Human Immunodeficiency Virus Type 1 RNA in Peripheral Blood Mononuclear Cells of Patients Receiving Prolonged Highly Active Antiretroviral Therapy

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The levels of early spliced mRNA and genomic RNA of human immunodeficiency virus (HIV) type 1 in peripheral blood mononuclear cells (PBMC) of 14 patients who were receiving highly active combination antiretroviral therapy for \( \geq 116 \) weeks were determined. The level of viral genomic RNA was below the level of detection in the plasma of these patients (\(<50 \text{ copies/mL} \)) but cell-associated viral \( \text{tat}, \text{rev}, \text{and nef} \) mRNA were detected in 86% (12 of 14) of the patients. Cell-associated viral genomic RNA was detected in 57% (8 of 14) of the patients. Early viral spliced mRNA was detected in the PBMC of all patients who had positive results of testing for HIV-1 genomic RNA, and the level of viral genomic RNA in these patients was 34–2214 copies per \( 10^6 \) cells.

Combination therapy involving drugs that inhibit human immunodeficiency virus (HIV) type 1 reverse transcriptase (RT) and protease decreases the virus load in patients’ plasma and increases the survival of patients with AIDS [1–3]. Although viral RNA levels in plasma are suppressed below the limit of detection (\(<50 \text{ copies/mL} \)) for a prolonged period of time, replication-competent HIV-1 can still be grown after activation of resting CD4\(^+\) lymphocytes [4, 5]. Detection of cell-associated viral RNA has been used in other studies [6, 7] and provides a measure of the effectiveness of combination therapy in controlling viral replication in different viral reservoirs in vivo.

Transcription of viral spliced mRNA occurs early in HIV-1 infection, and transcription of genomic RNA occurs late in the viral replication cycle [8–11]. Early viral spliced mRNA (\( \text{tat}, \text{rev}, \text{and nef} \)) is transcribed 12–18 h after infection [8, 9]; it is doubly spliced subgenomic RNA present in the cells as dominant 200- and 400-bp transcripts, as detected by RT polymerase chain reaction (RT-PCR) assay [8–12]. Early viral spliced mRNA encodes the HIV-1 accessory proteins, which include Tat, Rev, and Nef. These viral proteins accelerate HIV-1 replication and down-regulate the host immune system, playing cardinal roles in viral pathogenesis and development of AIDS [13–15]. After the level of spliced mRNA reaches a peak, the transcripts of HIV-1 genomic RNA begin to accumulate in the cytoplasm.

The viral genomic RNA appears in abundance 24 h after infection as a 9.2-kb species [8, 10, 11]. This pattern of viral RNA transcription has been examined in the laboratory and also detected in AIDS patients [6–9, 11].

In this study, we measured viral RNA expression in peripheral blood mononuclear cells (PBMC) from 14 patients who had received highly active antiretroviral therapy (HAART) for \( \geq 2.2 \) years.

Patients and Methods

Patients. Fourteen HIV-1–seropositive patients from the Merck 060/Intercompany Collaboration 004 study at Beth Israel Deaconess Medical Center and Massachusetts General Hospital were enrolled in this study. The patients were asymptomatic, had baseline CD4\(^+\) cell counts of \( >500 \text{ cells/mL} \), and had received combination antiretroviral therapy (indinavir, zidovudine, and lamivudine) for \( \geq 116 \) weeks. Patients were selected in whom the combined drug regimen produced continuous suppression of plasma HIV-1 RNA levels to \( \leq 50 \text{ copies/mL} \) for \( \geq 116 \) weeks; at that point, PBMC were examined for HIV-1 spliced RNA and genomic mRNA.

Specimen collection and RNA extraction. Blood samples were obtained when the patients had received HAART for \( \geq 116 \) weeks. Plasma and PBMC were isolated from whole blood, using EDTA as anticoagulant. The plasma was collected after a low-speed spin and stored at \( -70^\circ \text{C} \) before it was tested for viral genomic RNA. PBMC were isolated from whole blood using Ficoll-Hypaque (Amersham Pharmacia Biotech AB) gradients, and the total cellular RNA was extracted by use of the RNeasy kit (Qiagen). The RNA was purified with DNase after the RNeasy clean-up protocol (Qiagen) was carried out and before examination using RT-PCR.

