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Kinetic Analysis of Intravirion Reverse Transcription in the Blood Plasma of Human Immunodeficiency Virus Type 1-Infected Individuals: Direct Assessment of Resistance to Reverse Transcriptase Inhibitors In Vivo

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Intravirion reverse transcripts have been identified in the blood plasma of human immunodeficiency virus type 1 (HIV-1)-infected individuals. In the present studies, the kinetic processes of intravirion HIV-1 reverse transcription, in the blood plasma of HIV-1-infected persons treated with nevirapine, were investigated. Nevirapine is a nonnucleoside inhibitor of reverse transcriptase (RT) which decreases the level of HIV-1 viral particles in the blood plasma of infected individuals. By analyzing HIV-1 virions at different time points prior to and after initiation of nevirapine therapy in vivo, the levels of intravirion reverse transcripts have been demonstrated to be dramatically susceptible to this anti-RT agent, out of proportion to effects on plasma virion load. The intravirion reverse transcripts were also documented to rebound to the pretreatment levels, concomitant with the development of resistant viral mutants. In addition, the infectivity of HIV-1 virions dramatically decreased after nevirapine treatment, further indicating that the effects of this anti-RT agent begin within the cell-free virions. Since the levels of intravirion reverse transcripts were altered according to the susceptibility or resistance of the HIV-1 RT enzyme to this inhibitor, these data demonstrate that the formation of intravirion reverse transcripts is a dynamic process in vivo. Moreover, because the alteration in ratios between intravirion HIV-1 reverse transcripts and viral genomic RNA directly reflects the efficiency of reverse transcription, we propose that the determination of these ratios in the blood plasma of HIV-1-positive patients may be a useful and, most importantly, a direct assay to monitor the efficacy of anti-RT agents in vivo.

Nevirapine is a nonnucleoside inhibitor of the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) (15). This compound can significantly decrease the level of virions in the blood plasma of HIV-1-infected individuals in a very short time period after initiation of therapy (19, 24). On the basis of these characteristics, nevirapine, as well as other antiretroviral agents, have been used to study the dynamics of HIV-1 replication in vivo. It has been recognized that the maintenance of HIV-1 virions in the blood plasma is a rapid, dynamic process, and the viral strain(s) circulating in the peripheral blood plasma are likely to represent the actively replicating virus in the body at that particular point in the clinical infection (10, 19, 24). In addition, the quantity of HIV-1 particles in the blood plasma is inversely correlated with the CD4-positive lymphocyte count in the blood (9, 10, 19, 24). Therefore, consistently decreasing the virion load in blood plasma and the virions' infectivity may slow the progression of clinical HIV-1 disease. Of importance, viral mutants resistant to many anti-HIV-1 agents, including nevirapine, can rapidly develop. As a result, the quantity of HIV-1 virions in the blood plasma can rebound to the pretreatment levels in a very short time after initiation of anti-RT therapy, accompanied by decreasing levels of CD4-positive lymphocytes (10, 19, 24).

Nevirapine-resistant mutations in the HIV-1 RT gene occur notably at codons 103, 106, and 181 in vitro and additionally at codons 108, 188, and 190 in vivo (19, 20). All of these mutated codons are in the two β-sheets (amino acids 100 to 110 and 180 to 190, respectively), where a hydrophobic pocket structure is formed in the HIV-1 RT enzyme. This structure is close to the polymerase active site of HIV-1 RT (12). Nevirapine binds to the HIV-1 RT enzyme through this structure to block polymerization without interfering with nucleotide binding or the nucleotide-induced conformational changes in the enzyme (21). These HIV-1 virus resistance mutations may individually or in combination exist in the blood plasma of nevirapine treated-patients. The mutations can even sequentially replace one another, as nevirapine is consistently administered (19). However, the Y181C mutation is most frequently (79%) associated with the loss of anti-RT activity for nevirapine monotherapy, since Y181 and Y188 are the key sites where nevirapine binds to the HIV-1 RT enzyme (19). Interestingly, coadministration of zidovudine (AZT) completely prevents the occurrence of Y181C mutants, indicating that this combination of agents forces the selection of alternative mutations (19).

