NOTES

Decay of Human Immunodeficiency Virus Type 1 Unintegrated DNA Containing Two Long Terminal Repeats in Infected Individuals after 3 to 8 Years of Sustained Control of Viremia

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Covert human immunodeficiency virus (HIV) replication was ongoing during the first 3 years of aviremia in 22 patients, as determined by detection of DNA containing two long terminal repeats (2LTR DNA). Although total HIV DNA was detected in 60 2LTR DNA-negative samples, the absence of 2LTR DNA in 90% of patients following 7 to 8 years of highly active antiretroviral therapy suggests suppression of cryptic viral replication.

Measurement of plasma RNA levels is considered the best prognostic marker for predicting clinical outcome in human immunodeficiency virus type 1 (HIV-1) infection and is also a marker of therapy efficacy. Treatment guidelines suggest that viremia should be measured every 3 to 6 months in treated patients (14), yet following highly active antiretroviral therapy (HAART), viremia is drastically reduced to undetectable levels and the treatment strategy is focused on maintaining this aggressive and seemingly efficient therapy for the longest possible period (12). The existence of latent reservoirs of integrated proviral DNA in resting CD4+ T lymphocytes provides a long-lived source of replication-competent virus in patients undergoing potent HAART (3, 5, 13, 17). In the absence of integration, the extrachromosomal viral DNA can remain in the linear form or circularize to produce circular DNA forms containing one or two long terminal repeats (LTRs), respectively (1, 15). It was thought that unintegrated DNA had a transient existence in infected cells due to its rapid degradation once formed (16) and that its detection was consistent with de novo infection. The accumulation of unintegrated DNA was observed in infected individuals with high plasma RNA levels (10), and several studies evaluated the use of DNA containing two LTRs (2LTR DNA) as a marker for HIV disease progression (11, 18). A decrease in 2LTR DNA levels was seen in a group of patients following the initiation of antiretroviral therapy (4) in correlation with therapy efficacy, and later studies used 2LTR assays to monitor ongoing viral replication in subjects with suppressed plasma viremia (2, 8). However, few studies document whether HAART is able to arrest covert virus replication in well-suppressed patients.

The objective of this study was to monitor HIV total DNA and unintegrated 2LTR DNA levels in peripheral blood mononuclear cells (PBMCs) from aviremic subjects receiving HAART for very long periods and to assess whether there was a correlation between cryptic HIV replication, total DNA, and unintegrated 2LTR DNA levels.

Three hundred twenty-six sequential PBMC samples were obtained from 22 aviremic subjects on HAART during an 8-year period. The PBMCs were separated from whole blood, and total cellular DNA was extracted by cell lysis as previously described (7). The cell lysates were stored at −20°C until assayed. The amplification, detection, and quantification of HIV total DNA were performed using a previously described colorimetric assay (6, 9). For the detection of unintegrated 2LTR DNA, the U3-U5 junction between the two LTRs was amplified in a final reaction volume of 100 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 200 μM deoxynucleoside triphosphates (dNTPs), 0.15 μM each of

### TABLE 1. HIV DNA forms in PBMC samples collected from 22 aviremic patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total HIV DNA</th>
<th>2LTR DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Samples</td>
</tr>
<tr>
<td>Positive</td>
<td>21</td>
<td>188</td>
</tr>
<tr>
<td>Undetectable*</td>
<td>1</td>
<td>138</td>
</tr>
<tr>
<td>Not tested</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>326</td>
</tr>
</tbody>
</table>

* Detection limits: total HIV DNA = 50 copies/10⁶ PBMCs and 2LTR DNA = 25 copies/10⁶ PBMCs.
primer 477 and biotinylated primer 516 (1), 1.5 U of Taq DNA polymerase, and 50 μl of cell lysate sample. Following an initial denaturation step of 95°C for 2 min, amplification proceeded for a total of 50 cycles: 5 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s; 45 cycles at 94°C for 10 s, 55°C for 10 s, and 72°C for 10 s; and a final extension at 72°C for 5 min. Two LTR PCR products were detected by hybridization to a fluorescein-labeled specific probe, 569 (1), followed by colorimetric detection (9).

