SPECIAL REPORT

Plasma HIV-1 Load and Disease Progression in HIV-Infected Patients in Hungary

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Nucleic Acid Sequence Based Amplification (NASBA) is a suitable method for the quantification of HIV-1 RNA in plasma and serum samples. Since determination of the viral load appears to be a valuable marker for the prediction of disease progression and for monitoring the efficiency of antiretroviral therapy, the National AIDS Committee initiated the introduction of NASBA in Hungary at the end of 1996. We obtained plasma samples from patients with ARC and AIDS of the Szt. László Hospital, Budapest. We found an increased viral burden in untreated AIDS (CDC group C) patients compared to untreated ARC (CDC group B) patients. In plasma samples of clinically stable ARC and AIDS patients treated with antiretroviral drugs we detected relatively low HIV-1 RNA copy levels while similarly treated ARC and AIDS patients with progressive disease had high HIV-1 RNA copy numbers. The CD4^{+} T-cell count was lower in AIDS patients compared to ARC patients, as expected. In general, there was an inverse correlation (r = -0.487, P < 0.0001) between CD4^{+} T-cell counts and HIV-1 RNA levels. We concluded that measurement of HIV-1 RNA plasma level has an important role in assessing prognosis and effects of antiretroviral therapy in HIV-infected patients. (Pathology Oncology Research Vol 4, No 1, 52–55, 1998)

Key words: plasma HIV-1 RNA copy number, NASBA, AIDS, AIDS Related Complex

Introduction

Since the prognosis of HIV-1 infected individuals is variable, it is of utmost importance to predict the development of AIDS in virus carriers. In addition to CD4^{+} T-cell count determination, measurement of plasma HIV-1 RNA levels can give valuable information for this purpose.[15] Nucleic acid amplification techniques such as RT-PCR and NASBA directly test for the presence of HIV-1 RNA and permit the monitoring of antiretroviral treatment. For these reasons the National AIDS Committee initiated the introduction of NASBA in Hungary.

The NASBA method mimics the process of retroviral replication. The procedure relies on cycles of reverse tran-

scription and RNA polymerase mediated replication to generate single-stranded RNA copies of the target sequence. Quantification of RNA is based on coamplification of HIV-1 plasma sample RNA together with internal calibrators. The quantity of amplified RNA is measured by means of electrochemiluminescence (ECL).[1] We report here our initial experiences using NASBA to assess whether plasma HIV-1 RNA levels correlate with the progressive or stable course of ARC and AIDS.

Materials and Methods

Patients

Plasma samples were obtained from 84 HIV-1-infected patients of the Szt. László Hospital, Budapest, representing ARC (AIDS Related Complex, corresponding to CDC group B or preAIDS) and AIDS (CDC group C) stages of HIV-1 infection.[5,11] Both groups were further subdivided according to the clinical course (progressive or stable).
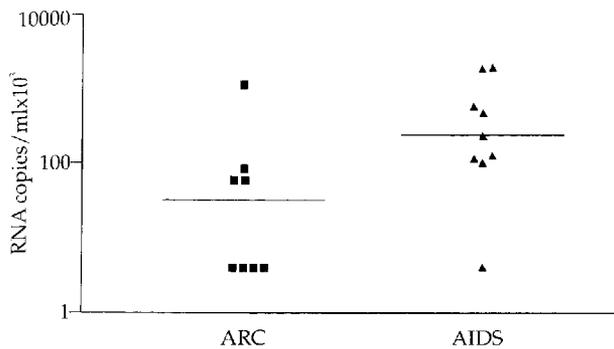
Figure 1. *Plasma HIV-1 RNA copy levels in untreated patients with ARC and AIDS. Each symbol represents RNA copy number of an individual patient.*

Sample collection and processing

Whole blood was collected into blood collection tubes containing sodium citrate anticoagulant (final concentration: 0.33 percent). Plasma was separated by centrifugation at 10000 g for 1 min. Plasma samples were immediately stored as coded specimens at −70°C until processing.

Determination of HIV-1 RNA copy number

Measurements were performed by Akzo Nobel Quantitative NASBA HIV-1 RNA QT.

1. Nucleic acid release and isolation − Nucleic acids from plasma were isolated according to the method of Boom et al.[2] Briefly, 100 μl plasma samples were lysed by 0.9 ml lysis buffer containing guanidine thiocyanate and Triton-X-100. The quantitative NASBA method applicates three internal calibrator RNAs (Q RNAs). Q RNAs are differing from each other and from the HIV-1 wild-type (WT) RNA in a 22 nucleotide long randomized sequence which permits their specific detection. 10^6, 10^5, 10^4 copies of the Q_A, Q_B and Q_C internal standard RNA molecules, respectively, were added to the sample before nucleic acid isolation. Thus, the ratio of Q_A: Q_B: Q_C: WT
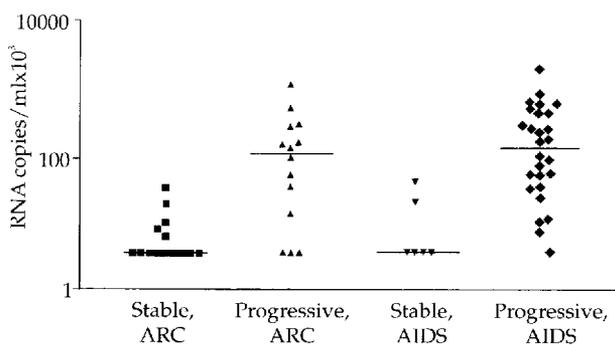

