, Bio Rad Laboratories, Richmond, CA, USA) as previously described. To ensure that sufficient DNA was present in the PBMC preparations and that inhibitors were absent from the reaction mixtures, the β-globin gene was amplified in the same DNA samples.[22] Parallel reactions were carried out in the absence of the reverse transcription step as a control for RNA specificity.

In these experimental conditions, the lower limit of detection of the assay was 50 RNA copies/mL of plasma and 2 DNA or RNA copies/10^5 PBMCs. Quantitative data of cellular parameters were then normalized to the percentage of CD4+ T lymphocytes in PBMCs; data were expressed as relative copy number of nucleic acid species per 10^6 CD4+ cells and then log transformed.

Statistical Analysis

Linear regression analysis was performed to evaluate correlations between quantitative parameters, and quantitative variations between baseline and 1-year follow-up were evaluated using Student's t-test for dependent variables. Changes in values during the study period were analyzed by repeated measurement analysis of variance (ANOVA), whereas comparison between frequencies of qualitative variables was performed using chi-squared test.

RESULTS

From October 1996 to May 1997, 82 patients were enrolled in this study and consecutive blood samples were obtained from all subjects. All enrolled patients showed good adherence to the therapy; no relevant side effects or intolerance to drug regimens were observed, and all patients completed the study period. Patients were divided in two groups according to CD4 cell count obtained at baseline. CD4 cell counts were below 350/mm^3 (mean, 267 ± 65) in 43 (52.4%), and between 351 and 700/mm^3 (mean, 475 ± 78) in 39 (47.6%) subjects. According to the CDC classification system, 70 of 82 (85.4%) patients were at stage A2, 4 of 43 with CD4 counts below 350/mm^3 and 8 of 39 with CD4 counts between 351/mm^3 and 700/mm^3 were at stage A3 and A1, respectively; neither AIDS-defining illness nor clinical progression of disease was observed during the study.

At baseline, in the group with CD4 count 350/mm^3 or less (n = 43), 18 patients (41.9%) had undetectable levels of plasma HIV-RNA (<50 copies/mL) and 5 (11.6%) and 2 (4.6%) had undetectable levels (<2 copies/10^5 PBMCs) of proviral DNA and RNA transcripts, respectively. The corresponding figures among the 39 subjects with CD4 count between 351/mm^3 and 700/mm^3 were 16 (41.0%), 1 (2.6%), and 1 (2.6%), respectively. The mean values for each parameter are shown in Table 1.

In patients with CD4 count of 350/mm^3 or less, HIV-RNA copy number in plasma was correlated with concentrations of proviral DNA and RNA in PBMCs (r = .45, P = 0.002; r = .61, P = 0.00001, respectively). In patients with CD4 counts between 351/mm^3 and 700/mm^3, HIV-RNA copy number in plasma was correlated with concentration of RNA specific transcripts (r = .64, P = 0.00001) but not with proviral DNA concentration (r = .14, P = 0.40).

Plasma HIV-RNA concentrations were evaluated every 3 months for 1 year. There were persistent decreases from the baseline values in both groups. After 1 year of three-drug therapy, in groups with CD4 count 350/mm^3 or less and between 351/mm^3 and 700/mm^3, the mean HIV-RNA decreases in plasma were 0.75 ± 0.87 log (range, -0.48 to -2.3; P = 0.000001) and 0.77 ± 0.89 log (range, -0.60 to -2.3; P = 0.000004), respectively, and patients with undetectable virema were 34 of 43 (79.1%) and 33 of 39 (86.4%) (chi-squared test with baseline values P = 0.00042 and P = 0.00007, respectively).

Among patients with undetectable viremia at baseline, 13 of 18 (77.2%) in the group with CD4 count 350/mm^3 or less and 12 of 16 (75%) in the group with

Table 1. Laboratory Parameters at Baseline and after 1 Year of Highly Active Antiretroviral Therapy