RT-PCR. Three RT-PCR assays were used in this study: (1) An assay for detecting HIV-1 early spliced mRNA was used to detect \( \text{tat}, \text{rev}, \text{and nef} \) mRNA expression in patient PBMC; (2) an HIV-1 genomic RNA detection assay was used to detect viral genomic RNA in patient plasma and PBMC; and (3) an in-house
RT-PCR assay was used to qualitatively examine the HIV-1 genomic RNA expression in patient cells. Published primers were used to detect early viral spliced mRNA [8]. The primer pair US and ART7 spans known splice junctions to amplify the doubly spliced mRNA for the regulatory proteins Tat, Rev, and Nef [8, 12]. The sequences of the primers are 5′-TCTCTGACGAGCGCTGCTG-3′ and 5′-TTCTATTCTCTCGGCACTGTCG-3′ [8, 12]. The primers for detection of cellular β-globin mRNA were the same as those described in another study [16]. RT-PCR was done using the Qiagen OneStep RT-PCR kit as described in the manufacturer’s instructions. In brief, the RT reaction was carried out at 50°C for 30 min. The initial PCR activation was done at 95°C for 15 min and was followed with 45 cycles of denaturation at 94°C for 0.5 min, annealing at 50°C for 0.5 min, and extension at 72°C for 1 min. The final PCR products were examined on a 2% agarose gel after electrophoresis.

The Ultra-sensitive Amplicor HIV-1 Monitor test (Roche) was used to examine HIV-1 genomic RNA expression in patient plasma. The assay was done as described in the manufacturer’s instructions. A modification of this test was used to examine HIV-1 genomic RNA in patient PBMC specimens.

The in-house RT-PCR assay that was used to qualitatively examine the HIV-1 genomic RNA expression in patient cells used primers other than those in the Monitor assay to detect viral genomic RNA. The primer sequences are 5′-ACAGGGACTTGAAAGCGAAA-3′ and 5′-CTTTAGCTGCTTCGACCCATC-3′. These primers amplify a 172-bp fragment containing the 5′ starting sequences of the HIV-1 gag gene. The RT-PCR conditions used in this test were similar to those described above, and the final PCR product was determined on 2% agarose gel after electrophoresis.

Results

Detection of HIV-1 early spliced mRNA in patient PBMC. First, we examined the HIV-1 spliced mRNA expression in patient PBMC, using an RT-PCR assay specific for detection of HIV-1 tat, rev, and nef mRNA [8, 9]. tat, rev, and nef mRNA were detected in 12 (86%) of the 14 patients examined. In these patient samples, rev and nef mRNA (219 or 225 bp and 203 bp, respectively) was observed more clearly on the gel than was tat mRNA (402 bp) (figure 1A). The expression of tat, rev, and nef mRNA in a time-course experiment using T cells infected with HIV-1 laboratory strain HIV-1b was shown in figure 1B. These data show that the HIV-1 early mRNA that encodes viral regulatory proteins is actively transcribed in the PBMC of patients who have received HAART for ≥2 years, even though the HIV-1 RNA level in the plasma of such patients is undetectable (<50 copies/mL).

Detection of HIV-1 genomic RNA in patient plasma and PBMC. HIV-1 genomic RNA species were examined in patient plasma and PBMC samples obtained at the same time. All 14 patients had undetectable HIV-1 genomic RNA (<50 copies/mL) in their plasma specimens, as determined using the Monitor assay. The HIV-1 genomic RNA in PBMC was detected by use of an in-house RT-PCR assay and the Monitor assay, modified to permit detection of genomic RNA in cells (table 1).

RNA extracted from PBMC was treated with DNase before amplification of viral RNA by RT-PCR assays. HIV-1 genomic RNA was detected in 8 (57%) of 14 patients by use of the in-house RT-PCR assay. The 8 patients in whose samples HIV-1 genomic RNA was detected all tested positive for early viral spliced mRNA. When samples from these patients were examined with the modified Monitor assay, viral genomic RNA was detected in 8 of 14 patients, the same number in whom viral genomic RNA was detected by our in-house RT-PCR assay. In addition, the same individuals who tested positive for HIV-1 genomic RNA by the in-house RT-PCR assay also tested positive by the Monitor RT-PCR assay.

