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# Dynamics of Molecular Parameters of Human Immunodeficiency Virus Type 1 Activity In Vivo

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**The dynamics of viral activity during different phases of human immunodeficiency virus type 1 (HIV-1) infection were investigated by competitive PCR methods. In particular, we studied the time course of three quantitative molecular parameters of viral activity (genomic RNA copy number in plasma and provirus and late HIV-1 transcript molecule copy numbers in peripheral blood CD4$^+$ T lymphocytes) in untreated patients and patients treated with specific anti-HIV-1 compounds. The results shown here indicate that direct RNA parameters are quantitative molecular indices sensitive enough to be used for a more accurate evaluation of the natural history of this infection and that an indirect parameter, the mean transcriptional activity for each provirus in CD4$^+$ T lymphocytes, may be important in studying this infection in vivo at the molecular level. A dramatic decrease of the indices was evident at seroconversion, but the quantitative values were virtually stable throughout the time the untreated patients were studied during the clinical latency phase. Furthermore, the results indicate that an early response to antiretroviral compounds is detected in most subjects as a decrease in the viral activity level.**

Visna virus, the prototype of the lentivirus group, causes chronic pneumonia and a progressive form of demyelinating disease in sheep months to years after the initial infection (19). In the human immunodeficiency virus type 1 (HIV-1) infection, a variable clinical course has been described (27), and AIDS develops within 10 years of the primary infection in about 50% of patients (24). In this infection, initial viremia is followed by antibody production and a decrease in the virus replication level, as evidenced by the detection of the HIV-1 p24 antigen in serum (18), by qualitative and quantitative viral isolation assays of plasma samples (1, 12, 20), and more recently, by quantitative molecular methods (3, 26, 33). Nevertheless, HIV-1 replication is never totally blocked (2, 3, 6, 26, 33, 35), and direct evidence of ongoing infection has recently been obtained from the lymphoid tissues of clinically asymptomatic patients by molecular hybridization (30) and in situ PCR (13). Although high individual variability in viral load has been detected by all the quantitative molecular studies (3, 26, 33), it has been observed that depletion of circulating CD4$^+$ T lymphocytes and disease progression are strongly correlated with increased levels of viral burden (3).

Despite significant advancement over the past decade, our present knowledge of the pathogenic mechanisms of HIV-1 infection remains incomplete. However, it is currently believed that a better understanding of the relationship between levels of viral activity and disease progression may be the key for a more correct approach to answering most of the questions on AIDS pathogenesis that have arisen over the past few years (14); consequently, attempts have been made to investigate quantitatively different indices of HIV-1 infection in vivo at the molecular level (15, 21, 26, 28, 37–41). This concerted effort has been based on the availability of semiquantitative or quantitative molecular methods that were capable of gaining insight into previously unchecked virological aspects of the natural history of HIV-1 infection in vivo, thus also providing a more solid theoretical basis for specific antiretroviral treatment.

At present, several lines of evidence (7) indicate that a modified version of PCR amplification, competitive PCR (cPCR) (17), is the method of choice for quantitative detection of both viral (7) and eukaryotic (11) nucleic acids present in very small amounts in clinical samples. To study HIV-1 infection in vivo at the molecular level and competitive reverse transcription PCR (cRT-PCR), procedures have been planned and optimized (25, 32, 35). Applications of these techniques to investigating HIV-1 infection in samples from asymptomatic and symptomatic patients have supplied direct evidence on the level of viral activity in vivo at different phases of the infection (2, 6, 33).

In this study, we have aimed to evaluate the dynamics of different molecular parameters of HIV-1 activity during the natural course of this infection. Sequential clinical samples from untreated HIV-1-infected patients were used, including patients at seroconversion and subjects studied during the clinical latency phase. The following molecular parameters were monitored by cPCR and cRT-PCR and compared with the immunological and clinical status: (i) HIV-1 genomic RNA copy number in plasma (HIV-1 viremia); (ii) late HIV-1 transcript copy number in peripheral blood mononuclear cells (PBMCs); (iii) HIV-1 provirus copy number in PBMCs; and (iv) mean transcriptional activity (the RNA/DNA ratio; HIV-1-specific late transcript copy number/provirus copy number for $10^5$ CD4$^+$ PBMCs). In addition, the same techniques were used to monitor directly the effect of specific antiretroviral treatments in a group of patients treated with anti-HIV-1 compounds.