Patients	*Time of Therapy*		*P-value**
	Baseline	*1 Year*	
CD4 ≤ 350/mm^3 (n = 43)	267 ± 65 (200–372)	332.6 ± 93.3 (198–550)	<0.001
HIV–RNA in plasma†	2.34 ± 0.89 (1.39–3.69)	1.59 ± 0.43 (1.40–3.18)	<0.001
HIV–DNA in PBMCs‡	2.49 ± 0.69 (1.00–3.48)	1.69 ± 0.66 (1.00–2.70)	<0.001
HIV–RNA in PBMCs‡	2.12 ± 0.72 (1.00–3.48)	1.17 ± 0.66 (1.00–2.18)	<0.001
CD4 = 351–700/mm^3 (n = 39)	475 ± 78 (359–629)	533.9 ± 90.0 (395–720)	0.11
HIV-RNA in plasma†	2.33 ± 0.88 (1.39–3.69)	1.56 ± 0.44 (1.40–3.25)	<0.001
HIV-DNA in PBMCs‡	2.38 ± 0.45 (1.00–3.83)	1.54 ± 0.59 (1.00–2.54)	<0.001
HIV-RNA in PBMCs‡	2.03 ± 0.44 (1.00–3.11)	1.32 ± 0.45 (1.00–2.32)	<0.001

Data are mean ± SD (range). *By Student's t-test for dependent variables; †log copies/mL; ‡log copies/10^6 CD4+ T cells.

CD4 count between 351/mm^3 and 700/mm^3 maintained plasma RNA levels below 50 copies/mL at each time point.

To evaluate both CD4 counts and proviral DNA and RNA specific transcripts copy number in PBMCs during 1 year of therapy, patients in each group were divided into two subgroups according to their HIV-RNA levels in plasma: subgroup 1, with HIV-RNA copy numbers below 50 copies/mL at baseline and at each time point; subgroup 2, with detectable viremia at least once during the study period.

In subgroup 2, the mean decrease of HIV-RNA levels in plasma was 1.02 ± 0.63 log and 1.06 ± 0.62 log in patients with CD4 350/mm^3 or less and between 351/mm^3 and 700/mm^3, respectively ($P = 0.0000001$ for each group).

The mean CD4+ T-cell counts were 332.6 ± 93.3 and 533 ± 90.9 after 1 year of therapy ($P = 0.000226$ and $P = 0.11$, respectively), and in each group, no significant differences in the mean increase of CD4 count were observed between the two subgroups ($P = 0.11$ for ≤350/mm^3 CD4 group and $P = 0.96$ for 351/mm^3 to 700/mm^3 CD4 group).

The changes of proviral DNA concentrations owing to treatment are graphically depicted in Figure 1. There are persistent decreases from the baseline values in the two groups of patients ($P < 0.001$, for each group). The mean decreases in proviral DNA copy number in patients of subgroup 1 were 0.67 ± 0.7 and 1.03 ± 0.53, respectively in patients with CD4 count 350/mm^3 or less and with CD4 count between 351/mm^3 and 700/mm^3. In each group, no significant differences in proviral DNA decreases were observed between patients in subgroups 1 and 2. Proviral DNA was higher in patients of subgroup 2 than in patients of subgroup 1 both in the group with CD4 count of 350/mm^3 or less and between 351/mm^3 and 700/mm^3 ($P = 0.0025$ and $P = 0.02$, respectively). In subgroup 1, 9 of 13 and 11 of 12 patients with CD4 count 350/mm^3 or less and between 351/mm^3 and 700/mm^3, respectively, had detectable levels of proviral DNA in PBMCs at baseline. After 1 year of therapy, the corresponding figures were 4 of 13 and 4 of 12, respectively (chi-squared test with baseline values $P = 0.049$ and $P = 0.003$, respectively).

In subgroup 2 of each group, 1 of 30 patients with CD4 count 350/mm^3 or less and 0 of 27 with CD4 count between 351/mm^3 and 700/mm^3 had undetectable levels of proviral DNA in PBMCs at baseline. The corresponding figures were 10 of 30 and 12 of 27 at the end of the study period (chi-squared test with baseline values $P = 0.003$ and $P = 0.00009$, respectively).

In subgroup 2 no significant correlations were observed between decreases of HIV-RNA in plasma and proviral DNA in PBMCs ($P = 0.60$ and $P = 0.62$ in groups with ≤ 350 and 351–700/mm^3 CD4, respectively).