Studies of certain cell culture systems have now demonstrated that cell-free HIV-1 virions contain significant levels of reverse transcripts (14, 23, 29). Recently, our laboratory has identified intravirion reverse transcripts in the blood plasma of both asymptomatic HIV-1-seropositive individuals and patients with AIDS (26) by a virion immunocapture methodology conjugated with quantitative DNA-PCR and RT-PCR assays (8, 26). The majority of intravirion HIV-1 DNA is formed secondarily to partial reverse transcription. The in vivo intra-

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TABLE 1. Pertinent clinical data for HIV-1-infected individuals

Patient no.	CDC clinical classification stage[a]	Concn of HIV-1 p24 antigen in serum before treatment (pg/ml)	CD4-positive lymphocyte count (cells/mm^3) before treatment	Daily dose of nevirapine (mg)
164-018	B-2	111	212	12.5
164-028	B-2	493	228	12.5
164-021	B-3	318	134	12.5
164-019	A-2	93	213	50
164-035	B-3	306	102	50
164-042	C-3	102	10	50
164-051	B-2	38	317	200
164-043	C-2	216	395	200

[a] CDC, Centers for Disease Control.

virion HIV-1 DNA/RNA ratios in the strong-stop negative-strand region are approximately $1{:}10^{-2}$ to $1{:}10^{-3}$ and in the *gag* and near full-length region are approximately $1{:}10^{-4}$ to $1{:}10^{-5}$. Interestingly, although the majority of the patients analyzed in this initial study were treated with AZT, the levels of intravirion HIV-1 reverse transcripts still remained relatively high (26). This phenomenon is contrasted with the in vitro observation, in which the addition of AZT to cell cultures decreases the levels of intravirion reverse transcripts in HIV-1 virions produced by these treated cells (references 23 and 27 and unpublished data). However, since AZT resistance may have developed in vivo in the presence of AZT after long-term treatment, the intravirion HIV-1 reverse transcripts could have arisen from AZT-resistant viral strains (26).

In vitro studies have demonstrated that increasing virion-associated DNA levels potently increases retrovirus infectivity and, therefore, may alter intra- and interhost transmission of retroviruses (27–29; unpublished data). These effects have been demonstrated for both HIV-1 and simple murine amphotropic retroviruses (8, 23, 27). Further, RT inhibitor(s) is able to decrease the levels of intravirion HIV-1 reverse transcripts and, thus, to inhibit the infectivity of virions upon target cells, especially quiescent cells (27). Because of these biological and potentially clinical ramifications, a sequential, longitudinal cohort study of intravirion HIV-1 reverse transcripts in the presence of the RT inhibitor nevirapine was performed. These analyses demonstrated correlations between the production of resistant viral mutants and the levels of intravirion HIV-1 reverse transcripts. Because nevirapine treatment produces a prompt antivirus response that is followed by loss of antiviral activity in conjunction with the rapid development of drug-resistant mutants, it is possible to observe the sequential alterations of intravirion reverse transcripts in the presence of nevirapine within a relatively short time period. Analysis of a cohort of patients with AIDS treated with nevirapine, whose clinical data have already been well-documented (3, 19), helps reveal the dynamics of intravirion reverse transcription in vivo and demonstrates the potential utility of intravirion HIV-1 DNA quantitation as a measure of virus resistance to anti-RT agents.

TABLE 2. HIV-1 RNA copies in blood plasma

Patient no.	No. of copies after the following no. of wks of nevirapine treatment (10^5/ml):				
	−1	1	4	8	12
164-018	51.7	24.2	48.3	54.1	54.0
164-028	39.8	8.1	18.2	41.3	42.5
164-021	28.7	11.3	28.4	29.0	29.0
164-019	6.6	1.2	4.0	6.1	6.1
164-035	26.7	12.1	14.2	27.1	25.8
164-042	50.1	7.7	44.6	50.2	50.2
164-051	25.1	6.7	20.1	13.2	24.8
164-043	27.8	5.5	19.3	28.2	28.0