## MATERIALS AND METHODS

**Patients.** Sequential clinical samples from two groups of HIV-1-infected patients were used in this research study. All 23 subjects enrolled in this study were patients at the Clinic of Infectious Disease, University of Ancona, Ancona, Italy.

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Group A consisted of 12 untreated patients (8 males and 4 females; mean age, 31 years; age range, 21 to 45 years). The risk factors associated with HIV-1 infection were as follows: intravenous drug addiction, 7 subjects; homosexual activity, 1 subject; and heterosexual contact with an HIV-1-infected partner, 4 subjects. Two patients were studied at seroconversion (Centers for Disease Control [CDC] class I) and monitored for 23 and 12 months, respectively. Ten patients (all CDC class II with the single exception of patient 10 [CDC class III]) were studied during the clinical latency phase and monitored for a mean period of 11 months (range, 6 to 19 months). Group B contained 11 patients (patients 13, 14, and 23, CDC class II; patients 15, 16, 17, and 22, CDC class III; patients 18, 19, 20, and 21, CDC class IV) studied at the beginning of antiviral treatment with two specific anti-HIV-1 compounds (zidovudine [formerly called azidothymidine] [AZT] or dideoxyinosine [ddI]) (time zero) and monitored for 3 to 11 months during therapy. For these patients (9 males and 2 females; mean age, 29 years; age range, 21 to 33 years), the risk factors associated with HIV-1 infection were as follows: intravenous drug addiction, 7 subjects; homosexual activity, 3 subjects; and heterosexual contact with an HIV-1-infected partner, 1 subject.

**Clinical samples and nucleic acid purification.** For this study, peripheral blood samples (EDTA treated) were centrifuged over a Ficoll density gradient. Plasma samples were recovered from the upper phase, and PBMCs were recovered from the top of the Ficoll layer after centrifugation. The PBMCs were washed three times with phosphate-buffered saline. To clear the platelets and cell debris, each plasma sample was centrifuged again (2,800 × *g* for 10 min); 1 ml of this supernatant was diluted in 9 ml of RPMI 1640 (Whittaker, Walkersville, Md.) and ultracentrifuged at 150,000 × *g* for 2 h in a swing-out rotor (Kontron Instruments, Zurich, Switzerland). RNA samples were extracted from the virion pellet and PBMC pellet by the guanidinium thiocyanate method, as previously described (2). To minimize unintegrated DNA contamination, DNA samples were extracted from the nuclei of PBMCs (2).

**Molecular analysis of HIV-1 activity in vivo.** In accordance with our previous study, cPCR-based methods (cPCR and cRT-PCR) (3, 25) were used to analyze quantitatively the following substrates: HIV-1 genomic RNA from plasma, virus-specific late transcripts from PBMCs, and proviral HIV-1 DNA from nuclei. A fragment of the HIV-1 *gag* gene (nucleotides 1551 to 1665) was analyzed by using primer pair SK38 and SK39 (29) in each sample. Competitive analysis was performed by using plasmid pSKAN (25) as an internal competitor; this plasmid is a derivative of plasmid pBS (Stratagene, La Jolla, Calif.) in which the *gag* fragment with a 18-bp deletion (internal to the primer binding sites) was inserted downstream from the T3 RNA polymerase promoter. Competitor RNA was obtained after linearization of the pSKAN plasmid and transcription in vitro; the competitor was then purified, treated with DNase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and quantified by spectrophotometric reading, gel electrophoresis, and end-point dilution followed by Poisson analysis of positive scores. DNA and RNA competitors were stored at −80°C in stock solutions containing 100,000 copies per 10 μl or 31,250 copies per 2 μl, respectively, and the integrity of stored competitors was verified monthly by using known amounts of the wild-type template in a cPCR or cRT-PCR assay. The exact competitor amounts were added to the mixture for cPCR or cRT-PCR; by using our experimental protocol (25), four competitor preparations were made for each sample to be tested. In particular, each RNA sample (10 μl; equivalent to 100 μl of plasma or to 200,000 PBMCs) was reverse transcribed along with 2 μl of increasing copy numbers of competitor RNA (50 to 6,250) and amplified as previously described (3); DNA samples (20 μl; equivalent to 100,000 nuclei) were amplified in reaction tubes containing 10 μl of the competitor plasmid pSKAN at increasing copy numbers (20 to 2,500). Row data on cellular parameters (provirus and transcript copy numbers per $10^5$ PBMCs) were normalized to the percentage of $CD4^+$ T lymphocytes in PBMCs. The amplification profile (1 cycle consisted of 15 s of denaturation at 94°C, 15 s of annealing at 60°C, and 30 s of extension at 72°C) was repeated for a total of 50 cycles with a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). For competition analysis, 5 μl of each 100-μl reaction sample was run on a 10% polyacrylamide gel and analyzed (Fig. 1) with a video densitometer (Ultra Violet Products Ltd., Cambridge, United Kingdom) after ethidium bromide staining, as previously described (3, 25).

