Shifts in cell-associated HIV-1 RNA but not in episomal HIV-1 DNA correlate with new cycles of HIV-1 infection in vivo

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HIV-1 replication in vivo is denoted by the production of infectious virus and concomitant new rounds of infections. To date, quantification of viral particles in plasma provides the most sensitive method for monitoring HIV-1 replication due to the high turnover rates of both free virions and of productively infected cells [1,2]. Upon initiation of effective combination antiretroviral therapy, plasma viraemia can be reduced to levels that are below the detection limits of current ultrasensitive tests (10 copies/ml) [3,4]. However, residual HIV replication may persist in a fraction of patients on long-term antiretroviral therapy despite suppression of plasma viraemia. This was demonstrated by sequence evolution of HIV-1 at the nucleotide level in PBMC and lymphoid tissue in patients undergoing successful antiretroviral therapy [5–7]. Assessment of viral evolution by sequence analysis is costly, time-consuming and technically very demanding. Therefore, alternative surrogate markers to detect and monitor residual low-level HIV-1 replication have been sought for.

Measurements of HIV-1 transcription in PBMC [8–10] and production of cell-associated viral particles [11] have been proposed as tools to monitor residual HIV-1 replication in vivo. However, the interpretation of measurements of HIV-1 transcription is ambiguous. Cell-associated HIV-1 transcripts detected need not necessarily be a consequence of newly infected target cells but can instead be products of reactivated latently infected cells [7]. In addition, basal viral transcription may also occur in HIV-1-infected cells independently of ongoing viral replication [11]. Recently, it has been proposed that 2-LTR episomes, nonessential byproducts of reverse transcription, due to their postulated short half-life, may be used to detect ongoing HIV replication [12–14]. However, the efficacy of 2-LTR DNA measurements to monitor for the presence of recently infected cells has been questioned by in vitro experiments revealing high stability of 2-LTR circles in some HIV-1-infected cell lines [15,16].

To further assess the value of 2-LTR circles and other potential surrogate markers for residual active replication in vivo, we used short STI as a model system resembling limited and in some cases low-level HIV-1 replication. HIV-1 replication in PBMC was monitored by measuring viral transcription, late HIV-1 DNA reverse transcripts encompassing all integrated and most unintegrated forms of HIV-1 DNA, and HIV-1 2-LTR circles. These cell-associated parameters were then analysed in relation to plasma viraemia to assess their efficacy in reflecting ongoing HIV-1 replication.
Materials and methods

Patients and patient specimens

Patients were enrolled in a substudy of the Swiss Spanish Intermittent Therapy Trial (SSITT) [17], which included an extended study protocol with frequent blood sampling. Inclusion criteria comprised combination antiretroviral therapy with two classes of drugs (non-nucleoside reverse transcription inhibitors excluded), plasma viraemia ≤50 HIV-1 RNA copies/ml for >6 months and CD4 T cell count >300 cells/ml. Patients provided written informed consent according to the guidelines of the University Hospital Zurich Ethics Committee. The current report addresses the first 28 days of the study, which comprised 2 weeks of therapy followed by 2 weeks on therapy. Blood samples were obtained on days 0, 4, 8, 14, 18, 21, 25 and 28. PBMC and plasma were separated from anticoagulated blood by ficoll gradient purification (Lymphoprep, Nycomed Roskilde, Denmark) and frozen at −80°C. Due to limited sample availability, the number of patient specimens tested was ≤14 on the following study days: day 4: HIV-UsRNAPBMC (n=13), day 18: HIV-UsRNAPBMC (n=13) and late-DNA/2-LTR circles (n=12), day 21: all tests n=13; day 25: HIV-UsRNAPBMC (n=10) and late-DNA/2-LTR circles (n=11), day 28: late-DNA/2-LTR circles (n=13).

