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Quantitative Molecular Monitoring of Human Immunodeficiency Virus Type 1 Activity during Therapy with Specific Antiretroviral Compounds

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Methods for the absolute quantitation of nucleic acids present in small amounts in biological samples (competitive PCR and competitive reverse transcription PCR) were applied to the direct monitoring of specific anti-human immunodeficiency virus type 1 (HIV-1) therapy. With these techniques, different parameters of HIV-1 activity (including genomic RNA copy numbers in plasma, proviral and late transcript copy numbers in peripheral blood lymphocytes, and mean transcriptional activity per each HIV-1 provirus) were monitored during therapy with azidothymidine or ddI. In most of these treated patients, a direct response to the antiretroviral compounds employed was detected during the first few weeks of treatment, as documented by a fast decrease of all molecular indexes of HIV-1 activity. However, residual viral replication (albeit at minimal levels) was documented during therapy in all subjects monitored in this study. In a minority of the patients under study (3 of 12), the drug-dependent viral inhibition was maintained throughout the observation time (213 to 791 days), but in 9 patients a rebound in viremia level was detected during therapy with competitive reverse transcription PCR. Sequencing analysis of a portion of the HIV-1 gene *pol* from cell-free virions showed that circulating viral variants bearing at least two mutations compatible with azidothymidine or ddI resistance were detectable in the patients who exhibited a rebound in cell-free HIV-1 genomic RNA copy numbers in plasma but not in one patient who maintained (for 455 days) lowered levels of viral load during ddI treatment.

Different strategies to investigate quantitatively various indexes of human immunodeficiency virus type 1 (HIV-1) activity in vivo have recently been planned and developed (10, 14, 20, 21, 27, 28, 30–32). Currently, several technical aspects (6, 8, 9) indicate that competitive PCR (cPCR) is the method of choice for the quantitative detection of both viral and eukaryotic nucleic acids present at low concentration in biological samples. In this context, cPCR and competitive reverse transcription (cRT)-PCR procedures have been planned, optimized (19, 23, 26), and used to test clinical samples from HIV-1-infected patients at the different clinical phases of this infection (2, 3, 7, 24). Overall, the results of these quantitative in vivo studies indicated that a significant association exists between levels of viral activity and disease progression, suggesting that quantitative molecular methods could be a powerful tool for a better understanding of the natural history of the infection and AIDS pathogenesis. Nonetheless, the availability of quantitative methods that provide insight into previously unchecked virological and molecular aspects of HIV-1 infection in vivo has also supplied a theoretical basis for specific antiretroviral treatments. Furthermore, principally due to the considerable plasticity of the HIV-1 genome that enables this virus to escape the host's immune response or to generate drug-resistant viral mutants during specific antiviral treatments, the precise evaluation of the efficacy of anti-HIV-1 therapy requires direct quantitative assay systems.

In this study, we aimed to evaluate the adaptability and reliability of cPCR-based techniques to monitor the effect of specific antiretroviral treatments on HIV-1 expression and replication directly in infected patients. Sequential clinical samples from treated patients were used in this study, and different virological parameters (HIV-1 genomic RNA copy number in plasma [HIV-1 viremia], late HIV-1 transcript copy number in peripheral blood mononuclear cells [PBMCs], HIV-1 proviral copy number in PBMCs, and mean transcriptional activity [RNA/DNA ratio; HIV-1-specific late transcript copy numbers per each provirus copy number]) were assayed at each time point in samples from HIV-1-infected patients treated with azidothymidine (AZT) or ddI. Finally, the ability of quantitative molecular methods used in this study to provide information on the selection of drug-resistant viral mutants was also assayed by direct sequencing of the HIV-1 gene *pol* from cell-free plasma virions from three patients with documented rebound of viral load.

MATERIALS AND METHODS

Patients, clinical specimens, and nucleic acid purification. Sequential clinical samples were collected from 12 HIV-1-infected patients (8 males and 4 females; mean age, 29.5 years [range, 24 to 33 years]). The risk factors associated with HIV-1 infection were distributed as follows: intravenous drug addiction, eight subjects; homosexual activity, three subjects; heterosexual contact with a HIV-1-infected partner, one subject. The patients (four Centers for Disease Control and Prevention [CDC] class II, five CDC class III, and two CDC class IV patients and one patient who progressed from class III to IV during treatment) were studied at the beginning of antiretroviral therapy (time zero) with specific anti-HIV-1 compounds (AZT, ddI, and ddC) and monitored for 213 to 791 days during therapy.