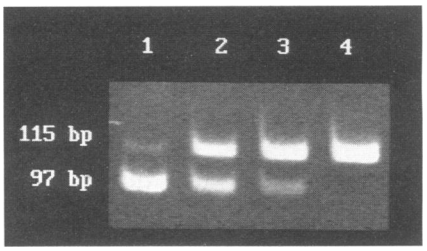


FIG. 1. Quantitation of HIV-1 genomic RNA in plasma samples by cRT-PCR. cRT-PCR was performed with RNA equivalent to 100 μl of plasma and 6,250, 1,250, 250, and 50 competitor RNA molecules (lanes 1 to 4, respectively). After RT, amplification (obtained by using the primer pair SK38 and SK39), and gel electrophoresis, followed by ethidium bromide staining, the amplified wild-type sequence is observed as the upper 115-bp band and the deleted competitor is observed as the lower 97-bp band. Gels were analyzed with a video densitometer to calculate the ratio between peak areas of fluorescent emission of amplified fragment carrying a deletion (corrected for its lower ethidium bromide incorporation) and wild-type sequence.

**Other methods and statistical analysis of data.** Primers used in this study were synthesized in our laboratory by using solid-phase phosphoramidite chemistry in a DNAsm synthesizer (Beckman Instruments, Inc., Fullerton, Calif.).

The Spearman rank correlation coefficient was determined to analyze association between molecular and clinical parameters from asymptomatic untreated subjects and to assess the correlation between changes in virological and clinical parameters after 6 to 13 weeks of treatment.

## RESULTS

**Molecular data from untreated HIV-1-infected patients.** The molecular results from sequential samples of untreated HIV-1-infected patients (group A) are reported in Fig. 2 and 3. High variability for all the parameters investigated is evident among the 12 HIV-1-infected patients included in this group. However, in CDC class I patients (patients at seroconversion, patients 1 and 2) (Fig. 2), the dynamics of the indices studied give a direct measure of the early decrease in both HIV-1 viremia and mean transcriptional activity in PBMCs occurring soon after seroconversion. In these two cases, (i) infected peripheral blood cells were detectable at any given point in time, (ii) late viral transcripts were present in infected lymphocytes, and (iii) low residual viremia levels were always detectable after seroconversion. Furthermore, in both patients the