Concentrations of RNA-specific transcripts decreased significantly after 1 year of therapy in patients with CD4 counts 350/mm^3 or less and those with between 351/mm^3 and 700/mm^3 ($P < 0.001$ for each group) (see Table 1). In each group, a significant decrease of RNA transcripts also was observed in patients of subgroup 1 ($P = 0.0004$ for both groups). The mean values of RNA-specific transcripts were constantly higher in patients of subgroup 2 than in patients of subgroup 1, in both groups, CD4 count 350/mm^3 or less and between 351/mm^3 and 700/mm^3 ($P < 0.001$ for each group). In subgroup 2 of each group, the decrease of US-RNA transcript levels was correlated with that of HIV-RNA in plasma ($P = 0.00003$ for both groups). At baseline, in subgroup 1 RNA-specific transcripts had detectable levels in 11 of 13 and in 11 of 12 patients with CD4 count 350/mm^3 or less or between 351/mm^3 and 700/mm^3, respectively, and significant correlations were observed between decreases of US-RNA transcript

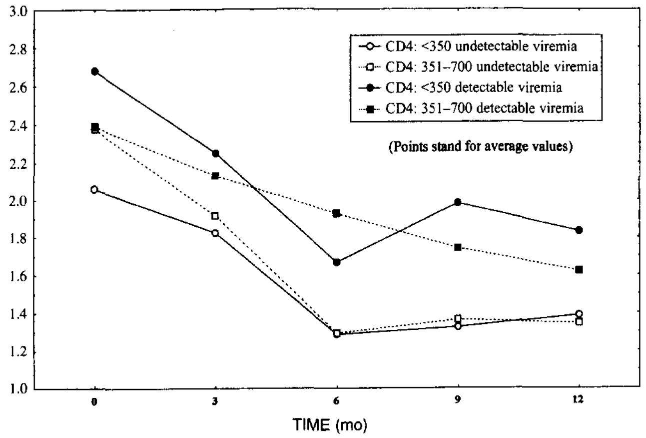

Figure 1. Mean change in proviral DNA copy number from baseline for patients with undetectable and detectable viremia and CD4 350/mm^3 or less or between 351/mm^3 and 700/mm^3.

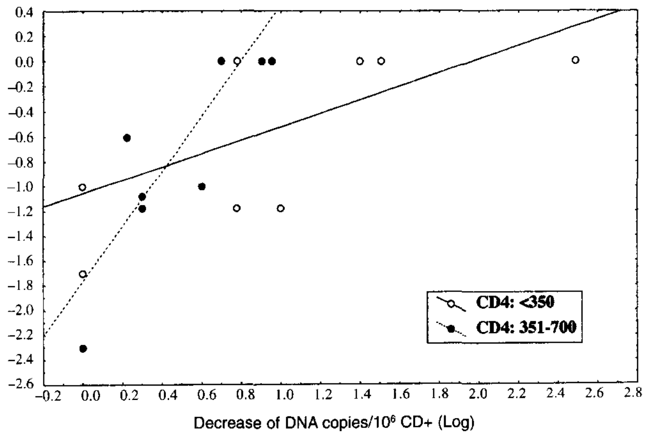

Figure 2. Correlations between decreases of US-RNA transcript and proviral DNA levels in patients with undetectable viremia and CD4 350/mm^3 or less or between 351/mm^3 and 700/mm^3.

Table 2. Relation between HIV-DNA and US-RNA Transcript Detection PBMCs after 1 Year of Therapy in Patients with Undetectable Viremia

		*US-RNA Transcripts**	
Patients	*Number Tested*	*Positive*	*Negative*
CD4 ≤ 350 cells/mm^3 (n = 13)			
DNA positive	4	0	4
DNA negative	9	0	9
CD4 351–700 cells/mm^3 (n = 12)			
DNA positive	4	2	2
DNA negative	8	0	8

*Positive or negative by cRT-PCR. US-RNA = unspliced RNA.

and proviral DNA levels in PBMCs (P = 0.0085 and P = 0.00024, respectively) (Figure 2). For these patients, the relation between HIV-DNA and US transcript detection in PBMCs after 1 year of therapy is presented in Table 2. In the group with CD4 count 350/mm^3 or less, proviral DNA remain detectable in 4 of 13 patients, whereas all samples were negative for presence of US-RNA transcripts. Four of 12 patients with CD4 count between 351/mm^3 and 700/mm^3 were positive for proviral DNA, and two of these also were positive for presence of US-RNA transcripts.

