Evaluation of Cell-Free and Cell-Associated Peripheral Blood Human Immunodeficiency Virus Type 1 RNA Response to Antiretroviral Therapy

Laura Romano, Giulietta Venturi, Marinunzia Catucci, Angelo De Milito, Pier E. Valensin, and Maurizio Zazzi

Plasma human immunodeficiency virus (HIV) type 1 RNA load is the reference marker for response to antiretroviral therapy. To compare peripheral blood mononuclear cell (PBMC)-associated and plasma HIV-1 RNA response to treatment, HIV-1 RNA was quantified by reverse transcription–competitive polymerase chain reaction in 20 patients at 0, 12, and 24 weeks following addition of saquinavir to their treatment regimens. HIV-1 RNA was undetectable in 15 plasma samples but in only 2 PBMC samples (P = .002) and CD4 cell counts correlated more with PBMC than with plasma HIV-1 RNA load. Changes in HIV-1 RNA load in PBMC and in plasma were correlated, and the decrease was higher in plasma than in PBMC at weeks 12 (P = .002) and 24 (P = .017). Moreover, PBMC, but not plasma HIV-1 load, at week 12 was predictive of HIV-1 RNA levels at week 24 in both plasma (P = .004) and PBMC (P < .001). Thus, measurement of PBMC HIV-1 RNA may be useful during antiretroviral therapy.

Because the amount of cell-free genomic human immunodeficiency virus (HIV) type 1 RNA in plasma is a strong predictor of disease progression [1], quantitation of plasma HIV-1 RNA has been the key marker for treatment management of HIV-infected subjects [2]. There is evidence that plasma HIV-1 RNA levels promptly decrease in response to successful antiretroviral therapy and, accordingly, rebounding plasma HIV-1 RNA titers are interpreted as a marker for anti–HIV-1 treatment failure [2, 3]. However, the possibility that other measurable virologic parameters promptly respond to antiretroviral therapy and provide valuable information for management of infected patients has not been fully investigated. Because circulating cell-free HIV-1 RNA is released from infected cells in lymphoid tissues and in the bloodstream, cell-associated HIV-1 RNA levels should theoretically be an appropriate direct indicator of viral replication during antiretroviral treatment. Short-term follow-up studies of small numbers of previously untreated (drug-naive) patients have suggested that potent combination therapy with reverse transcriptase and protease inhibitors may significantly lower both cell-free and cell-associated HIV-1 RNA [4, 5]. However, HIV-1 replication has been less effectively blocked in lymphoid tissues than in peripheral blood mononuclear cells (PBMC) [4], and combined reverse transcriptase inhibitors have failed to decrease both peripheral blood and lymphoid tissue cell-associated HIV-1 RNA levels while significantly reducing plasma HIV-1 RNA load [6]. While lymphoid tissue biopsies are not suitable for large-scale routine examination, there are no major obstacles to developing commercial kits for titration of HIV-1 RNA in PBMC.

In vitro studies have shown that HIV-1 RNA expression in PBMC is regulated by a complex mechanism of alternative splicing producing three major size classes of transcripts: unspliced RNA coding for gag and pol proteins and constituting new genomes, singly spliced RNA coding for env and vpu proteins, and multiply spliced RNA coding for most of the regulatory proteins [7–9]. Several studies have addressed a possible in vivo correlation between differential expression of HIV-1 transcripts and disease progression. Saksela et al. [10] detected increased expression of both unspliced and multiply spliced HIV-1 mRNA in patients with subsequent disease progression and a transient decrease in unspliced and multiply spliced HIV-1 RNA titers following zidovudine therapy. Other studies [11, 12] reported a relative increase in the ratio between unspliced and multiply spliced HIV-1 mRNA associated with disease progression. Undetectable levels of multiply spliced HIV-1 mRNA were found in most long-term nonprogressors [13]. The complexity of HIV-1 mRNA expression and the heterogeneity of singly and multiply spliced transcripts make it difficult to establish a definite correlation between a specific HIV-1 mRNA splicing pattern and the clinical course of HIV-1 infection. Nevertheless, the amount of a single class of HIV-1 RNA in PBMC could be conveniently investigated as a possible marker for assisting with treatment of HIV-infected patients.

We did a retrospective quantitative analysis of plasma and PBMC unspliced HIV-1 RNA in 20 subjects receiving combined...
treatment with reverse transcriptase inhibitors and the protease inhibitor saquinavir. Titration of cell-free and cell-associated HIV-1 RNA was done by in-house reverse transcription (RT) competitive polymerase chain reaction (cPCR) systems on plasma and PBMC RNA stored at weeks 0, 12, and 24 after saquinavir was added to treatment regimens.