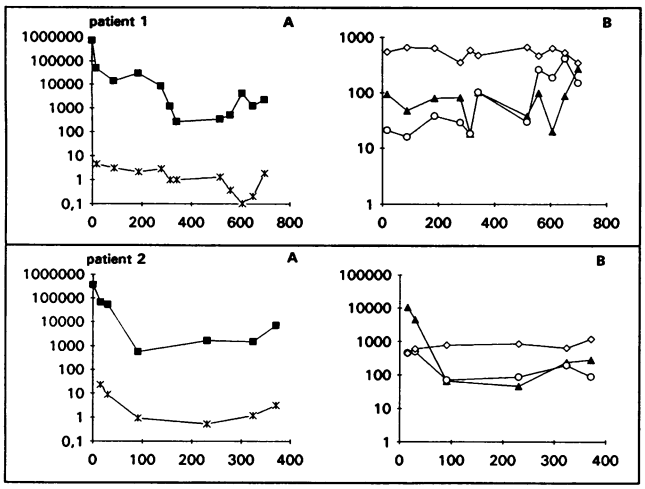


FIG. 2. Dynamics of molecular parameters of HIV-1 infection in two patients studied at seroconversion (CDC class I) and monitored during the clinical latency phase. All $x$ axes show the number of days; the $y$ axes are shown in log scale and are defined below. (A) HIV-1 genome copy number per milliliter of plasma (HIV-1 viremia) (■) and mean transcriptional activity (transcript copy number/provirus copy number per $10^5$ $CD4^+$ T lymphocytes [RNA/DNA ratio]) (*). (B) HIV-1 transcript (▲) and provirus (○) copy numbers per $10^5$ $CD4^+$ T lymphocytes and number of $CD4^+$ T lymphocytes per cubic millimeter of blood (◇). At day 0, both patients had detectable p24 antigen levels in plasma (82 and 40 pg/ml for patients 1 and 2, respectively). Reactivity to HIV-1 gp160 and p24 in patient 1 was assessed by immunoblotting; in patient 2 (tested antibody negative at day 0), seroconversion could be observed in the second sample available.

time course of viremia levels was paralleled by that of the RNA/DNA ratio at all times.

For 5 of the 10 asymptomatic subjects (patients 3 to 12) (whose awareness of their HIV-1 seropositive status averaged 4.8 years and ranged from 2 months to 8 years), the study was carried out for more than 1 year; in these patients, the molecular parameters were virtually stable throughout the observation time or fluctuated within a narrow range (Fig. 3). At this phase of the infection, viremia levels show high patient-to-patient variability. Similarly, sharp individual differences were observed among these patients in terms of provirus copy numbers and specific HIV-1 transcripts. Nevertheless, the dynamics of HIV-1 viremia on the one hand and of late transcript and provirus copy numbers per $10^5$ $CD4^+$ T lymphocytes on the other hand were parallel over time in most patients at this preclinical stage (Fig. 3) with only a few exceptions. Interestingly, in one case (patient 8), a continuous progressive increase in viremia levels (from 250 at day 0 to 7,730 genomes per ml of plasma at day 288) was perfectly paralleled by an increase in the number of late transcript molecules per $10^5$ $CD4^+$ T lymphocytes (from 28 at day 0 to 116 on day 288), but not by provirus copy numbers. On the other hand, the increase in provirus molecules observed in patients 3 and 7 was not paralleled by any significant change in viremia levels. The mean transcriptional activity was observed to be substantially stable in all patients followed. In 3 of the 10 subjects (Fig. 3, patients 5, 7, and 12), the RNA/DNA ratio persisted at values higher than 1.0, independently of viremia levels.

**Molecular data from HIV-1-infected patients treated with specific antiretroviral compounds.** The cPCR-based methods were used to monitor a group of 11 HIV-1 patients treated with AZT (10 patients) or ddI (1 patient [patient 18]). As shown in Fig. 4, the molecular data provide insight into the dynamics of viral activity in vivo during therapy. In fact, an early decrease in viremia levels (mean decrease, 95.35% [compared with the initial value]) is evident for almost all the patients at the first point during treatment. Interestingly, an early and efficient reduction of viral load was documented in several patients with high basal viremia levels (patients 15, 17, 18, 19, 20, and 22); in the majority of these patients, the decrease in viremia levels was paralleled by early decreases in transcript copy numbers and mean transcriptional activity (RNA/DNA ratio).

In several cases (patients 13, 17, 18, and 20), the decrease in provirus molecules per $10^5$ $CD4^+$ T lymphocytes seemed to be slightly delayed compared with the viremia level or transcript copy number, and at the same time, $CD4^+$ T-cell counts generally failed to indicate any significant change during the course of treatment (Fig. 4, panels B).

Changes observed in the numbers of provirus and transcript molecules in infected cells, genome copy numbers per milliliter of plasma (as percentage decrease from the initial value; data not shown) and $CD4^+$ T lymphocyte counts were compared after 6 to 13 weeks of therapy in 10 of the 11 patients studied. The results showed that in these treated patients, the viremia levels and the late transcript amounts in infected cells are significantly correlated ($P = 0.021$), while the number of provirus DNA molecules per $10^5$ $CD4^+$ T cells is the only virological marker that correlates with the $CD4^+$ cell number (Spearman correlation; $P = 0.023$).

In several patients (Fig. 4, patients 13, 14, 15, and 16), a rebound in the viremia level was seen. In two patients (patients 17 and 21), the viremia levels rebounded to levels higher than those observed before therapy; this phenomenon was also seen in two patients (patient 22 [Fig. 5] and patient 23 [Fig. 4]) who received inconstant therapy. In patient 22 (Fig. 5), all the virological parameters evaluated were reduced after 105 days of treatment, but progressive increases in HIV-1 viremia, late viral transcript molecules in PBMCs, and provirus copy number were evidenced after interruption of therapy with AZT.