Quantitation of HIV-1 RNA

Plasma HIV-1 RNA was quantified using a ultrasensitive modification of the HIV-1 monitor test (Roche, Rotkreuz, Switzerland) [18]. Cell-associated unspliced HIV-1 RNA was extracted and measured as described previously [9,11] using a modification of the HIV-1 monitor test (Roche, Rotkreuz, Switzerland).

Quantitation of HIV-1 DNA

Extraction of PBMC DNA

Cell extracts were prepared by direct lysis as previously described [19] and total cellular DNA was measured by fluorometry (Picogreen, Molecular Probes, Eugene, Oreg., USA) against a standard curve prepared from diluted PBMC lysates with known cell content. The nonintegrated fraction of PBMC-DNA was extracted as described previously [13].

Quantitation of late-DNA

PCR strategies to detect HIV-1 DNA are shown in Figure 1. Late-DNA from clinical specimens was measured with a modification of the HIV-1 monitor test (Roche, Rotkreuz, Switzerland) as previously described [20]. Late-DNA in PBMC cultures infected in vitro was measured by real-time PCR using primers and probe described previously [20]. PCR reactions were performed in duplicate. Serial dilutions of known amounts of linearized full-length HIV-1 plasmid pNL4-3 [21] were used as external standard. PCR reactions were performed as follows: DNA (5 µl) was added to 40 µl HotStartTaq Master-mix (QIAGEN, Hilden, Germany) supplemented with 1 µM of each primer, 0.3 mM of the fluorescent probe and a final concentration of 3 mM MgCl₂. Cycling and detection were performed in an i-cycler (Biorad, Basel, Switzerland). Cycling conditions were: 95°C 15 min followed by 40 cycles of 95°C 10 sec, 60°C 1 min). Copy numbers interpolated from the standard curve were calculated using the i-cycler software provided by the manufacturer.

Quantitation of 2-LTR circles

2-LTR circles were amplified with LTR-specific primers and PCR products were detected by hybridization requiring internal binding of two oligonucleotide probes (Figure 1). Serial fivefold dilutions of DNA extracts (5 µl) were tested in quadruplicates. DNA was amplified in 55 µl HotStartTaq Master mix (QIAGEN, Hilden, Germany) supplemented with 1.5 µM of the primers f56(tggtaacctagatcccctc) and m55c(acccctggcctggtgtgta) [94°C 10 min, 48°C 10 sec, 60°C 10 sec, 72°C 20 sec]. PCR products were denatured with 150 µl of 150 mM NaOH and specific 2-LTR circles were then detected by PCR ELISA with microtitre plates coated with capture-oligonucleotide m57(5′-amino-C6-ctaccaacacaaggtgactctgccgattggc-gaacc). Detection was dependent on hybridization of the capture oligonucleotide and a biotinylated probe (m58 5′-biotin-tagtcagtggaggaaatctctagcagtactggaagggctatt) to the plus-strand of the amplified DNA (Figure 1). Hybridization and detection of PCR products by ELISA was performed as described [11]. Detection limit of the measurement was 1 DNA copy/PCR as calculated by end-point dilution of a subcloned 2-LTR PCR product from patient 116. This PCR product was confirmed to represent genuine 2-LTR circles by sequencing (data

Figure 1. PCR strategies for measurement of HIV-1 nucleic acids

Locations of PCR primers and probes used for detection are indicated by arrows (5′→3′). Bio, 5′-biotin residues used for detection; capt, 5′-amino residues used to anchor capturing probes to solid supports. Fluorophore (f) and quencher (q) on the internal probe used to monitor real-time PCR.
Correlates of HIV-1 replication

not shown). Copy numbers of 2-LTR circles were calculated using 50% end-points [22]. When 2-LTR circles were only detected in one single PCR reaction, copy numbers were estimated assuming that one copy of 2-LTR circular DNA had been present in a total of four reactions. Detection limits of PCR-negative specimens for 2-LTR circles were calculated as <1 copy of 2-LTR circular DNA normalized to the input of cell equivalents into the four replicates tested.