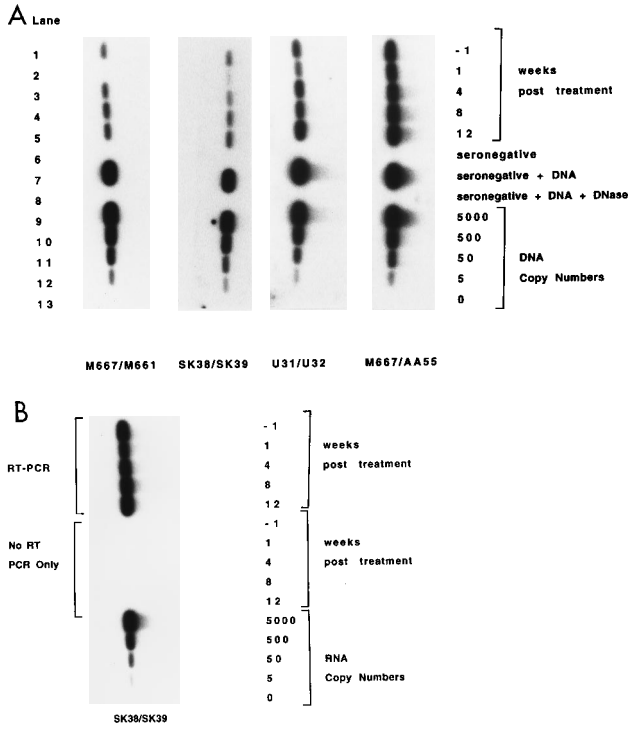

FIG. 1. Detection and analysis of intravirion HIV-1 DNA and RNA in vivo by quantitative DNA-PCR and RT-PCR. HIV-1 virions were captured by anti-gp120 and anti-gp41 antibody-coated latex beads from the blood plasma of an HIV-1-seropositive patient (no. 164-019), who received nevirapine treatment. As a control, blood plasma from an HIV-1-seronegative person was treated at the same time. (A) The captured HIV-1 virions were then treated with DNase, and the viral DNA was extracted and amplified by PCR with different primer pairs. The PCR products were analyzed by Southern blot assays. Lanes 1 to 5, intravirion HIV-1 DNA from different time points prior to and after nevirapine therapy; lane 6, sample from the HIV-1-seronegative patient; lane 7, sample from the HIV-1-seronegative patient plus HIV-1 DNA from ACH-2 cells (one HIV-1 proviral copy per cell) (26); lane 8, sample from the HIV-1-seronegative patient plus HIV-1 DNA from ACH-2 cells, which was then treated with DNase (control for DNase activity); lanes 9 to 12, standard curve of HIV-1 DNA extracted from ACH-2 cells (copy numbers). (B) The viral RNA was extracted from HIV-1 virions and reverse transcribed into cDNA. The cDNA was then diluted (1:100) and amplified by PCR with primer pair SK38/SK39. The PCR products were analyzed by Southern blot assays. As a control, the samples analyzed without an RT step were also amplified to confirm the absence of DNA contamination. The standard unspliced HIV-1-specific RNA curve (copy number) was prepared from in vitro transcribed HIV-1 RNA, as previously described (26, 29). This figure illustrates a representative patient from the analyzed cohort.

Intravirion HIV-1 reverse transcripts are altered by nevirapine treatment in vivo. All patients in this study were treated with nevirapine as part of AIDS Clinical Trials Group protocol 164 (3), approved by the institutional review board of the University of California, San Diego. Informed consent was obtained from all individuals in this study. Four weeks prior to administration of nevirapine, the patients in this study discontinued all other antiviral agents. The pertinent clinical data for the eight individuals in this study are listed in Table 1.

HIV-1 virions in the blood plasma (400 μl) were isolated by using anti-gp120 and gp41 antibody-coated latex particles and

TABLE 3. Comparison of intravirion HIV-1 DNA/RNA ratios in various HIV-1 genomic regions before and after nevirapine treatment

Patient no.	Ratio for the following regions[a]									
	RU5 at the following wks (10^3):					U3 at the following wks (10^4):				
	−1	1	4	8	12	−1	1	4	8	12
164-018	8.4 (100)	8.3 (98.5)	6.3 (74.5)	7.8 (91.9)	9.3 (109.4)	13.5 (100)	8.2 (60.7)	6.8 (50.4)	12.0 (88.9)	14.1 (104.4)
164-028	21.5 (100)	20.0 (93.0)	21.2 (97.9)	21.1 (97.6)	20.8 (96.7)	17.5 (100)	13.1 (74.9)	10.5 (60.1)	14.6 (83.4)	16.2 (92.6)
164-021	1.5 (100)	1.3 (86.7)	1.4 (93.3)	1.4 (93.3)	1.5 (100)	3.1 (100)	2.0 (64.5)	2.1 (67.7)	2.4 (77.4)	3.0 (96.8)
164-019	1.8 (100)	1.4 (82.3)	1.6 (92.2)	1.8 (100)	1.9 (107.1)	3.0 (100)	0.8 (26.7)	1.9 (63.3)	2.5 (83.3)	3.1 (103.3)
164-035	12.1 (100)	11.0 (93.4)	10.0 (82.0)	12.1 (100.)	12.2 (100.8)	71.0 (100)	12.1 (17.0)	12.2 (17.2)	14.3 (20.1)	30.2 (42.5)
164-042	1.6 (100)	1.5 (92.9)	1.6 (97.2)	1.5 (92.5)	1.5 (92.5)	2.0 (100)	1.5 (75.0)	1.8 (90.0)	1.8 (90.0)	1.8 (90.0)
164-051	6.7 (100)	6.4 (95.3)	6.0 (88.5)	6.1 (90.6)	6.7 (100)	12.8 (100)	4.5 (35.2)	11.1 (86.7)	10.0 (78.1)	13.1 (102.3)
164-043	5.4 (100)	4.9 (90.1)	4.6 (85.1)	5.0 (86.9)	5.6 (103.1)	17.1 (100)	6.0 (35.1)	12.3 (71.9)	13.5 (70.9)	18.5 (108.2)
Mean[b]	100	91.4	88.8	94.3	101.3	100	43.2	63.4	74.0	92.5
SD[b]	0.0	5.2	8.0	4.8	5.7	0.0	26.7	22.9	22.7	21.1