## DISCUSSION

In this research, the level of HIV-1 activity has been evaluated in sequential samples from patients soon after primary infection, from asymptomatic infected subjects, and from patients (including AIDS patients) treated with antiretroviral compounds. The data presented here are in agreement and extend previous molecular studies of HIV-1 infection in vivo, indicating that the molecular counterpart of infection progression is a continuous (and in most cases, probably slow) change in the mean viral activity rate (2, 3, 26, 28, 33, 36), rather than an "on/off" switch from latency to virus expression.

Two patients were monitored after primary infection and seroconversion; the results from these subjects indicate that although the immune response appears relatively efficient in reducing viral load after this very early and active phase of HIV-1 infection, viral replication is never silenced. This molecular evidence does not exclude the possibility that real latency may occur at the level of individual infected cells, as recently shown by the chimpanzee model system (34), but the evidence does suggest that viral replication occurs at any point in time during the natural course of this infection, which is in complete agreement with the recent observation that lymphoid germinal centers are an important reservoir of HIV-1 and support active viral replication during the asymptomatic phase of the infection (13, 30). In the last few years, it has been

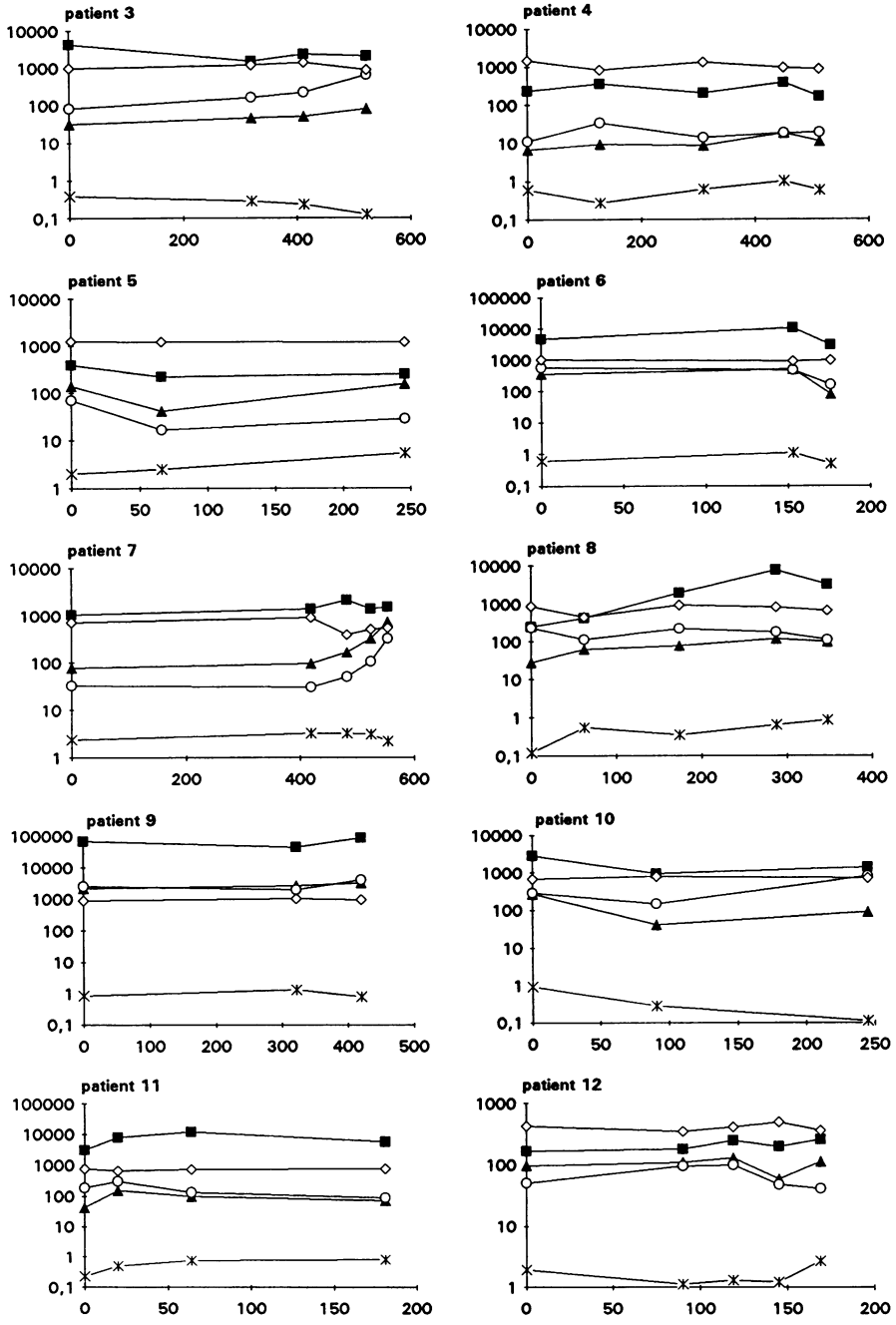