Infection of PBMC in vitro
PBMC from HIV-1-negative donors (Schweizerisches Rotes Kreuz, Zürich, Switzerland) were depleted of CD8 cells (RosetteSep, StemCell technologies, Vancouver, BC, Canada) according to the manufacturer’s description and stimulated with 5 µg/ml phytohaemagglutinin (PHA) for 72 h. Cells were then adjusted to 2×10^6/ml in RPMI 1640, 10% FCS, 100 U/ml IL-2 and used for infection experiments. Infections with a primary X4 viral isolate 2044 [23] and a R5 isolate Case C 1/85 [24] were performed using a multiplicity of infection of 0.1. After 48 h, HIV-1 replication was arrested using a mixture of AZT, nelfinavir and efavirenz (10 µM each, obtained from the NIH AIDS Research and Reference Reagent Program). Medium with antiretrovirals was replaced every 2–4 days. In parallel, infected controls were cultivated in the absence of antiretrovirals.

Calculations and statistics
HIV-1 RNA measurements were normalized to the input of total cellular RNA (expressed in copies/10^6 cell equivalents) assuming that 1 µg of total cellular RNA represents 10^6 cell equivalents as previously shown [9]. Copy numbers of HIV-1 DNA were normalized to cellular input assuming that 10^6 cells contained 7 µg of genomic DNA [25].

Unless stated otherwise, statistical analyses were performed using log_{10} transformed values utilizing GraphPad Prism software (AMPL Software Turramurra, Australia). In order to represent PCR-negative specimens, in our analysis detection limits of individual tests were used. Overall, 79% (A), 95% (B), 100% (C) and 71% (D) of measurements (A: HIV-UsRNA\text{\textsubscript{plasma}}, B: HIV-UsRNA\text{\textsubscript{PBMC}}, C: late-DNA, D: 2-LTR circles) were PCR-positive.

Results
Longitudinal analysis of HIV-1 nucleic acid levels in vivo
The aim of the current study was to assess potential surrogate markers of recent limited HIV replication, which would allow detection of residual virus replication during antiretroviral therapy. To this end we quantified distinct species of cell-associated HIV-1 nucleic acids and analysed their performance as predictors of recent HIV-1 replication. Levels of HIV-RNA\textsubscript{plasma}, HIV-UsRNA\textsubscript{PBMC}, 2-LTR circles and late-DNA were monitored longitudinally in peripheral blood of 14 HIV-1-infected patients during 2 weeks of structured treatment interruption and during 2 weeks after reinitiation of therapy (Figure 2A, B). At baseline, plasma viraemia was suppressed to <50 RNA copies/ml in all patients. Hence, HIV-RNA\textsubscript{plasma} was still detectable in 43% of patient samples at lower levels (mean 31 ±6 copies/ml). Cell-associated HIV-UsRNA\textsubscript{PBMC} and late-DNA were detected in all patients at baseline (mean values of 279 ±118 and 266 ±89 copies/10^6 cells, respectively). 2-LTR circles were detected in 71% of specimens at baseline (mean 21 ±6 copies/10^6 cells). Viral rebound during STI led to a significant elevation in levels of HIV-RNA\textsubscript{plasma} between baseline (day 0) and day 14 (P<0.0001; paired t-test). Simultaneously, levels of HIV-UsRNA\textsubscript{PBMC} were also significantly increased (P=0.003), while changes in levels of late-DNA and 2-LTR circles did not reach significance (P>0.2) (Figure 2B).