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. PBMCs were washed three times with phosphate-buffered saline. To clear the platelets and cell debris, the plasma was centrifuged again (3,000 × *g* for 10 min). One milliliter of this supernatant was diluted in 9 ml of RPMI 1640 (Whittaker, Walkersville, Md.) and ultracentrifuged at 150,000 × *g* for 1 h in a swingout rotor (Kontron Instruments, Milan, Italy). RNA samples were extracted from the

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virion pellet and PBMC pellet by using the guanidinium thiocyanate method, as previously described (1), and to minimize unintegrated DNA contamination, DNA samples were extracted from the nuclei of PBMCs (1).

cPCR and cRT-PCR. cPCR and cRT-PCR procedures (2, 19) were used to analyze the following HIV-1-specific substrates directly and quantitatively: viral genomic RNA from plasma, virus-specific late transcripts from PBMCs, and proviral DNA from cell nuclei. Briefly, competitive analysis was performed by using plasmid pSKAN (19); this plasmid is a derivative of plasmid pBS (Stratagene, La Jolla, Calif.) in which the *gag* fragment with an 18-bp deletion (internal to the primer binding sites) is inserted downstream from the T3 RNA polymerase promoter. Competitor RNA was obtained after linearization of pSKAN 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 endpoint dilution followed by Poisson analysis of positive scores. Each RNA sample (10 μl, equivalent to 100 μl of plasma or 200,000 PBMCs) was reverse transcribed along with 2 μl of increasing copy numbers (50 to 6,250) of competitor RNA and amplified as previously described (2); 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). The amplification profile (15 s of denaturation at 94°C, 15 s of annealing at 60°C, and 45 s of extension at 72°C) was repeated for 50 cycles by using a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). All RNA and DNA samples were tested in a series of four different reactions. For competition analysis, 5 μl of each 100-μl reaction sample was run on a 10% polyacrylamide gel and analyzed after ethidium bromide staining, as previously described (2, 3, 19).

Direct sequencing of the HIV-1 gene *pol* from cell-free plasma virions. The 769-bp fragment of the HIV-1 *pol* gene (in which mutations relevant to the development of AZT and ddI resistance occur) was specifically amplified, using the primers *pol*A and *pol*NE1 (13, 17, 19) (Fig. 1). Briefly, 10 μl of each RNA sample (equivalent to 100 μl of plasma) maintained at −80°C after nucleic acid purification, and in which the amount of genomic molecules was previously determined by cRT-PCR, was reverse transcribed for 15 min at 42°C in a final volume of 20 μl containing 100 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 40 U of RNasin (Boehringer Mannheim), 20 pmol of *pol*NE1 primer, 0.2 mM (each) deoxynucleoside triphosphates, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl), and DEPC-treated water. The viral cDNA was denatured for 5 min at 94°C, and 1× PCR buffer containing 80 pmol of primer *pol*NE1, 100 pmol of primer *pol*A, and 2.5 U of *Taq* DNA polymerase was added. The PCR profile (denaturation for 30 s at 94°C; annealing for 30 s at 40°C, and extension for 90 s at 72°C) was repeated for 50 cycles in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus) followed by a final incubation at 72°C for 10 min; the PCR product was analyzed with a 1% agarose gel in Tris-borate buffer. In some instances, the 769-bp RT-PCR product was subjected to further PCR amplification, using the following internal primer pairs to obtain an adequate amount of product for direct sequencing: *pol*4 and *pol*C (position 5′-3′ 2,596 to 2,619 and 2,913 to 2,893 of the AIDS-associated retrovirus type 2 (ARV2) nucleotide sequence, respectively) (25), and *pol*D and *pol*E (position 5′-3′ 3,034 to 3,052 and 3,255 to 3,239, respectively) (Fig. 1). Amplification with primers *pol*4 and *pol*C generates a 318-bp fragment in which mutations determining changes at amino acid residues 24 to 112 of reverse transcriptase protein can be analyzed; nested PCR using primers *pol*D and *pol*E gives a 222-bp-long product in which mutations occurring at amino acid residues 167 to 267 can be recognized. Briefly, 10 μl of DNA obtained after RT-PCR were reamplified in a mixture (final volume, 100 μl) containing 1× PCR buffer, 0.2 mM (each) deoxynucleoside triphosphates, 100 pmol of each primer, and 2.5 U of *Taq* DNA polymerase. The reactions were subjected to 50 amplification cycles (15 s at 94°C, 15 s at 50°C, and 60 s at 72°C, using primers *pol*4 and *pol*C; 15 s at 94°C, 15 s at 45°C, and 60 s at 72°C, using primers *pol*D and *pol*E) followed by a final incubation at 72°C for 10 min. The products generated by RT-PCR (and in some cases by nested PCR) were separated electrophoretically through a Tris-acetate gel of 2% Nusieve; DNA was than rescued from the gel (by cutting the specific ethidium bromide-stained band), extracted with phenol-chloroform, precipitated with 2 volumes of ethanol–Na-acetate (1/10, vol/vol), and dried under vacuum.