FIG. 3. Molecular parameters of HIV-1 activity in infected patients monitored during the clinical latency phase (CDC classes II and III). All *x* axes show the number of days; the *y* axes are shown in log scale and are defined below. Symbols: ■, HIV-1 genome copy number per milliliter of plasma (HIV-1 viremia); *, mean transcriptional activity (transcript copy number/provirus copy number per $10^5$ $CD4^+$ T lymphocytes [RNA/DNA ratio]); ▲, HIV-1 transcript copy number per $10^5$ $CD4^+$ T lymphocytes; ○, HIV-1 provirus copy numbers per $10^5$ $CD4^+$ T lymphocytes; ◇, number of $CD4^+$ T lymphocytes per cubic millimeter of blood.

clearly established that an early burst of viremia precedes seroconversion in primary HIV-1 infection (8, 10). However, substantial differences in the dynamics of the different molecular indices in these two patients may be observed. In fact, although in both patients the decrease in HIV-1 viremia paralleled the decrease in mean transcriptional activity in peripheral blood $CD4^+$ T lymphocytes, these decreases occurred in two patients with real differences in the provirus DNA copy numbers. Although one cannot exclude the possibility that primary HIV-1 infection of one of the two patients was evidenced earlier, these differences may suggest that regulation of viral expression during and after primary infection is an individual (probably multifactorial) variable.

In most persistent viral infections, viral load is an individual variable, as are viral tropism, organ pathology, and host's immune response. In human lentivirus infections, it has been