Correlation analysis of HIV-1 nucleic acids in vivo
To further probe the efficacy of the different parameters in reflecting recent viral replication and to determine their interdependencies, cross-sectional correlation analysis was performed for each time point (Figure 3). A highly significant correlation between levels of HIV-RNA\textsubscript{plasma} and HIV-UsRNA\textsubscript{PBMC} was observed at the peak of viraemia (day 14). This correlation remained significant at all time points tested after reinitiation of treatment with one exception (day 25). However, at this time point only specimens for 10 of the 14 enrolled patients were available. Interestingly, although there was no correlation between HIV-RNA\textsubscript{plasma} and late-DNA contents during rebound of viraemia, these parameters were significantly correlated during the decay of viraemia (days 18–28). Similarly, 2-LTR circles and HIV-RNA\textsubscript{plasma} were not correlated during rebound. However, in contrast to late-DNA, 2-LTR circles correlated only weakly with plasma RNA levels during the decay of viraemia. Significance in the correlation of these two parameters was only reached on sampling days 18 and 28. Of note is that we observed a highly significant correlation of late-DNA with both, HIV-UsRNA\textsubscript{PBMC} and 2-LTR circles. A correlation of HIV-UsRNA\textsubscript{PBMC} with 2-LTR circles was observed but did not reach significance at all time points tested.

Specificity of the 2-LTR circles detection
In contradiction to previous reports [12,13], which proposed measurements of 2-LTR circles as surrogate
marker of ongoing viral replication, we found that levels of 2-LTR circles did not specifically reflect recent rounds of infection. In fact, levels of 2-LTR circles remained unchanged in the 4-week study period despite ongoing HIV replication. Of note is that previously 2-LTR circles were quantified in purified extracts of unintegrated episomal DNA [13], whereas our method is based on the analysis of total lysates of PBMC. To verify that the observed differences between our and previous studies are not due to the use of different assay systems, we compared both extraction methods. Altogether, 2-LTR circles were measured in episomal DNA extracts in PBMC derived at baseline and day 14 from eight patients (104, 111, 116, 120, 125, 121, 127, 128). We noticed a loss in sensitivity of the 2-LTR circles measurement, when the selective DNA extraction procedure was performed. Overall, only 50% of specimens were PCR-positive after episomal DNA extraction compared to 68% using total lysates. It is noteworthy that the frequency of 2-LTR circles detection after episomal DNA extraction was similar to the frequency described by Sharkey et al. [13]. Importantly, no increase of 2-LTR circles upon cessation of therapy was observed when the specific episomal extraction procedure was performed. Mean changes between days 0 and 14 in 2-LTR circle contents estimated using either episomal extraction or total extracts did not differ significantly (P=0.51, t-test on absolute values). Total DNA extracts include both chromosomal and episomal DNA. Therefore, an obligatory prerequisite of our method is the specificity of our probes for the detection of 2-LTR circles independent of proviral HIV DNA contents. Our assay combines an amplification step with LTR-specific primers and detection of the PCR products with two internal probes, one spanning the 2-LTR ligation junction and one mapping to U3 (Figure 1). We confirmed the specificity of this technique using 10^6 copies of linearized cloned proviral DNA as target in our test. No PCR products could be detected with our 2-LTR assay from this high input of proviral DNA (data not shown). Hence, assay differences or assay inadequacies did not account for the discrepancy in observations regarding the dynamics of 2-LTR circles in vivo.

Assessment of HIV-1 DNA stability in vitro

The hypothesis that levels of 2-LTR circles are an indication of recent low-level HIV replication had been mainly based on the finding that 2-LTR circles in HIV-infected T cell lines rapidly decay (1 log_{10}/day) [13]. Analogous to this, we tested whether 2-LTR circles are similarly unstable in primary cells. To this end, mitogen-stimulated primary CD4 T cells were infected with a X4 using primary isolate (2044, Figure 4A) or a R5 using primary isolate (Case C 1/85, Figure 4B). HIV replication was arrested after 2 days by addition of a mixture of three antiretroviral drugs and 2-LTR circles and late-DNA was monitored over a period of 7 days. Both viral isolates were confirmed to be fully sensitive to the individual antiretroviral drugs as well
as the combination of drugs in vitro by monitoring p24 production in independent assays (data not shown). No decrease of absolute levels in 2-LTR circles in antiretroviral-treated PBMC infected with the X4 isolate between day 2 and day 9 after infection was observed, whereas in cultures infected with the R5 isolate a slight reduction was noticed (0.07 log10/day as determined by linear regression). However, this decrease was indistinguishable (P=0.09) from the decay of late-DNA in the same culture (0.11 log10/day), indicating that infected cells were lost during the assay period. We further analysed the interdependencies of 2-LTR circles and late-DNA to control for cell death in the infected cell population. We found that relative amounts of 2-LTR circles/late-DNA did not decrease after addition of antiretroviral drugs (data not shown). Instead, we found a high correlation between 2-LTR circles and late-DNA in both drug-treated and control cultures (Figure 4C). Thus, 2-LTR circles remained highly stable in infected primary PBMC over a 7-day assay period.