Sequencing reactions of double-stranded PCR products were performed directly, as previously described (18), by the chain termination method, using a Sequenase kit (U.S. Biochemicals, Denver, Colo.) with minor modification; the samples were heat denatured (90°C for 3 min) in the presence of 10% dimethyl sulfoxide and the sequencing primer and quickly cooled in dry ice for 60 s. The labeling reaction was carried out at room temperature for 1 min; the termination reaction was carried out at 37°C for 3 min. Ten percent dimethyl sulfoxide was added to both labeling and termination mixtures. After the sequencing products were separated on a denaturing 6% polyacrylamide gel, the gels were washed, dried, and autoradiographed. Furthermore, to rule out the possibility that mutations could be introduced by the PCR assay, sequencing reactions were performed twice and bidirectionally for all samples from independently amplified products.

Statistical analysis of data. Statistical analysis of quantitative data was performed with the nonparametric Mann-Whitney-Wilcoxon test.

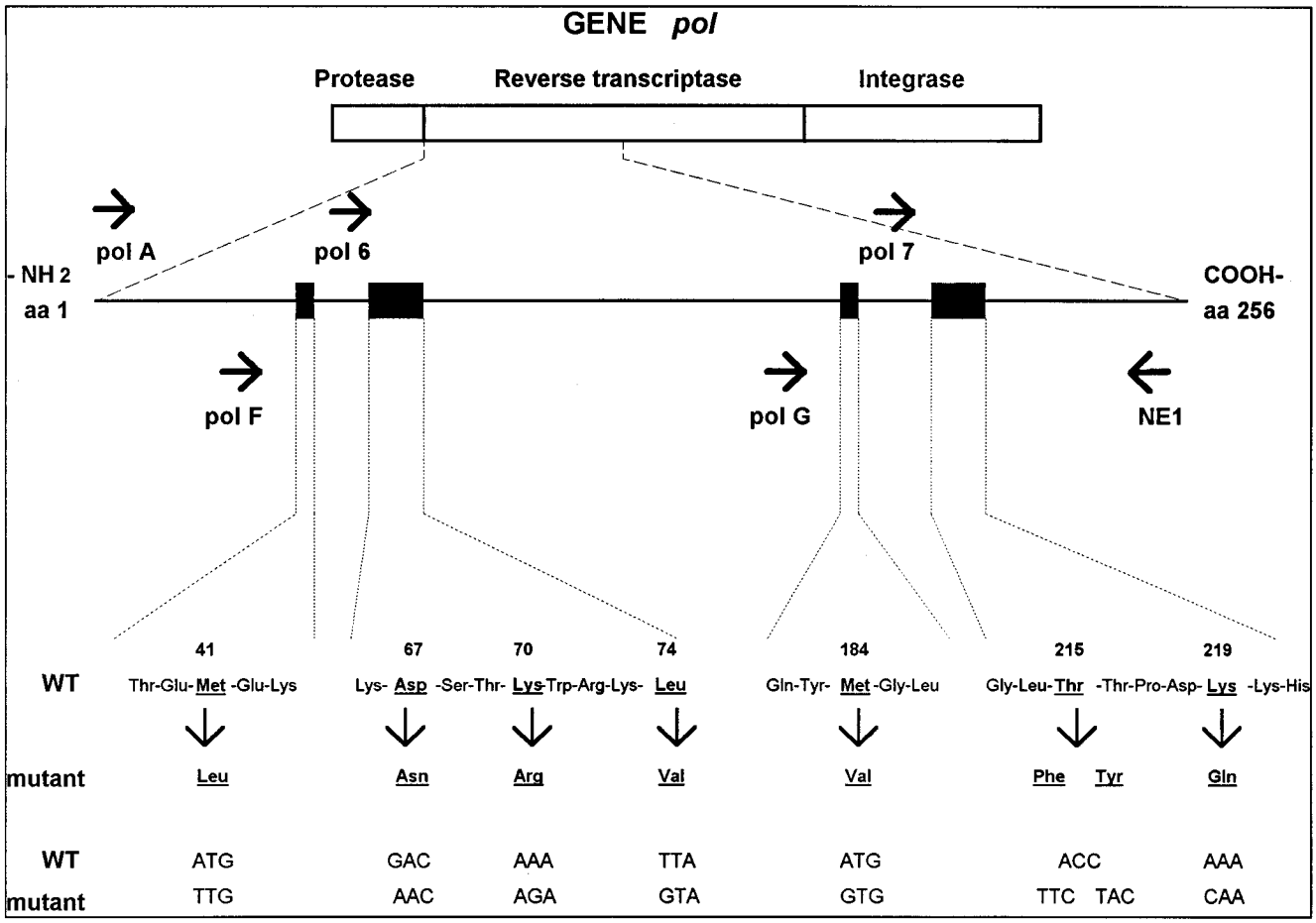
RESULTS

Molecular monitoring of HIV-1 activity during antiviral therapy. We studied a group of 12 HIV-1-infected patients under treatment with AZT (six patients [Table 1]) and ddI (two patients [Table 2]) or in whom treatment had been discontinued (one patient treated with AZT from days 1 to 394 and then with AZT and ddC; two patients treated with AZT for 230 and 660 days, respectively, and then with ddI; and one patient who received AZT for 170 days and interrupted the therapy from days 170 to 632 [Table 3]).