Materials and Methods

Patients and samples. A retrospective quantitative analysis of PBMC and plasma HIV-1 RNA was done on 60 blood samples from 20 HIV-1-infected adults. At baseline, 3, 4, 8, 1, and 4 patients were classified as CDC stage A2, A3, B3, C2, and C3, respectively; all had <350 × 10⁶/L CD4 cells (range, 34–340). Therapy with saquinavir (600 mg 3 times/day) was added to their treatment protocols—zidovudine plus zalcitabine or zidovudine plus didanosine, which had been given 8–20 months. Samples were obtained from each patient at weeks 0, 12, and 24 after initiation of saquinavir therapy. PBMC were separated from 5 mL of citrated blood by standard Ficoll centrifugation and washed twice with PBS. Cellular RNA was purified by use of trigeneric BD (Molecular Research Center, Cincinnati) and resuspended in 40 μL of diethylpyrocarbonate-treated water. Plasma RNA was isolated by spin columns with a kit (QiAamp Viral RNA kit; Qiagen, Hilden, Germany). Both PBMC and plasma RNA were stored at −70°C for up to 12 months before testing.

Construction of HIV-1 DNA competitor and HIV-1 RNA control. A deleted competitor HIV-1 DNA fragment was generated by PCR amplification of a pol sequence of the HIV-1 Z6 genome in plasmid pSYC1857 (Perkin-Elmer, Emeryville, CA) using antisense primer T52/LR62 and sense primer T52. Primer T52 (5'-CTATCAATACATGGATGATTTGTATGT-3') hybridizes to position 3163–3182 of the HIV-1 ARV2/SF2 isolate (GenBank accession no. K04007) with its 3' terminal 20 bases and contains a 27-base 5' tail (underlined) identical to position 3096–3122. PCR amplification with primers LR52 (5'-CTATCAATACATGGATGATTTGTATGT-3') and LR62 (5'-CTGTCATGTGATGTGACTGAGAGCAGACA-ACATCT-3') hybridizes to position 3163–3182 of the HIV-1 ARV2/SF2 isolate (GenBank accession no. K04007) with its 3' terminal 20 bases and contains a 27-base 5' tail (underlined) identical to position 3096–3122. PCR amplification with primers LR52 and LR62 (5'-CTGTCATGTGATGTGACTGAGAGCAGACA-ACATCT-3') generates a 237- or 197-bp product when wild type HIV-1 RNA-derived cDNA or the T52/LR62 competitor DNA, respectively, is used as the template. Concentration of the purified T52/LR62 DNA fragment was estimated by agarose gel electrophoresis and spectrophotometry. A wild type HIV-1 RNA standard was generated by insertion of the wild type LR52/LR62 HIV-1 DNA fragment into plasmid pCRJ1 (Invitrogen, Leek, The Netherlands), linearization at a unique SalI site, and transcription in vitro using T7 RNA polymerase. The purified wild type HIV-1 RNA standard was titrated by spectrophotometer, added to HIV-1-negative plasma, and used in preliminary experiments in order to estimate the yield of RNA extraction and RT.

Quantification of HIV-1 RNA in plasma. Features and clinical application of the in-house RT-cPCR will be described in detail elsewhere. The procedure involves RNA extraction by spin columns, ready-to-use bead-mediated RT, cPCR in a microtiter plate, agarose gel electrophoresis of the reaction products, and densitometric analysis of the digitized image of the gel. Comparative tests showed that this system has the same sensitivity (400 RNA copies/mL) and intertest variability (41.9%, calculated by quadruplicate testing of 12 samples) as the reference HIV-1 monitor kit (Amplisor; Roche Molecular Systems, Branchburg, NJ). Results obtained with the two assays in a panel of 45 clinical samples were in good agreement (mean difference, 0.36 ± 0.25 log₁₀). In brief, RT was done for 30 min at 37°C by resuspending the first-strand cDNA synthesis beads (Ready-to-Go You-Prime; Pharmacia, Uppsala, Sweden) with 20 μL of template RNA and 6 pmol of primer LR62. Four identical aliquots of the cDNA mixture were then used as templates for a 44-cycle PCR in the presence of 10 pmol of the primer pair LR52/LR62 and of increasing amounts (10, 60, 360, and 2160 copies) of the competitor T52/LR62 DNA fragment. PCR products were electrophoresed on 2.4% NuSieve–0.6% Seakem (FMC, Rockland, ME) agarose gels, stained with ethidium bromide, and analyzed by densitometric scanning.