DISCUSSION

An important goal in the quantification of viral load in patients infected with HIV-1 is direct measurement of the impact of antiviral therapy. Previous studies have demonstrated that HIV-1 proviral DNA in PBMCs provides a direct measurement of the number of infected cells and correlates with disease progression.[23-25] Furthermore, proviral DNA remained detectable in PBMCs of infected individuals despite the fact that some, including HAART-treated patients, had undetectable plasma RNA levels for over 1 year.[10,11,26]

The present study quantified viral RNA in plasma, US-RNA transcripts, and proviral DNA in PBMCs in 82 HIV-1-infected patients treated with HAART for up to 1 year. Results confirm previous studies, indicating that treatments with potent antiretroviral regimens can produce significant reduction of HIV-RNA in plasma. Both in patients with CD4 count 350/mm^3 or less and in those with CD4 count between 351/mm^3 and 700/mm^3, a significant decrease of HIV-RNA in plasma and a persistent and significant decrease of proviral DNA concentrations in PBMCs were noted. At the same time, a parallel increase of CD4 T-cell count was observed, although it reached statistical significance only in those with CD4 cell counts of 350 cells/mm^3 or less. When the two groups of patients were divided into subgroups according to their HIV-RNA levels in plasma, no significant differences in proviral DNA decreases were observed between patients with undetectable viremia at baseline and at each time point and those with low but detectable viremia. In the past few years, PBMC proviral DNA quantitation has been used to assess the viral load response to therapy with immunomodulators and nucleoside compounds. However, these studies came to different conclusions about the efficacy of nucleoside inhibitors in reducing proviral DNA copy number in these cells.[27-33] The significant decrease of proviral load in PBMCs during HAART observed here could be explained by specific inhibition of both reverse transcriptase and protease viral functions that affect the production of infectious virus.

The results of the subgroups of patients with undetectable viremia were analyzed in detail. At baseline, proviral DNA had detectable levels in the majority of patients: 9 of 13 (69%) and 11 of 12 (91%) patients with CD4 count 350/mm^3 or less and CD4 count between 351/mm^3 and 700/mm^3, respectively. A persistent and significant decrease in proviral DNA copy number was observed in these patients. Despite this decrease, at the end of the study period proviral DNA still was detectable by quantitative PCR in 4 of 13 (31%) and 4 of 12 (33%) patients. Results concerning persistence of proviral DNA are consistent with recent studies in which a small but relatively stable compartment of CD4+ T cells carrying replication-competent provirus was detected in aggressively treated patients by limiting dilution virus culture of highly purified resting cells.[10] However, although more sensitive and specific than proviral DNA quantitation by PCR, the enhanced co-culture method currently is not available in routine clinical practice to measure the viral reservoir. Although part of proviral DNA detected using PCR may consist of unintegrated or defective genomes, the circulating PBMCs (and those in lymph nodes) propagate their defective proviral copies by cell division, a process that is not inhibited by antiretroviral therapy. The defective genomes are found in a high proportion of proviral DNA from PBMCs,[34,35] but a low frequency of resting CD4+ T cells harboring replication-competent provirus still can be detected.[10] In addition, cells that contain unintegrated provirus may transform to virus-producing cells when provirus is extended and integrated into the cellular genome.[36] This implies that even if HAART abolishes or reduces the production of new viral particles to undetectable levels, HIV-1 infection can be monitored as long as proviral DNA remains detectable.

In patients of subgroups with undetectable viremia, levels of US-RNA transcripts in PBMCs decreased significantly after 1 year of therapy, and this reduction was correlated with that of proviral DNA. At the end of the study period, RNA transcripts were still detectable in two patients. Although quantitative analyses of US-RNA transcripts in PBMCs have been used to assess viral RNA expression levels,[18-20] they can represent either genomic or mRNA, and whether this persistence is attributable to low viral transcriptional activity or a reduction of packaging (and hence release) of infectious virus could not be determined in this study. However, Natarajan and colleagues demonstrated that prolonged treatment with HAART does not result in total suppression of viral replication despite suppression of plasma HIV-RNA to undetectable levels.[37]

Results obtained in this work demonstrate that proviral DNA quantitation in PBMCs meets some requirements for good monitoring of HAART: (1) at baseline it is measurable in the majority of patients, including those with undetectable RNA in plasma; (2) it permits evaluation of the number of infected cells that represent an important viral reservoir; (3) it allows detection of few DNA copies in 10^5 PBMCs; and (4) it decreases even in patients with persistent suppression of plasma viremia.

The use of proviral DNA as a surrogate marker of HIV infection still is to be established. However, data from this study suggest that it could be useful in monitoring HIV-infected patients with undetectable viremia, in whom persistence of proviral DNA can lead to an intensification of therapy regimen.[38] Further evaluation is needed to correlate the observed trend of proviral DNA with clinical endpoints.

In conclusion, in HAART-treated patients, quantification of proviral DNA in PBMCs could be used to determine the number of infected cells and may represent the next step to monitor patients with suppression of plasma viremia.

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