Discussion

Treatment of HIV-1 infection with potent antiretroviral regimens can result in a substantial reduction of viral replication below the detection limit of current ultrasensitive assay systems. However, based on sequence evolution, ongoing low-level virus replication has been demonstrated in some patients on successful antiretroviral therapy [5,6]. Being able to accurately and easily gauge the extent and dynamics of this persisting viral replication is very important for the future development as well as management of antiretroviral therapy. Since this low-level replication cannot be detected by conventional viral RNA measurements in plasma, surrogate markers have been sought for. Several groups have suggested that 2-LTR circles...
circular DNA reverse transcripts due to their postulated instability are suitable markers of recent low-level replication [12,13].

In the present report, a group of patients was studied through a 2-week interval of STI and the subsequent 2 weeks of recommenced antiretroviral treatment. The short-term interruption of antiretroviral treatment led to a substantial rebound of viral replication. However, in agreement with an earlier study of HIV DNA levels in patients with intermittent virological failure of therapy [26], this transient increase in plasma viraemia was not accompanied by a measurable increase in the pool size of HIV-infected cells in the periphery. Therefore, this setting allowed us to assess the potential of possible surrogate markers for recent HIV replication.

Although the pool size of HIV-infected cells as measured by late-DNA was not indicative for ongoing HIV-1 replication it appeared to predict the magnitude of viral rebound in plasma as evidenced by a trend for correlation of late-DNA levels with levels of 2-LTR circles in vitro. Linear regression analysis of the data from untreated cultures (open symbols, n=6) and cultures treated with antiretroviral drugs (closed symbols, n=4) resulted in curves with slopes and y-intercepts that were not significantly different from each other (P>0.16 and 0.11, respectively) allowing the combination of data sets. Closed lines show the predicted curve from a linear regression and the corresponding 95% confidence intervals (dashed lines) using all data depicted in panel A and B (r²=0.87, slope=1.01 ±0.09, y-intercept=–0.85 ±0.51, P=0.0001, n=10).

Figure 4. Stability of HIV DNA in primary CD4 cells infected in vitro

Primary CD4 cells were infected with viral isolates 2044 (A), and Case C 1/85 (B). Two days after infection HIV replication was arrested by addition of reverse transcriptase inhibitors (zidovudine and efavirenz) and a protease inhibitor (nelfinavir). One of three independent experiments is shown. Time points were tested in duplicates. Mean values and error bars are shown. Closed symbols depict data from cultures treated with antiretroviral drugs and open symbols show data from control cultures. A, B: triangles denote late-DNA and circles depict 2-LTR circles. C: correlation of late-DNA levels with levels of 2-LTR circles in vitro. Linear regression analysis of the data from untreated cultures (open symbols, n=6) and cultures treated with antiretroviral drugs (closed symbols, n=4) resulted in curves with slopes and y-intercepts that were not significantly different from each other (P>0.16 and 0.11, respectively) allowing the combination of data sets. Closed lines show the predicted curve from a linear regression and the corresponding 95% confidence intervals (dashed lines) using all data depicted in panel A and B (r²=0.87, slope=1.01 ±0.09, y-intercept=–0.85 ±0.51, P=0.0001, n=10).