The log₁₀ of the ratio between the intensity of the competitor band (corrected for the lower length in base pairs) and that of the wild type band was plotted against the log₁₀ of the number of copies of corresponding DNA added. Linear regression was used to calculate the wild type cDNA copy number at the equivalence between the corrected competitor and wild type band intensities [14, 15]. Repeated experiments with different amounts of the titrated HIV-1 RNA standard allowed us to determine that the proportion of RNA that is reproducibly obtained from plasma and reverse transcribed into cDNA is 40% ± 5%. Thus, a 2.5 correction factor was applied to the calculated cDNA titer to obtain the estimated number of copies of RNA in the starting material. HIV-1 RNA load in plasma was expressed as copies per milliliter.

Quantitation of HIV-1 RNA in PBMC. Since PBMC contain both HIV-1 DNA and RNA, an RNA-specific (RS)-PCR system was developed to avoid amplification of background DNA. RS-PCR [16] is a modification of conventional RT-PCR in which RNA is reverse transcribed with a primer containing a 3' target-specific sequence and a 5' unique sequence unrelated to the target. Thus, all cDNA molecules generated contain a 5' tail not hybridizing to background DNA but stabilizing annealing to amplification products obtained from cDNA. When an appropriately high annealing temperature is used, the RNA-derived cDNA but not the corresponding DNA is efficiently amplified by PCR. Control experiments using titrated [15] HIV-1 RNA from infected H9 cells and the HIV-1 RNA standard showed that primer RS1 (5'-TCTG-TCTGTTGACATAACTTCTGTATGT-3') can be used as the RT primer in an RS-PCR amplification of PBMC HIV-1 RNA in the presence of up to 10⁶ background HIV-1 DNA copies. Primer RS1 hybridizes to HIV-1 RNA positions 3323–3339 with its 3' 17 bases and contains a 13-base unrelated sequence (underlined) at its 5' end. Following completion of the RT step, cPCR was done with primers LR52 and RS1 as described above, except that a T52/RS1 DNA fragment was used as the competitor target. PCR products derived from amplification of wild type and competitor targets are 217 and 257 bp long, respectively. Data analysis was done as above, and HIV-1 RNA load in PBMC was expressed as copies per 10⁶ CD4 cells. Reconstruction experiments with titrated HIV-1–positive plasma and uninfected PBMC were done to check that plasma HIV-1 RNA did not copurify with and affect measurement of PBMC HIV-1 RNA. These experiments showed that uninfected PBMC RNA does not contain detectable levels of HIV-1 RNA.
Figure 1. CD4 cell counts and HIV-1 RNA levels in plasma and peripheral blood mononuclear cells (PBMC) in 20 subjects after addition of saquinavir (SQV) to treatment with nucleoside reverse transcriptase inhibitors.
Table 1. Spearman’s correlation of CD4 cells, plasma, and peripheral blood mononuclear cell (PBMC) HIV-1 RNA titers at weeks 0, 12, and 24 of saquinavir therapy.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Correlation coefficient (P)</th>
<th>Week 0</th>
<th>Week 12</th>
<th>Week 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma HIV-1 RNA vs. PBMC HIV-1 RNA</td>
<td>0.579 (.0075)</td>
<td>0.381 (.0960)</td>
<td>0.648 (.0020)</td>
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<tr>
<td>CD4 cells vs. PBMC HIV-1 RNA</td>
<td>-0.453 (.0437)</td>
<td>-0.553 (.0111)</td>
<td>-0.454 (.0437)</td>
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<tr>
<td>CD4 cells vs. plasma HIV-1 RNA</td>
<td>-0.322 (.1610)</td>
<td>-0.233 (.3172)</td>
<td>-0.346 (.1324)</td>
<td></td>
</tr>
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Figure 2. Scatter plots of correlation between variation in plasma HIV-1 load (log10 HIV-1 RNA copies/mL) and peripheral blood mononuclear cell (PBMC) HIV-1 load (log10 HIV-1 RNA copies/10^6 CD4 cells) after 12 and 24 weeks of saquinavir therapy.
If a >1 log₁₀ reduction in virus load with respect to baseline is considered a sign of successful therapy, then effective treatment was detected in 10 (50%) and 4 (20%) of the 20 patients at week 24 as assessed by quantification of HIV-1 RNA in plasma and PBMC, respectively. Of interest, PBMC HIV-1 RNA levels at week 12 were predictive of both PBMC and plasma HIV-1 RNA load at week 24 (P<.001 and P = .004, respectively), while the predictive value of plasma viremia at week 12 was none or limited for PBMC or plasma HIV-1 RNA titers, respectively, at week 24 (figure 3).