[a] Numbers in parentheses are HIV-1 DNA/RNA ratios normalized to pretreatment points as a baseline (100%).
[b] Only the normalized HIV-1 DNA/RNA ratios were used in these calculations.

were subsequently treated with DNase, as previously described (26). These isolated virions were then utilized for DNA- and RT-PCR analyses. The viral DNA extraction procedures used in these studies have been previously described (23, 29). Viral RNA extraction was conducted, as previously described, with minor modifications (2, 4, 11, 25). Briefly, half of an aliquot of the pelleted bead-virion complex was mixed with 400 μl of solution D (6.3 M guanidine thiocyanate, 42 mM sodium citrate [pH 4.0], 0.83% *N*-lauroyl sarcosine, 0.2 mM β-mercaptoethanol). After 5 min, 40 μl of 2 M sodium acetate (pH 5.5) was added. The viral RNA was then extracted with phenol-chloroform (1:1) and precipitated with ethanol. The resuspended RNA samples were subjected to RT-PCR with HIV-1 *gag* primer pairs/probe as previously described (26, 29). A control lacking RT was used to confirm the absence of viral DNA contamination in the RNA preparations. For copy number determinations, the HIV-1 DNA and RNA standard curves were prepared as previously described (29). It was notable that, although reverse transcription may lead to degradation of viral RNA via RNase H, quantitation of HIV-1 virions by using *gag* primers with RT-PCR would not lead to spuriously low RNA levels, since only a minority of viral particles harbor reverse transcripts containing *gag* regions.

A new set of primer pairs (U31 and U32)/probe (U33), which can detect HIV-1 DNA synthesis just following the first template switch during reverse transcription, was also used in the present study. The sequences and locations in the HIV-1 proviral genome for primers U31 and U32 are: 5′-GCCCG AGAGCTGCATCCG-3′ (nucleotides 294 to 311) and 5′-GG CAAAAAGCAGCTGCTT-3′ (nucleotides 430 to 447) and for probe U31 are CCAGGGAGGCGTGGCCTGGGCGGG ACTGGGGAGTGGCG (nucleotides 372 to 409). As described in previous studies, the RU5 region (negative-strand strong-stop DNA) was detected by using the primers/probe set M667/AA55/SK31; the *gag* region was detected by using the primers/probe set SK38/SK39/SK19; and the region in RU5-primer-binding site (PBS)-5′ noncoding region (5NC), which amplifies nearly full-length reverse transcripts, was detected by the primers/probe set M667/M661/SK31 (26). PCR was performed for 30 cycles. The products of PCR were analyzed by standard Southern blotting. The ^{32}P-labeled blots were analyzed and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and copy numbers were calculated by comparison with standard curves (26).

The differences of normalized intravirion HIV-1 DNA/RNA ratios at various time points were compared by analysis of variance (via Systat). If the analysis revealed significance, paired Student's *t* tests were then utilized. Correlation analyses were performed (via the SAS system [version 6.0]) to determine the relationships between normalized intravirion HIV-1 DNA/RNA ratios and the $\log_{10}$ of the 50% inhibitory concentration (micromolar). All tests were two-tailed. A P value of 0.05 or less was considered statistically significant.