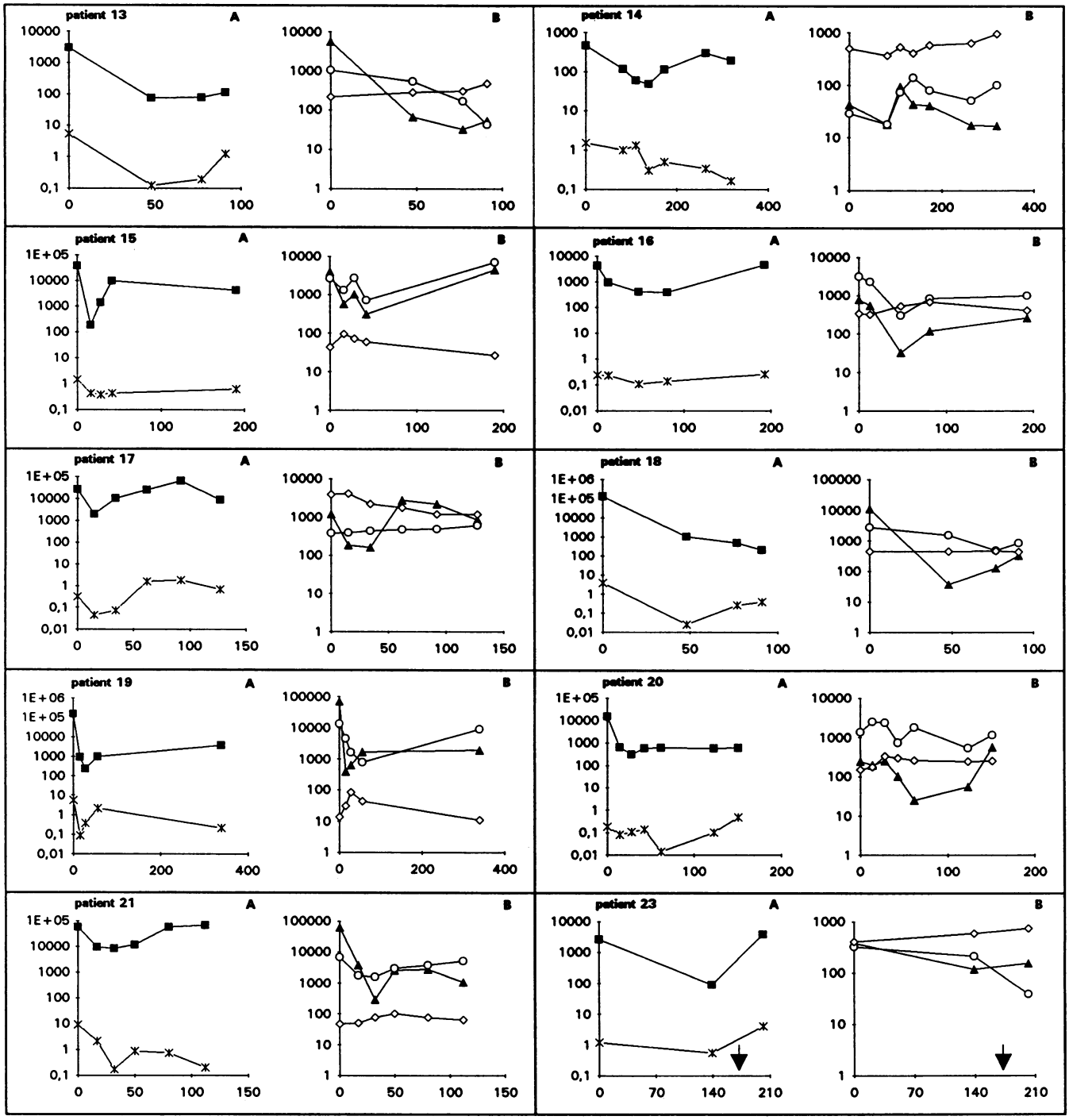


FIG. 4. Time course of molecular indices of viral activity and CD4$^+$ T-lymphocyte counts in patients treated with specific antiretroviral compounds. All $x$ axes show the number of days; the $y$ axes are shown in log scale and are defined below. (A) HIV-1 genome copy number per milliliter of plasma (HIV-1 viremia) (■) and mean transcriptional activity (transcript copy number/provirus copy number per $10^5$ CD4$^+$ T lymphocytes [RNA/DNA ratio]) (*). (B) HIV-1 transcript (▲) and provirus (○) copy numbers per $10^5$ CD4$^+$ T lymphocytes and number of CD4$^+$ T lymphocytes per cubic millimeter of blood (◇). All patients but one were treated with 500 mg of AZT per day (patient 20 was treated with 400 mg of ddI per day). Patients 19 and 21 initially received 750 mg of AZT per day, which was reduced to 500 mg per day after 56 and 32 days, respectively. For patient 23, the arrows indicate the interruption of therapy with AZT at day 175.

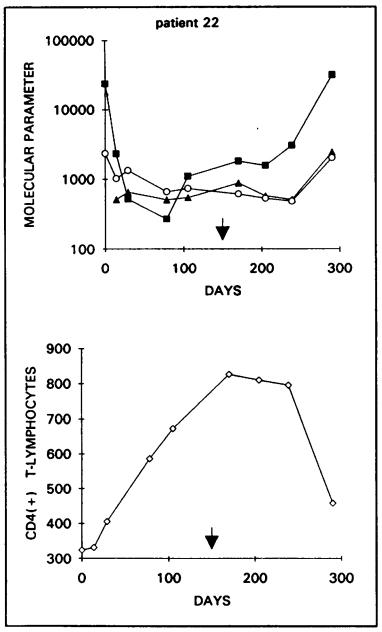


FIG. 5. Dynamics of molecular parameters of HIV-1 activity in one patient under treatment with AZT who discontinued therapy (arrows). Molecular parameters and $CD4^+$ T lymphocyte counts are shown. Symbols: ■, HIV-1 genome copy number per milliliter of plasma (HIV-1 viremia); ▲, HIV-1 transcript copy number per $10^5$ $CD4^+$ T lymphocytes; ○, HIV-1 provirus copy number per $10^5$ $CD4^+$ T lymphocytes; ◇, number of $CD4^+$ T lymphocytes per cubic millimeter of blood.