Discussion

Recent evidence suggests that HIV-1 protease inhibitors are substantially decreasing HIV-1–related morbidity and the incidence of AIDS-defining events [17, 18]. However, virologic failure of protease inhibitor treatment, as documented by plasma HIV-1 RNA load, appears to be more common than previously thought [19]. Failure to maintain HIV-1 viremia at undetectable or low levels is ultimately expected to critically favor disease progression and the need to change antiretroviral drugs [2]. Early detection of virologic treatment failure avoids prolonged use of ineffective drugs with the potential for adverse side effects and the selection of HIV-1 variants with reduced sensitivity to other compounds. Indeed, cross-resistance patterns have been documented during therapy with protease inhibitors [20, 21]. While commercial kits for measuring plasma HIV-1 RNA have played a key role in the management of infected persons, it remains to be established whether additional virologic markers can be measured that sensitively and promptly mark HIV-1 replication during antiretroviral therapy [22]. Failure to decrease cell-associated HIV-1 RNA levels as effectively as plasma HIV-1 RNA has been documented in small studies of “drug-naive” persons subjected to antiretroviral combination therapy [4, 6]. Although lymphoid tissues should be considered the material of choice to study HIV-1 replication, PBMC can be easily obtained from patients and may provide useful information for regular monitoring of antiretroviral therapy.

To comparatively study the course of plasma and PBMC HIV-1 RNA during changes in antiretroviral treatment, we retrospectively analyzed patients treated with saquinavir after prior treatment with combined reverse transcriptase inhibitors. Persons treated with saquinavir experience an early rebound in plasma HIV-1 load more often than those treated with other protease inhibitors [2, 19, 23]. This group was thus expected to facilitate detection of possible differences between time courses of HIV-1 RNA in plasma and PBMC during relapse of virus replication. Overall, HIV-1 RNA load in PBMC and plasma followed similar dynamics during the 24-week observation period, suggesting that both plasma and PBMC HIV-1 RNA respond to antiretroviral therapy. However, the HIV-1 RNA load decrease was significantly more substantial and sustained in plasma than in PBMC. Indeed, residual antiviral activity was still present at week 24 as assessed by reduction in plasma HIV-1 RNA load with respect to baseline, while PBMC HIV-1 RNA levels rebounded to baseline values. It is of interest that PBMC HIV-1 RNA titers were measurable in 13 (86.7%) of the 15 blood samples with undetectable plasma HIV-1 RNA, since availability of an alternative marker of viral replication may improve treatment when plasma viremia has been abated to undetectable levels. Moreover, the stronger correlation of CD4 cell counts with PBMC than with plasma HIV-1 RNA suggests that HIV-1 replication as detected by measurement of PBMC-associated viral RNA may be directly associated with damage to immune function.

It was of interest that PBMC but not plasma HIV-1 RNA at week 12 was predictive of both PBMC and plasma HIV-1 RNA levels at week 24. While this was derived in part from the lower RNA response in PBMC than with plasma HIV-1 RNA suggests that HIV-1 replication as detected by measurement of PBMC-associated viral RNA may be directly associated with damage to immune function.

Figure 3. Scatter plots of correlation between plasma (log₁₀ HIV-1 RNA copies/mL) or peripheral blood mononuclear cell (PBMC) (log₁₀ HIV-1 RNA copies/10⁶ CD4 cells) virus load at week 12 and plasma or PBMC virus load at week 24 of saquinavir therapy.
than plasma viremia, longer follow-up studies of persons under current therapeutic regimens are advisable.

Since cell-associated HIV-1 RNA appears to be maintained at a higher level than cell-free HIV-1 RNA in the bloodstream, long-lived circulating infected cells may play a role in generation of new viral genomes even in conjunction with undetectable plasma HIV-1 RNA titers. Peripheral blood HIV-1 DNA load is also minimally affected by antiretroviral therapy [24–26], confirming the persistence of infected PBMC during effective treatment. Although a variable proportion of these cell-associated HIV-1 genomes can be defective, maintenance of infectious HIV-1 titers has been shown by cocultivation of PBMC, while plasma HIV-1 RNA load was significantly reduced following reverse transcriptase inhibitor combination therapy in previously untreated persons [6]. Rescue of replication-competent HIV-1 from a reservoir of infected CD4 cells, both resident in lymph nodes and circulating in peripheral blood, has been reported in subjects with prolonged suppression of plasma HIV-1 RNA resulting from highly active antiretroviral therapy [27–29]. Further studies should be specifically aimed at defining the relationship between lymphoid tissue and PBMC-associated HIV-1 RNA and in determining whether measurement of PBMC HIV-1 RNA load can provide more information than quantitation of plasma viremia during highly active antiretroviral therapy. Should this be the case, no major obstacles are expected for adaptation of available kits [4] or development of reliable systems for measuring HIV-1 RNA in PBMC rather than in (or in addition to) plasma.

References


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