Virion-associated, DNase-resistant HIV-1 DNA and virion-associated RNA from all samples were analyzed by the quantitative DNA-PCR and RT-PCR assays as described above. Autoradiographs of a representative patient's samples (patient identification no. 164-019) are illustrated in Fig. 1. The other subjects were analyzed in the same fashion. Since decreases in HIV-1 virion levels in the blood plasma of patients soon after initiation of nevirapine treatment could confound analysis of intravirion reverse transcripts (Table 2), the ratios of viral DNA detected in different regions versus viral RNA (DNA/RNA ratio) were calculated (Table 3). As previously described (14, 23, 26, 29), the copy number of intravirion HIV-1 DNA was relatively high in the RU5 region (strong-stop DNA) and then sequentially lower in the U3, *gag*, and RU5-PBS-5NC regions. These data confirm that intravirion HIV-1 DNA is composed mainly, but not solely, of partial reverse transcripts (26, 29).

Since the number of viral particles per volume of blood plasma in different patients is variable and the levels of intravirion DNA differ along various regions of the HIV-1 genome, the viral DNA/RNA ratios were further normalized by comparing the DNA/RNA ratio at each time point, in each subject, with the specific DNA moiety's ratio at the pretreatment point, which was chosen as a baseline (100%) (Fig. 2). At 1 week posttreatment, the viral RNA levels decreased, ranging from 20 to 50% of the pretreatment level (Table 2), which is consistent with the observations previously described (7, 19). This reflects the rapid turnover rate of HIV-1 virions (10, 19, 24). However, the normalized DNA/RNA ratios indicated that intravirion HIV-1 reverse transcript levels decreased significantly more than plasma virion levels (Fig. 2; Table 3) ($P < 0.001$ for normalized HIV-1 DNA/RNA ratios in the U3, *gag*, and RU5-PBS-5NC regions). The later the HIV-1 DNA formation in the viral genome, the lower was the intravirion copy number of that DNA species observed after nevirapine treatment. Notably, levels of strong-stop intravirion HIV-1 DNA were only very modestly altered by nevirapine treatment ($P > 0.05$ for normalized HIV-1 DNA/RNA ratios in the RU5 region [see below]). These data demonstrate that intravirion reverse tran-

TABLE 3—*Continued*

Ratio for the following regions[a]									
gag at the following wks (10^5):					RU5-PBS-5NC at the following wks (10^5):				
−1	1	4	8	12	−1	1	4	8	12
15.4 (100)	11.5 (74.7)	68.2 (44.3)	12.4 (80.5)	11.1 (72.1)	11.4 (100)	8.0 (70.2)	4.6 (40.4)	9.5 (83.3)	9.5 (83.3)
10.0 (100)	3.3 (33.0)	5.3 (53.0)	7.8 (78.0)	9.4 (94.0)	20.0 (100)	6.7 (33.5)	9.8 (49.0)	13.5 (67.5)	17.0 (85.0)
6.2 (100)	3.4 (54.8)	4.3 (69.4)	5.0 (80.6)	6.0 (96.7)	4.1 (100)	1.7 (41.5)	2.5 (61.0)	3.5 (85.4)	4.0 (97.6)
6.8 (100)	2.4 (35.3)	4.4 (64.7)	5.1 (75.0)	7.1 (104.4)	2.5 (100)	0 (0.0)	1.7 (68.0)	2.5 (100)	2.6 (104.0)
27.9 (100)	4.2 (15.1)	3.5 (12.5)	4.8 (17.2)	6.8 (24.4)	13.0 (100)	1.7 (13.1)	2.7 (20.8)	4.5 (34.6)	5.9 (45.3)
16.0 (100)	7.6 (47.5)	9.2 (57.5)	14.6 (91.3)	14.4 (90.0)	1.6 (100)	0.6 (37.5)	0.9 (56.3)	1.2 (75.0)	1.4 (87.5)
32.0 (100)	10.0 (31.3)	20.2 (63.1)	38.4 (120)	40.8 (127.5)	16.1 (100)	5.2 (32.3)	2.6 (16.2)	19.0 (118.0)	20.0 (124.0)
39.8 (100)	22.2 (55.8)	30.9 (77.6)	40.1 (101)	42.5 (106.8)	2.8 (100)	1.1 (39.3)	0 (0.0)	2.3 (82.1)	3.2 (114.3)
100	43.3	55.3	80.5	89.5	100	33.4	39.0	80.7	92.6
0.0	18.5	20.0	29.6	30.7	0.0	20.7	24.2	24.3	24.0

scription was inhibited by an anti-RT agent in vivo, which is consistent with the previous in vitro observations (23, 27; unpublished data).