The molecular data show the dynamics of viral activity during specific antiretroviral therapy (Tables 1 to 3). In fact, an early fall in levels of cell-free (genomic) HIV-1 RNA copy numbers is evident for almost all patients (Fig. 2). In several patients with high basal viremia levels, a dramatic drop of viral load was observed within 14 to 17 days (Table 1, patients A02 and A03; Table 2, patient D02; Table 3, patients X01, X03, and X04); in some of these patients (A03, X01, and X04), the early decrease of viremia levels was paralleled by a fall in transcript copy numbers and mean transcriptional activity (RNA/DNA ratio). The decrease in proviral molecules per 10^5 PBMCs was delayed compared with the viremia level or transcript copy numbers; at the same time, $CD4^+$ T-cell counts generally failed to indicate any significant change during treatment, with only a few exceptions (patients A05, A06, and D01).

In seven cases (Table 1, patients A01, A03, A05, and A06; Table 3, patients X01, X02, and X04), a clear rebound of viremia levels was seen during the observation period; in most of these patients, a rebound to viremia levels higher than those observed before therapy was seen after 80 to 296 days, strongly suggesting that selection of drug-resistant HIV-1 strains occurred in these patients. Similarly, an evident rebound of HIV-1 viremia was observed in one patient (Table 3, patient X03) who interrupted AZT therapy at day 170. Interestingly, molecular parameters of viral activity other than HIV-1 viremia (specific HIV-1 transcript molecules in PBMCs, proviral DNA molecules, and mean viral transcriptional activity) generally paralleled the time course of cell-free genome copy numbers in plasma; however, in most of these treated patients, the RNA parameters (cell-free viral genomes per milliliter of plasma and viral transcripts per 2×10^5 PBMCs) seem to be highly sensitive indexes of the efficacy of a given antiretroviral compound. Finally, in several patients under therapy with AZT or ddI (patients A01, A02, A03, A04, D02, X01, and X04), sharp changes in viral activity levels were not paralleled by an evident modification of $CD4^+$ T-lymphocyte number per cubic millimeter of blood.

A statistical analysis of changes in viral load (genome copy numbers per milliliter of plasma) was performed in 11 of the 12 patients under treatment with AZT or ddI (patient X02 received AZT for 6 months during the year before the first evaluation [time zero] and was excluded from this analysis). The Mann-Whitney-Wilcoxon test employed for nonparametric statistical comparison of quantitative values at time zero (baseline) and after 4 to 13 weeks shows a significant change of HIV-1 genome copy number ($P = 0.00032$) and transcript molecules in PBMCs ($P = 0.023$) but not of HIV-1 proviral DNA ($P = 0.106$). Additionally, in 7 of the 11 patients (indentified as patients A01, A03, A05, A06, X01, X02, and X04) who continued antiviral treatment during the period under study (patient X03 interrupted AZT therapy at day 170), a rebound of viral load was observed; the comparison between the baseline level of the genome copy number (HIV-1 viremia) and that determined in the last control (after 35 to 71 weeks from time zero) shows a significant increase ($P = 0.023$) in



PCR PRIMERS	position (ARV2 sequence; Ref. 25)	PCR PRODUCTS (bp)
POL A (5'-3')	2555-2571 (17 nt)	POL A / NE 1 (769)
NE 1 (5'-3')	3323-3307 (17 nt)	

SEQUENCING PRIMERS:	nucleotide position (ARV2 sequence)	starting from aa	mutation analysed (aa)
POL F	2626 - 2643 (18 nt)	24	41
POL 6	2696 - 2713 (18 nt)	47	67; 70 ; 74
POL G	3057 - 3074 (18 nt)	168	184
POL 7	3132 - 3149 (18 nt)	192	215; 219

FIG. 1. The presence of mutations relevant to the development of resistance to AZT and ddI was evaluated by sequence analysis of a fragment of the HIV-1 gene *pol*. A 769-bp fragment of *pol* was amplified with primers *pol*A and *pol*NE1 (in some instances, this sequence was further amplified with internal primer pairs [*pol*4 and *pol*C or *pol*D and *pol*E]; see Materials and Methods). Sequencing reactions of double-stranded PCR products were determined directly by the chain termination method, using primers *pol*F, *pol*6, *pol*G, and *pol*7. aa, amino acid(s); WT, wild type; nt, nucleotide(s); ARV2, AIDS-associated retrovirus type 2.

these cases. Similarly, a significant increase of HIV-1 transcript copy number ($P = 0.021$) and not of proviral DNA molecules ($P = 0.250$) is observed.