Total HIV DNA was quantified in 188 (58%) samples, in a range from 53 to 27,386 HIV copies/10^6 PBMCs (data not shown), and was undetectable in the remaining 138 samples. Unintegrated 2LTR DNA was detectable in 103 (32%) samples and undetectable in 218 samples (Table 1). At the end of the 2nd year, one patient had interrupted day hospital attendance and three patients had died.

A two-time-point analysis of HIV total DNA levels was assessed in the remaining 18 patients by using the levels obtained in the first (t1) and last (t2) samples analyzed. As shown in Fig. 1, very high DNA levels (>2,000 copies/10^6 PBMCs, corresponding to 1 HIV-positive cell every 230 to 400 PBMCs) were observed at t1 in three patients and high levels (>1,000 DNA copies/10^6 PBMCs, corresponding to 1 HIV-positive cell every 690 to 950 PBMCs) were observed in five patients. Low levels of HIV DNA ranging from 185 to 871 DNA copies/10^6 PBMCs (1 positive cell every 1,148 to 5,405 PBMCs) were...
quantiﬁed in six patients, while undetectable levels of total HIV DNA (<50 DNA copies/10⁶ PBMCs) were observed in four subjects. At t₂, 11 subjects had undetectable total DNA levels (<50 copies/10⁶ PBMCs), 4 patients had low DNA levels (99 to 447 DNA copies/10⁶ PBMCs), and 2 patients had high DNA levels (1,149 and 1,411 DNA copies/10⁶ PBMCs, respectively). Very high total DNA levels (3,904 DNA copies/10⁶ PBMCs) were observed only for one patient who interrupted therapy due to pregnancy.

The numbers of samples positive for HIV total DNA and unintegrated 2LTR DNA were analyzed comparatively as depicted in Fig. 2. Fifty-ﬁve percent of the samples examined had detectable total DNA levels after 8 years of aviremia. In contrast, although 2LTR DNA was detectable in approximately 50% of the samples during the ﬁrst 3 years, the percentage of positive samples dropped drastically to 6% at the end of the 4th year and 2LTR DNA remained detectable in a small percentage of samples until the end of the study period. During this period, 2LTR DNA was undetectable in 60 (82%) of the 73 cell lysates analyzed, which all had detectable total DNA levels. Overall, the longitudinal monitoring of total HIV and unintegrated 2LTR DNA in patients revealed a general decay for HIV DNA levels to undetectable levels.

The two-time-point analysis of total HIV DNA levels showed that at the end of the observation period, the majority of patients (83%) monitored had undetectable or low total DNA levels, while very high or high DNA levels were quantiﬁed in only 3 (17%) patients. This decay in the number of infected cells suggests that long-term potent antiretroviral therapy may efﬁciently suppress HIV cryptic replication and maintain persistent aviremia leading to a regression of HIV infection.

At the start of this study, unintegrated 2LTR DNA was detectable in 56% of the patients monitored. After 3 years of HAART, a dramatic decrease was observed in the number of samples with detectable 2LTR DNA, and after 8 years of uninterrupted therapy, 2LTR DNA was detectable in only 9% of the tested samples. These observations support the concept that 2LTR circles are labile and do not persist over time.

Our ﬁndings indicate that covert HIV replication was ongoin during the ﬁrst 3 years of follow-up, conﬁrmed by the detection of 2LTR circles, produced by HIV de novo infection. The absence of 2LTR DNA in the majority of our patients following 7 to 8 years of HAART appears to be due to suppression of cryptic viral replication, which is conﬁrmed by the presence of detectable total HIV DNA in approximately 80% of the 2LTR DNA-negative samples. Cryptic replication seems to be almost completely suppressed after 3 years of HAART, suggesting that both total and unintegrated 2LTR DNA forms could be useful markers for the monitoring of regression of HIV infection in aviremic patients.

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