When monitoring the kinetics of 2-LTR circle formation in PBMC upon treatment interruption, we detected no increase in contents of 2-LTR circles, nor did we observe a significant decrease of this parameter after reinitiation of therapy. These observations suggest that although formation of 2-LTR circles is undoubtedly a consequence of viral infection and replication, this parameter does not efficiently reflect rapid changes of HIV replication in vivo. Previous reports have considered presence of 2-LTR circles to be indicative for newly infected cells due to their seemingly high instability in infected T cell lines [12,13]. However, we did not observe a direct impact of viral replication on 2-LTR circle concentration in vivo, nor did we find evidence that 2-LTR circles decay at a faster rate than late-DNA in primary T cells infected in vitro. In agreement with our observation, recent reports [15,16,27] showed that the decay of 2-LTR circles in infected T cell lines may be a result of cell division and cell death rather than caused by an intrinsic instability of 2-LTR circles. Similarly, the decay of T cell excision circles, other nonreplicating episomes, was reported to be mainly influenced by the division of T cells [28]. Thus, the stability of 2-LTR circles in infected PBMC in our in vivo and in vitro studies may be a consequence of the comparably low cell division rate of PBMC. In view of this, 2-LTR circles could be considered as a marker providing information on HIV persistence rather than functioning as an indicator of active replication. This
argument is substantiated by our observation that levels of 2-LTR circles both in vivo and in vitro were highly correlated with the total number of HIV-infected cells. In support of this, a recent study shows that levels of 2-LTR circles correlated with the frequency of the recovery of replication-competent virus from PBMC obtained from effectively treated patients [14]. Experimental conditions used allowed both recovery of virus from latently infected and from newly, productively infected cells. Thus, these data also strongly argue for a profound correlation between levels of total HIV DNA and non-integrated episomal DNA.

A key observation of our study is that in contrast to 2-LTR circles, increases in levels of cell-associated unspliced HIV RNA (HIV-UsRNAPBMC) reflect recent HIV replication. We have previously shown evidence that the vast majority of HIV-UsRNAPBMC detected during successful antiretroviral therapy stems from nonproductively infected cells, which express basal levels of unspliced HIV RNA but no HIV particles and presumably no HIV antigens [11]. In the current study we confirmed that absolute levels of HIV-UsRNAPBMC in PBMC were not a suitable measure of recent infection as they were highly correlated with the stable levels of late-DNA. However, the increase in HIV-UsRNAPBMC observed upon cessation of therapy – although low in magnitude – strongly correlated with viral rebound in plasma. It is conceivable that this increase in HIV transcription reflects a rise in the number of productively infected cells, which can consist of both newly infected cells and reactivated latently infected cells. The observation that HIV-UsRNAPBMC levels increased, whilst no impact on the pool size of infected cells was evident, is probably a consequence of the high turnover rate of productively infected cells in the periphery [1]. Productively infected cells have been reported to occur in frequencies at least 30-times lower than those of persistently HIV-infected cells [11]. Thus, the proportion of newly infected cells is comparably low in vivo and parameters such as measurements of late reverse transcribed or integrated HIV-1 DNA that account for both persistent and new infection will less sensitively reflect changes in the latter cell population.

Taken together, we found that the relative increase in transcriptional activity of HIV-infected cells as measured by the increase in cell-associated HIV RNA levels was the best cellular marker of recent HIV infection. However, absolute levels of cell-associated HIV RNA did not reflect ongoing active HIV-1 replication. Neither did levels of cell-associated episomal and total HIV-1 DNA. Based on these observations, other classes of cellular HIV RNA such as multiply spliced HIV RNA [11,29,30] or PBMC-associated virion RNA [11] may be viewed as possible candidates for surrogate markers of residual HIV replication during antiretroviral therapy. However, as shown here, the complex interdependencies of the different types of HIV-1 nucleic acids may complicate the search for specific, sensitive and reliable markers of ongoing low-level HIV replication. Nevertheless, their identification would provide us with valuable tools to gain further insights into the nature of HIV persistence and could largely impact on the future design and maintenance of antiretroviral treatment.

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