Selection of drug-resistant HIV-1 mutants in patients with documented rebound of viral load. We evaluated the correlation between the rebound of plasma viremia and the appearance of drug-resistant mutants in HIV-1-infected, treated patients. Sequencing analysis of a portion of the HIV-1 gene *pol* was carried out in three of the five patients with documented rebounds of viremia levels during therapy and in one patient (Table 2, patient D02) in whom a low viral load was maintained during therapy for more than 15 months. The results of sequencing analysis are summarized in Table 4. Two patients (A01 and A03) received AZT, 1 patient (X02) had received AZT before being switched to ddI, and one patient (D02) was treated with ddI. In all cases, the first and the last samples available were assayed; sequential samples from patient X02 were analyzed from the time AZT was stopped and therapy was continued with ddI. This patient received AZT for 3 months 1 year before the first evaluation; interestingly,

TABLE 1. Molecular and immunological data from HIV-1-infected patients treated with AZT[a]

Patient	Days of treatment	cRT-PCR		c-PCR (proviral DNA molecules per 2×10^5 PBMCs)	RNA/DNA ratio	$CD4^+$ T-lymphocytes per mm^3 of blood	CDC class
		Cell-free genomes per ml of plasma	HIV-1 transcripts per 2×10^5 PBMCs				
A01	0	39,260	81	898	0.090	432	II
	30	5,070	247	474	0.521	400	
	65	5,096	114	204	0.561	384	
	155	4,150	89	1,356	0.066	412	
	233	7,150	509	1,094	0.465	527	
	296	58,090	1,101	1,940	0.568	414	
	357	85,450	433	876	0.494	457	
	393	130,310	905	2,796	0.324	400	
A02	0	21,528	ND[b]	208	ND	276	II
	14	6,006	6	62	0.097	385	
	32	4,134	3	36	0.083	554	
	46	2,366	16	134	0.116	300	
	56	7,982	34	ND	ND	252	
	89	2,640	85	50	1.700	230	
	119	2,610	120	114	1.053	229	
	221	15,960	215	52	4.135	230	
A03	0	70,148	395	1,246	0.317	383	III
	15	5,044	60	1,360	0.044	400	
	34	26,702	56	780	0.072	450	
	62	66,534	2,223	536	4.147	478	
	92	172,198	1,869	388	4.818	500	
	127	23,582	887	482	1.839	630	
	295	14,898	283	1,216	0.233	266	
	358	127,738	2,124	1,034	2.054	584	
	390	413,770	4,325	542	7.980	400	
	419	287,430	4,280	1,398	3.062	358	
	455	360,930	6,549	1,416	4.625	300	
A04	0	9,875	75	120	0.628	476	III
	90	2,314	68	70	0.966	ND	
	118	920	13	46	0.283	333	
	213	416	13	150	0.087	396	
A05	0	4,420	170	694	0.245	337	III
	13	980	120	516	0.233	320	
	32	920	12	108	0.111	370	
	48	420	12	200	0.060	535	
	81	400	80	348	0.230	671	
	193	12,142	211	308	0.684	410	
	214	520	96	230	0.418	485	
	319	858	65	26	2.500	433	
	375	598	23	10	2.340	561	
	501	32,880	845	1,046	0.808	602	
A06	0	27,768	746	950	0.785	323	II
	14	22,160	ND	994	ND	340	
	44	4,810	ND	ND	ND	852	
	126	ND	707	954	0.741	ND	
	168	10,868	73	1,048	0.069	892	
	211	17,020	67	522	0.128	738	
	246	43,110	282	42	6.714	478	

[a] All patients received 500 mg of AZT per day.
[b] ND, not determined.

mutations at positions 41 (Met→Leu) and 215 (Thr→Phe and Tyr) (Table 4) were already selected at this time. At day 112 from the beginning of ddI therapy, a mutation at position 74 (Leu→Val; consistent with resistance to ddI) appeared as a mixed genotype population; this mutation became predominant 1 month later. Furthermore, a mutation at position 41 was seen in the last sample from patient A03, and in patients A03 and X02 a change at codon 39 (Thr→Ala; significance not investigated) was also detected. Mutations at positions 67 (Asp→His) and 70 (Lys→Arg) were documented in the last sample from patient A01, who received AZT for 13 months. Importantly, in this patient the maintenance of the wild-type genotype during the first 8 months of therapy parallels the decreased viremia levels. Similarly, a wild-type genotype was seen in samples from patient D02; in this patient, a reduced viral load was maintained during the observation period (455