To determine the level of viral genomic RNA in patient PBMC, we used the RNA extracted by the lysis buffer in the Monitor kit and by the in-house method. Examination of the samples was conducted in parallel with the Monitor RT-PCR assay (table 1). The Monitor HIV quantitation standard RNA was added to the lysis buffer used to extract RNA from plasma and PBMC samples. Quantitation standard was added directly to the PBMC samples from which RNA was extracted using the in-house assay [9]. A representative result of the comparison of HIV-1 genomic RNA levels in patient plasma and PBMC processed by use of the Monitor RT-PCR assay lysis buffer
and the in-house extraction assay is shown in Table 1. These results show that the HIV-1 genomic RNA was detected in patient PBMC samples by both methods (Table 1), whereas the plasma viral RNA level was below the limit of detection (<50 copies/mL). These data also show that the in-house RT-PCR assay for examination of cell-associated viral RNA can be modified to measure the viral copy number by using the Monitor kit, which was initially used to measure the viral copy number in patient plasma. By this method, we found 223 HIV-1 RNA copies in 10^6 PBMC for patient 1, <50 copies in patient 2, 219 copies in patient 3, 52 copies in patient 6, 278 copies in patient 10, 2214 copies in patient 11, 427 copies in patient 13, and 220 copies in patient 14. Levels were undetectable in the other 6 patients.

### Discussion

Our results show that patients who received HAART for ≥2 years had actively-transcribed viral spliced mRNA and viral genomic RNA in their PBMC, even when their plasma viral RNA levels were undetectable (<50 copies/mL). The level of viral genomic RNA in PBMC was 34–2214 copies per 10^6 cells, whereas the plasma HIV-1 RNA copy number was <50 copies/mL. Another study reported that viral spliced RNA and genomic RNA were detected in the PBMC of 5 patients who received HAART therapy for 80 weeks [7]. In our study, HIV-1 spliced mRNA was detected in 12 (86%) of 14 patients, and HIV-1 genomic RNA was detected in 8 (57%) of 14 patients who received HAART therapy for ≥116 weeks. Our study further shows that HIV-1 transcription persists in PBMC of patients receiving potent, extended antiretroviral therapy.

The proteins encoded from the early viral spliced mRNA play crucial roles in viral replication and AIDS progression. Tat is a transactivator that recruits cellular elongation factors to the HIV-1 promoter and dramatically boosts viral genomic RNA transcription [13]. Two other proteins translated from the early viral spliced mRNA are Rev and Nef. Rev is a key factor in accumulating HIV-1 genomic RNA in the cytoplasm and is essential for viral genomic RNA production [14]. Nef plays an important role in HIV-1 pathogenesis. Nef activates T cells to favor HIV-1 replication. Nef also down-regulates CD4 receptors and major histocompatibility complex I on the cell surface, an action that not only facilitates viral replication but also disrupts the host immune response to infected cells [15]. Taken together, the early viral spliced mRNA and the encoded proteins play a central role in HIV-1 pathogenesis and in the development of AIDS. Control of the early viral spliced RNA expression and its protein production is an important step in the control of viral replication and restoration of host immunity.

The relationship between expression of viral spliced RNA, genomic RNA, and infectious virus in the PBMC of these patients requires further study [4, 5]. Other studies have shown that infectious viruses are cultured from the PBMC of patients who have plasma viral RNA levels of ≤50 copies/mL after HAART [4, 5]. Establishing the relationship between viral spliced and genomic RNA transcription and infectious virus release may provide a quick and sensitive assay to measure viral replication in a patient’s PBMC. In contrast to the method of culturing infectious virus from these patient cells, examination of the early viral spliced mRNA and viral genomic RNA provides a quick, sensitive surrogate marker for measuring the efficacy of anti-HIV drugs and therapeutic vaccine treatment. The RT-PCR assay can process a large number of samples in a shorter time than can the viral culture assay, and the PCR assay is characterized by its sensitivity, specificity, and reproducibility. Measuring the cell-associated viral RNA expression in HIV-infected patients could provide sensitive parameters for measurement of prompt clinical progress and rapid adjustment of treatment options.

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