Figure 2. *Plasma HIV-1 RNA levels in antiretroviral drug treated patients with stable or progressive ARC and AIDS.*

RNAs, from which the initial input of WT-RNA was calculated remained constant independent of any loss occuring during nucleic acid isolation. Under high salt conditions the WT-RNA and the internal calibrator RNAs were bound to silicon dioxide particles. With repeated washings proteins and other contaminating materials were removed and finally the RNAs were eluted from the silica.

2. Nucleic acid preamplification − 10 μl NASBA reaction mixture containing 40 mM Tris, pH 8.5, 12 mM $MgCl_2$, 42 mM KCl, 15 % v/v DMSO, 1 mM each dNTP, 2 mM each NTP, 0.2 M gag specific Primer1 and Primer2 were added to 5 μl RNA, and incubated at 65°C for 5 min to allow destabilization of secondary structures in the RNA and subsequently cooled down to 41°C to allow primer annealing.[6,7,9]

3. Nucleic acid amplification − The amplification was started by adding 5 μl enzyme mixture (0.1 μl BSA, 0.1 units RNase H, 40 units T7 RNA polymerase and 8 units AMV-reverse transcriptase) to the samples and incubated at 41°C for 90 min. The present wild-type HIV-1 RNA in eluted nucleic acid was co-amplified with the three internal calibrators. The wild-type RNA and the calibrator RNAs served as templates for the extension of gag region Primer1 (containing the T7-RNA polymerase recognition site) by AMV-RT (Avian Myeloblastosis Virus reverse transcriptase). Primer1 created complementary DNAs (cDNAs) of the original target RNAs. The resulting double-stranded RNA-DNA hybrids were substrates for the enzyme RNase H, which degraded the RNA portion of RNA-DNA duplexes. The resulting double-stranded DNA molecule contained a functional T7-RNA polymerase recognition sequence. T7-RNA polymerase synthesized multiple single-stranded RNA copies of the DNA molecule. The produced RNA transcripts served as template for cDNA synthesis by AMV-RT using Primer2. Subsequent RNase H cleavage and second strand synthesis from Primer1 generated double-stranded DNA suitable for transcription by T7-RNA polymerase and the cycle started again.[6,7,8,9]

4. Nucleic acid detection − The basis of quantitative NASBA detection is ECL (electrochemiluminescence) reaction of $Ru(bpy)_3^{2+}$ [ruthenium (II) tris (bipyridyl)] with TPA [tripropilamine]. $Ru(bpy)_3^{2+}$, was used for labelling specific hybridization probes for WT and calibrator amplicons. It was oxidized at the surface of the electrode, forming the strong oxidant, $Ru(bpy)_3^{3+}$. Simultaneously, TPA, which was present in large molar excess in the assay buffer solution, was oxidized at the electrode to form the cation radical TPA^{+}, which rapidly and spontaneously lost a proton to form the radical $TPA^{.}$. $Ru(bpy)_3^{3+}$, a strong oxidant, and $TPA^{.}$, a strong reductant, react to form excited state of the ruthenium complex, $Ru(bpy)_3^{2+*}$. The excited state decays to the ground state through a normal fluorescence mechanism, emitting a photon having a wavelength of 620 nm. To separate the amplificates (WT, Q_A, Q_B,and

Q_C) aliquots of the amplified sample were added to four hybridization solutions, each specific for one of the amplificates. The respective amplificates were hybridized to a bead-oligo and a ruthenium-labeled probe (bead-oligo = biotin-oligo bound to streptavidin coated magnetic beads). The magnetic beads carrying the hybridized amplificate/probe complex were captured on the surface of the detector's electrode by means of a magnet. The analysis of signals was performed by a computer connected to the system.[1,8] The detection limit was: 4000 copies/ml.

Statistical analysis

The Mann-Whitney test and Spearman's test were used for statistical analysis.

Results

We obtained plasma samples from 84 HIV-1 infected patients of the Szt. László Hospital with ARC (CDC group B) and AIDS (CDC group C). 17 untreated patients (8 with ARC and 9 with AIDS) were inaccessible for antiretroviral therapy. 67 patients received antiretroviral chemotherapy. The following drugs were used alone or in combination: zidovudine, lamivudine, dideoxycytidine, dideoxyinozitole, ritonavir, indinavir, delavirdine. 33 of the 67 treated patients were in ARC stage and 34 in AIDS stage. From patients with ARC, 14 had progressive disease and 19 were clinically stable. 28 patients with AIDS had progressive disease and 6 AIDS patients were stable.