TABLE 2. Molecular data from HIV-1-infected patients treated with ddI[a]

Patient	Days of treatment	cRT-PCR		c-PCR (proviral DNA molecules per 2×10^5 PBMCs)	RNA/DNA ratio	$CD4^+$ T-lymphocytes per mm^3 of blood	CDC class
		Cell-free genomes per ml of plasma	HIV-1 transcripts per 2×10^5 PBMCs				
D01	0	35,334	127	102	1.249	539	II
	103	2,710	32	96	0.333	471	
	133	210	23	60	0.383	500	
	162	390	29	42	0.681	536	
	201	624	9	10	0.900	630	
	264	600	28	44	0.636	823	
	313	900	32	24	1.333	ND[b]	
D02	0	40,092	166	350	0.475	153	IVc2
	14	1,664	138	646	0.213	181	
	28	832	733	676	1.084	341	
	43	1,534	86	236	0.364	305	
	62	1,612	53	354	0.149	268	
	123	1,508	68	254	0.269	250	
	151	1,638	346	270	1.282	255	
	217	5,694	117	332	0.352	163	
	308	1,540	220	330	0.667	179	
	341	ND	200	340	0.588	336	
	455	6,940	598	558	1.072	ND	

[a] Patients were treated with 400 mg of ddI per day.
[b] ND, not determined.

days of therapy with ddI). Overall, mutations in at least two codons of the HIV-1 gene *pol* were documented in three patients whose viral loads (evaluated by cRT-PCR) increased during specific antiretroviral therapy.

DISCUSSION

In this study, different parameters of HIV-1 activity were monitored in patients under therapy with specific antiretroviral compounds. The data shown here confirm and extend recent observations that the molecular techniques employed in this study may efficiently be used for complete virological and clinical monitoring of treated HIV-1-infected patients. Importantly, although HIV-1 gene *pol* sequences were analyzed in only 5 of 12 patients, the results suggest that the processes of emergence and selection of drug-resistant HIV-1 mutants may be revealed with cRT-PCR.

Ideally, a method suitable for evaluating viral activity in vivo requires the characteristics of rapidity (the choice of a given therapeutic strategy may be dependent on the data obtained), flexibility (possibly different tissues or clinical specimens of different origin have to be used), high sensitivity and specificity, and finally, adaptability to absolute quantitation. Under these conditions, the advantages of using molecular methods appear to be evident, particularly in relationship to the genomic evolutionary potential documented for lentivirus infections (4, 11, 16). In these infections, a mixture of distinct genomes (22) is generally detected, and not only antigenic properties (5) but also gene expression (15), tissue tropism, pathogenicity, and drug resistance (13) of the infecting virus may change during the infection. In this context, it is reasonable to hypothesize that in vitro propagation of HIV-1 (viral isolation) increases the representation of the viral strains with a high replicative capacity, thus only approximating what is really occurring in vivo. Recently, we indicated that a cPCR-based strategy efficiently identifies those HIV-1-infected patients who (independently of clinical conditions) exhibit highly active infection (2). Subsequently, an additional study with a cPCR-based approach was carried out; it evaluated the HIV-1 viremia of untreated and treated subjects at different phases of the infection (24). Overall, the data suggested that this methodology may efficiently be used for not only studies of HIV-1 pathogenesis but also diagnostic applications, including monitoring of specific antiviral therapy. More recently (3), we documented that cRT-PCR-based techniques allow the dynamics of different virological parameters to be monitored for years in infected patients, thus obtaining a direct molecular profile of viral activity and replication during the various clinical phases of HIV-1 infection.

In the study presented here, we have specifically addressed the direct laboratory evaluation of therapy with specific anti-HIV-1 compounds at the molecular level. Using a cRT-PCR assay system, the degree of HIV-1 activity was evaluated in sequential samples from patients under treatment with antiretroviral compounds. Although the biochemistry of HIV-1 AZT and ddI resistance is not completely defined at present, AZT resistance was shown to be mediated by five amino acid substitutions in the viral reverse transcriptase protein (13, 17); similarly, resistance to ddI was found to be mediated by substitutions at codons 74, 69, and 184 (29).