observed that a virus infecting a host is in reality a mixture of distinct genomes (4, 16, 23) called quasispecies (31). This means that not only antigenic properties (by immune selection) (5) and drug resistance but also regulatory functions (22) and consequently gene expression, tissue tropism, and pathogenicity may change. In these conditions, particularly from a quantitative point of view, viral isolation only approximates what is really going on in vivo. In fact, although attempts to quantify HIV-1 load have been made in the past few years by analysis of the 50% tissue culture infective dose (9, 20), it is reasonable to suppose that in vitro propagation of HIV-1 increases the representation of the viral quasispecies with high replicative capacities. Otherwise, a method suitable for evaluating HIV-1 activity ideally requires not only high sensitivity but also adaptability to a quantitative evaluation of different molecular parameters. In this study, we used a cPCR-based technique and studied sequential samples from a group of symptomless HIV-1-infected patients (CDC classes II and III). In these patients, although high individual variability in the different viral activity markers was detected, the quantitative parameters were virtually stable throughout the observation time (with one exception, patient 8 [Fig. 3]), and the time course of quantitative parameters paralleled the time course of $CD4^+$ T lymphocyte counts and of stable clinical conditions. However, the real pathogenic and prognostic significance of the large subject-to-subject difference in viremia levels evidenced in asymptomatic individuals by this follow-up analysis remains to be clarified, and this aspect may be central to a more precise understanding of the natural history of this infection.

Recently, it has been observed that quantitation of HIV-1 viremia by cRT-PCR in samples from asymptomatic and symptomatic patients correlates with both the phase of the disease (the CDC clinical stage) and the $CD4^+$ T-lymphocyte counts (3). This correlation indicates that the competitive approach is capable of detecting differences in HIV-1 activity levels among these patients and that from a clinical point of view, this technique supplies a virological basis for identifying subjects with highly active HIV-1 infections independently of clinical conditions. More recently, an additional study on HIV-1 viremia in subjects at different phases of the infection has been performed by a competitive method (33); these investigators used a modified sequence of the HIV-1 *gag* gene for the competition assay and found very high levels of viremia (ranging from 50 to 22,000,000 HIV-1 genome copy numbers per ml of plasma). However, the values reported are generally higher by a factor of 10 (or more) than those observed by the methods described here and in other studies (3, 26, 35). In our opinion, the differences arise from technical reasons, and since these reasons may be central to the correct development of quantitative molecular techniques, some methodological aspects require attention. In particular, in competitive methods, synthesis, purification, and quantitation of the RNA competitor deserve the utmost care; it is advisable to check competitor quality and quantity not only by spectrophotometric analysis but also by gel electrophoresis and end-point dilution. Reconstruction curves may not be reliable when both competitor and wild-type synthetic RNAs lack such control and template integrity is not verified. In addition, even if the competitive method is optimized correctly, large differences in sequence length may give rise to undue differences in amplification efficiencies of the templates (7), thus greatly influencing the relative amounts of both products. This is the main reason why we constructed (25) a competitor in which the internal deletion is reduced to a minimum (18 bp) to obtain discrete bands in the amplification product after gel electrophoresis.

In the present study, we monitored patients treated with specific antiretroviral compounds; in this investigation, we did not intend to evaluate the different antiviral treatments, but we did analyze the adaptability and reliability of the competitive technique to study the impact of therapeutic intervention on viral activity in sequential samples. The molecular results from the 11 patients treated with AZT and ddI indicate that quantitative methods may supply direct and fast information on the effect of a given antiviral compound. In these cases, the effects of antiviral compounds on viral replication were shown not only by significant reductions in viremia levels but also by parallel decreases of the RNA/DNA ratio (Fig. 4). Furthermore, in a significant proportion of these patients, the dynamics of HIV-1 provirus copy numbers indicates that this parameter is a less sensitive index of changes in HIV-1 activity during treatment than viremia level or transcript copy number.

Significantly, the 2 patients that discontinued therapy exhibited dynamics of these molecular markers that correlated with the presence or absence of anti-HIV-1 treatment. Moreover, although residual viral replication was documented in all patients during therapy, the effects of the two compounds used in this study occurred very early, and a 90% drop in viremia levels was observed 14 to 40 days after the start of treatment. This viral inhibition may be maintained for long periods, but in several cases, a rebound was observed, thus indicating that quantitative molecular methods may supply fast and direct information of the efficacy of the treatment at any point in time during HIV-1 infection.

## ACKNOWLEDGMENTS

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