In plasma samples of untreated AIDS patients we detected significantly higher (p=0,0206) HIV-1 RNA copy levels (median: 220.000 copies/ml; range: 4.000-1.770.000 copies/ml) than in samples from untreated ARC patients (median: 30.500 copies/ml; range: 4.000-1.100.000 copies/ml) *(Figure 1)*. In plasma samples of clinically stable ARC and AIDS patients treated with antiretroviral drugs we detected relatively low HIV-1 RNA copy levels (median in stable ARC patients: 4.000 copies/ml, range: 4.000 – 36.000 copies/ml; median in stable AIDS patients: 4.000 copies/ml, range: 4.000–43.000 copies/ml). Similarly treated ARC and AIDS patients with progressive disease had high plasma HIV-1 copy levels (median in patients with progressive ARC: 120.000 copies/ml, range: 4.000-1.200.000 copies/ml; median in patients with progressive AIDS: 135.000 copies/ml, range: 4.000-2.000.000 copies/ml). *(Figure 2)*. The difference in HIV-1 plasma RNA levels between stable and progressor ARC patients receiving antiretroviral therapy is significant (p=0,006) like the difference between stable and progressor AIDS patients undergoing similar treatments (p=0,027).

We also analysed the CD4+ T-cell numbers of patients. As shown in *Table 1*, the CD4+ T-cell count also changed according to the stage and course of the disease, as expect-

Table 1. Mean CD4+ T-cell number (cells/mm³) in untreated and antiretroviral drug treated patients with ARC and AIDS

	Untreated patients	Treated patients	
ARC	392	progressive	294
		stable	382
AIDS	61	progressive	84
		stable	191

ed. We found an inverse correlation (Spearman correlation coefficient r: -0.4872, P<0.0001) between CD4+ T-cell counts and HIV-1 RNA levels *(Figure 3)*.

Discussion

Monitoring plasma HIV-1 RNA levels provides important data for patient management, including information on risk of disease progression, when to initiate therapy, the degree of initial antiretroviral effect achieved and when a drug regimen is failing.[15] Recent disease progression cohort data showed that there is a continuum of increased risk for AIDS and death as HIV-1 RNA levels increase. Patients with low HIV-1 RNA levels have a better clinical prognosis than those with higher RNA levels.[10,12,13] Since plasma HIV-1 RNA levels and CD4+ lymphocyte count determinations are independent predictors of clinical outcome, their combined use provides a more complete picture of an individual patient's status and response to therapy.[13] We found a broad range of plasma HIV-1 RNA levels in infected individuals who did not receive antiretroviral therapy. This is in agreement with the data obtained by others using similar HIV-1 RNA quantification methods.[13,14] Our finding that the median plasma HIV-1 RNA copy number was higher in untreated AIDS (CDC group
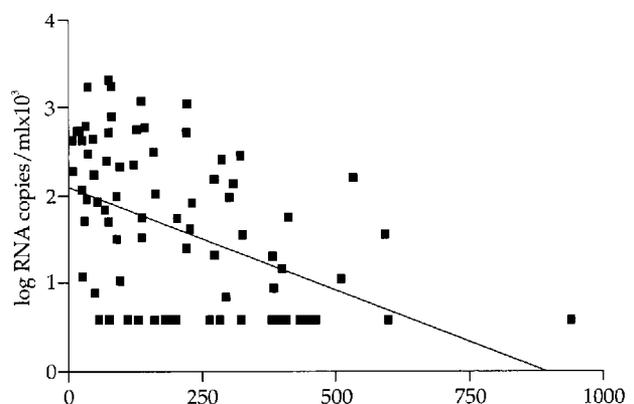

Figure 3. Scatter plot of plasma HIV-1 RNA level and CD4+ T-lymphocyte count (cell number/mm³) of all patients included in this study (Spearman's r = -0,4872, P<0,0001).

C) patients than in ARC (CDC group B) patients confirms earlier reports which demonstrated an increase in viral burden during disease progression.[3,14] Clinically stable ARC and AIDS patients receiving antiretroviral therapy had low plasma HIV-1 RNA levels in our study while patients with a progressive clinical course had considerably higher plasma HIV-1 RNA copy numbers in both groups. Thus, plasma HIV-1 RNA levels may correlate with and predict disease progression not only in asymptomatic patients but also in advanced (CDC group B and C) disease stages. Contrary to the results of Revets et al,[14] and in agreement with the data of Philips et al,[13] we found a good inverse correlation between HIV-1 plasma RNA levels and $CD4^+$ T-cell counts. In conclusion, our findings support earlier studies showing that disease progression and low $CD4^+$ T-cell counts are related to an increase of virus load in HIV infected individuals.

Acknowledgements

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