Different aspects of the results shown here may have diagnostic and clinical importance. First, during the early phase of treatment with AZT and ddI, a significant drop of all parameters of HIV-1 activity could be detected in almost all patients; nonetheless, a residual (low) viral replication level was always documented. Second, of the different parameters monitored in these patients, the copy number of cell-free genomes in plasma (HIV-1 viremia) was a more sensitive index of the drug-dependent fall in viral replication level than transcript (or provirus) copy numbers in PBMCs. Third, sequencing analysis of the HIV-1 gene *pol* suggests that the rebound of viral activity detected by the cPCR-based method employed in this study is associated with selection of drug-resistant viral mutants. This evidence may have medical importance, and it strongly indicates that quantitative molecular monitoring of treated patients by cRT-PCR represents a reliable strategy to evaluate directly in vivo the efficiency of a given antiviral therapy in any treated patient and at any time point. In fact, as

TABLE 3. Molecular data from HIV-1-infected patients treated with more than one antiretroviral compound[a] or from patients who discontinued therapy

Patient	Antiviral compound	Days of treatment	cRT-PCR		c-PCR (proviral DNA molecules per 2×10^5 PBMCs)	RNA/DNA ratio	$CD4^+$ T-lymphocytes per mm^3 of blood	CDC class
			Cell-free genomes per ml of plasma	HIV-1 transcripts per 2×10^5 PBMCs				
X01	AZT[b]	0	152,048	4,691	498	9.420	47	IVc1
		17	9,650	307	142	2.162	50	
		32	8,550	28	158	0.177	75	
		50	11,790	294	338	0.870	101	
		80	155,376	281	376	0.747	76	
		112	177,112	273	508	0.537	64	
		211	195,234	689	536	1.285	50	
		367	18,772	2,772	984	2.817	32	
	AZT + ddC	394	25,000	390	1,004	0.388	35	
		423	541,690	1,873	254	7.374	48	
		458	638,840	4,714	1,816	2.596	13	
		485	1,435,900	10,466	2,470	4.237	34	
X02	AZT	0	277,446	814	1,386	0.587	147	III
		20	142,064	1,027	1,466	0.701	212	
		34	31,694	1,794	1,890	0.949	129	
		62	37,258	1,563	1,200	1.302	130	
		90	526,942	403	1,512	0.267	89	
		118	143,936	333	2,970	0.112	116	
		147	15,392	29	2,192	0.013	110	
		174	65,286	190	448	0.424	43	
		203	41,158	1,230	3,250	0.378	34	
	ddI	230	68,250	312	456	0.684	41	
		258	75,740	3,395	2,786	1.219	36	
		285	527,280	2,835	ND[c]	ND	74	IVc1
		315	905,890	3,209	3,978	0.804	44	
		342	428,680	2,340	1,186	1.973	45	
		371	282,090	1,148	740	1.551	43	
X03	AZT	0	23,715	ND	604	ND	324	III
		14	2,310	156	310	0.503	331	
		29	520	240	482	0.498	405	
		78	271	195	256	0.762	586	
		105	1,104	320	432	0.741	673	
		142	1,680	485	452	1.073	700	
	Interrupted	170	1,800	485	340	1.426	826	
		205	1,560	335	308	1.088	810	
		239	3,010	369	350	1.054	795	
		290	31,780	922	764	1.207	457	
		575	63,220	1,050	1,358	0.773	656	
		583	397,500	7,274	1,148	6.336	802	
		632	774,360	1,524	688	2.215	741	
X04	AZT	0	38,352	246	166	1.482	43	III
		16	194	50	114	0.439	94	
		28	1,425	67	174	0.385	72	
		42	10,031	62	144	0.431	60	
		190	4,469	119	186	0.640	27	
		330	2,440	899	250	3.596	16	
	ddI	660	18,850	250	238	1.050	11	
		722	5,902	43	30	1.433	50	
		791	61,990	269	34	7.912	63	

[a] AZT, 500 mg/day; ddI, 400 mg/day.
[b] Inconstant therapy with AZT for 6 months before day 0.
[c] ND, not determined.

observed in three patients who showed a rebound of HIV-1 viremia during therapy (Table 4), emergence of viral strains bearing at least two mutations of the HIV-1 gene *pol* compatible with resistance to AZT or ddI were observed in cell-free plasma virus; in contrast, these mutations were undetectable in samples from a patient who maintained lowered HIV-1 viremia levels for 15 months during ddI therapy.

All patients included in this study received specific antiviral monotherapy. After an early phase of inhibition of viral replication, cRT-PCR indicated that this treatment was inefficient in a significant proportion of the subjects, as documented by a rebound of viremia levels. From this point of view, the molecular results may suggest that treatment with multiple compounds active at different steps of the viral replication

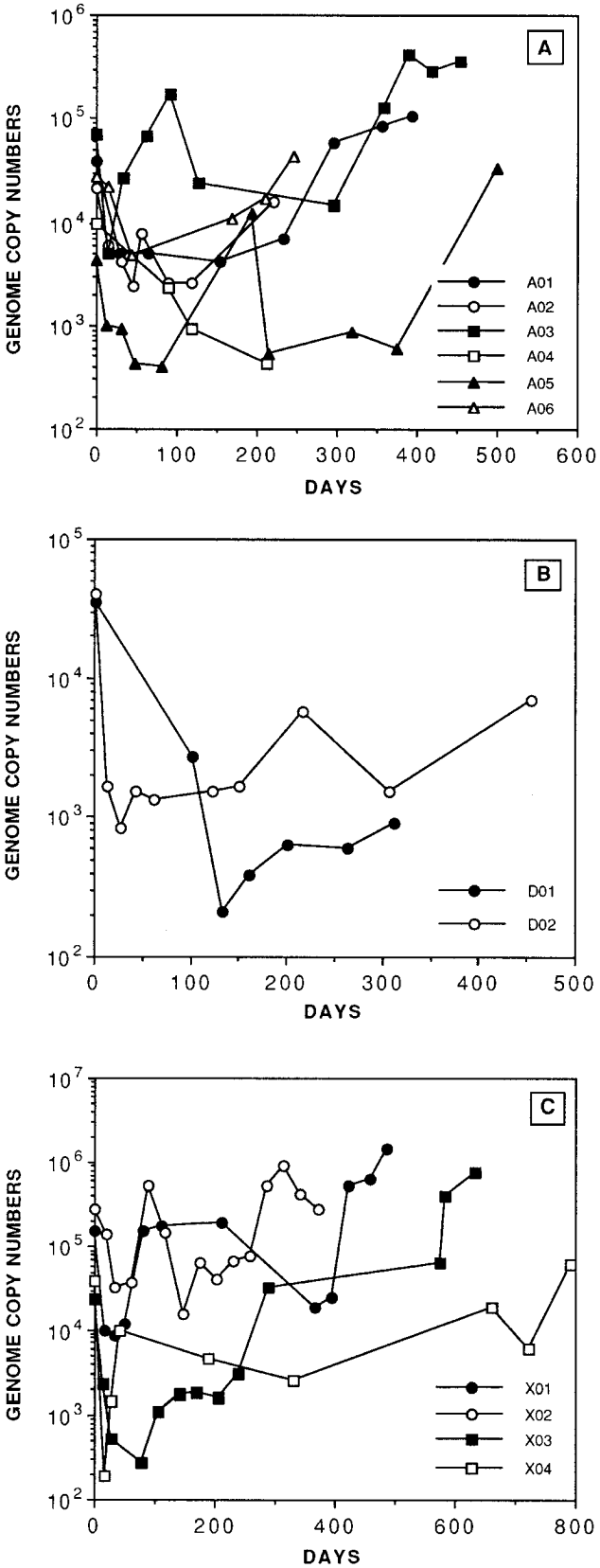


FIG. 2. Time course of HIV-1 genome copy numbers per milliliter of plasma in samples from patients treated with 500 mg of AZT per day (A) or 400 mg of ddI per day (B) and from subjects treated with more than one antiviral compound or patients who discontinued anti-HIV-1 therapy (C).

TABLE 4. HIV-1 viremia levels and selection of drug-resistant viral mutants in patients under treatment with antiretroviral compounds

Patient	Antiviral therapy			RT genotype at given codons[a] with sequencing primers:						
				*pol*F/*pol*6				*pol*G/*pol*7		
	Drug	Days	Viremia[b]	41	67	70	74	184	215	219
A02	AZT	0	39,260	WT	WT	WT	WT	ND	WT	WT
		233	7,150	WT	WT	WT	WT	ND	WT	WT
		393	130,310	WT	MU	MU	WT	ND	WT	WT
A03	AZT	0	70,148	WT	WT	WT	WT	ND	WT	WT
		455	360,930	MU	WT	WT	WT	ND	MU	WT
X02	AZT	0[c]	277,446	MU	WT	WT	WT	ND	MU	WT
	ddI	230	41,158	MU	WT	WT	WT	WT	MU	WT
		258	75,740	MU	WT	WT	WT	WT	MU	WT
		285	527,280	MU	WT	WT	WT	WT	MU	WT
		342	428,680	MU	WT	WT	MX	WT	MU	WT
		371	282,090	MU	WT	WT	MU	WT	MU	WT
D02	ddI	0	40,092	ND	WT	WT	WT	WT	ND	ND
		455	6,940	ND	WT	WT	WT	WT	ND	ND

[a] WT, wild type; ND, not done; MU, mutant; MX, detection of mixed genotypes.
[b] Genome copy numbers per milliliter of plasma.
[c] Inconstant therapy with AZT before day 0.

cycle or on different virus-specific targets is necessary in this infection. Further molecular investigation of traditional and new therapeutic strategies for HIV-1-infected patients will be necessary to address this aspect, and cPCR-based methodology may represent an efficient laboratory tool for both in vitro and in vivo research.

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