Rebounding of intravirion HIV-1 reverse transcript levels is concomitant with the development of resistant viral mutants. To identify the viral genotype with regard to nevirapine susceptibility or resistance, viral DNA from peripheral blood mononuclear cells was extracted. The RT gene was then PCR amplified and sequenced. Viral susceptibility to nevirapine and 50% inhibitory concentrations were also determined in cell culture. Both procedures were previously described in detail (19, 20). The results of these studies in the patients analyzed are illustrated below the *x* axes of the graphs in Fig. 2.

With continuing nevirapine treatment of all of the patients studied, the intravirion HIV-1 DNA/RNA ratios past the strong-stop region gradually increased to higher levels after a nadir during the first weeks of treatment (Table 3; Fig. 2). In many patients, the ratios approached pretreatment levels. This rebound began to occur at approximately 4 weeks after the initiation of therapy, even though nevirapine was still persistently administered. For the U3, *gag*, and RU5-PBS-5NC regions, the differences of normalized intravirion HIV-1 DNA/RNA ratios between the 1-week point and week 8 or between the 1-week point and week 12 were highly significant ($P < 0.01$). However, the differences between the pretreatment point and week 8 or the pretreatment point and week 12 were not significant ($P > 0.06$). These alterations suggest that nevirapine gradually lost its anti-reverse transcription effects for the entire virus population in plasma. Importantly, the genotype and phenotype analyses indicated that the resistant viral mutants developing in these patients occurred concomitantly with the rebounding in intravirion HIV-1 reverse transcript levels (Fig. 2; Table 3). Statistical analyses demonstrated significant correlations between normalized HIV-1 DNA/RNA ratios in the U3, *gag*, and RU5-PBS-5NC regions and the log of 50% inhibitory concentrations (micromolar) at various time points (U3 region, $r = 0.44$ and $P < 0.01$; *gag* region, $r = 0.48$ and $P < 0.01$; RU5-PBS-5NC region, $r = 0.63$ and $P < 0.01$). Thus, these kinetic analyses suggest a dramatic temporal relationship between the rebounding in intravirion HIV-1 reverse transcript levels and the acquisition of virus resistance.

HIV-1 virions develop decreased infectivity after initiation of nevirapine therapy. To study the biological consequences of alterations in intravirion HIV-1 reverse transcripts in vivo, the infectivity of HIV-1 virions in the blood plasma at different time points was analyzed. To compare the infectivity of the virions at different time points, the viral input in each infectivity assay was strictly normalized via quantitative RT-PCR. To determine the infectious titer of virions in the blood plasma samples at various time points, a previously described procedure was utilized (13). Briefly, the viral particles in the blood plasma from each time point were normalized by quantitative RT-PCR. The virions were then serially diluted 1:4 and added to 2×10^6 phytohemagglutinin- and interleukin-2-stimulated peripheral blood mononuclear cells from HIV-1-seronegative individuals. The infection was allowed to progress for 24 h. The unbound viruses were then washed off three times, and the cells were cultured in the presence of interleukin-2 (10 U/ml) for 28 days. The infectivity titer per specific quantity of virions was determined by measuring the amount of HIV-1 p24 antigen in the supernatants and was compared, in ratio form, to the quantity of input virions. The HIV-1 p24 antigen levels were quantitated by an enzyme-linked immunosorbent assay (Dupont, Wilmington, Del.). Virus infectivity dramatically decreased in each of three patients analyzed after nevirapine treatment (Fig. 3). However, when resistance developed, infectivity also increased. This increase in infectivity was concomitant with the resumption of intravirion HIV-1 reverse transcription. Thus, these data suggest a potentially important role for the subpopulation of HIV-1 virions harboring reverse transcripts in altering virus infectivity.

To exclude the possibility that nevirapine in the blood plasma carries over to the cell culture infectivity assays and significantly confounds these analyses, a series of control studies were conducted. The blood plasma samples obtained after initiation of nevirapine treatment were heated at 60°C for 1 h to inactivate HIV-1 virions and were then mixed (1:1) with blood plasma samples obtained at the pretreatment time point. These mixtures were then serially diluted, as described earlier, and used in parallel infectivity studies. Although nevirapine carryover had some very modest effects on slowing viral growth in culture, the infectivity titers of these mixed samples were not altered compared with those of the unmixed pretreatment samples. Importantly, it was also demonstrated that this heating step did not decrease nevirapine's activity in inhibiting exogenous RT reactions (data not illustrated).

In summary, there is a direct correlation of HIV-1 virion infectivity with alterations in levels of intravirion reverse transcripts. These data suggest that intravirion reverse transcrip-

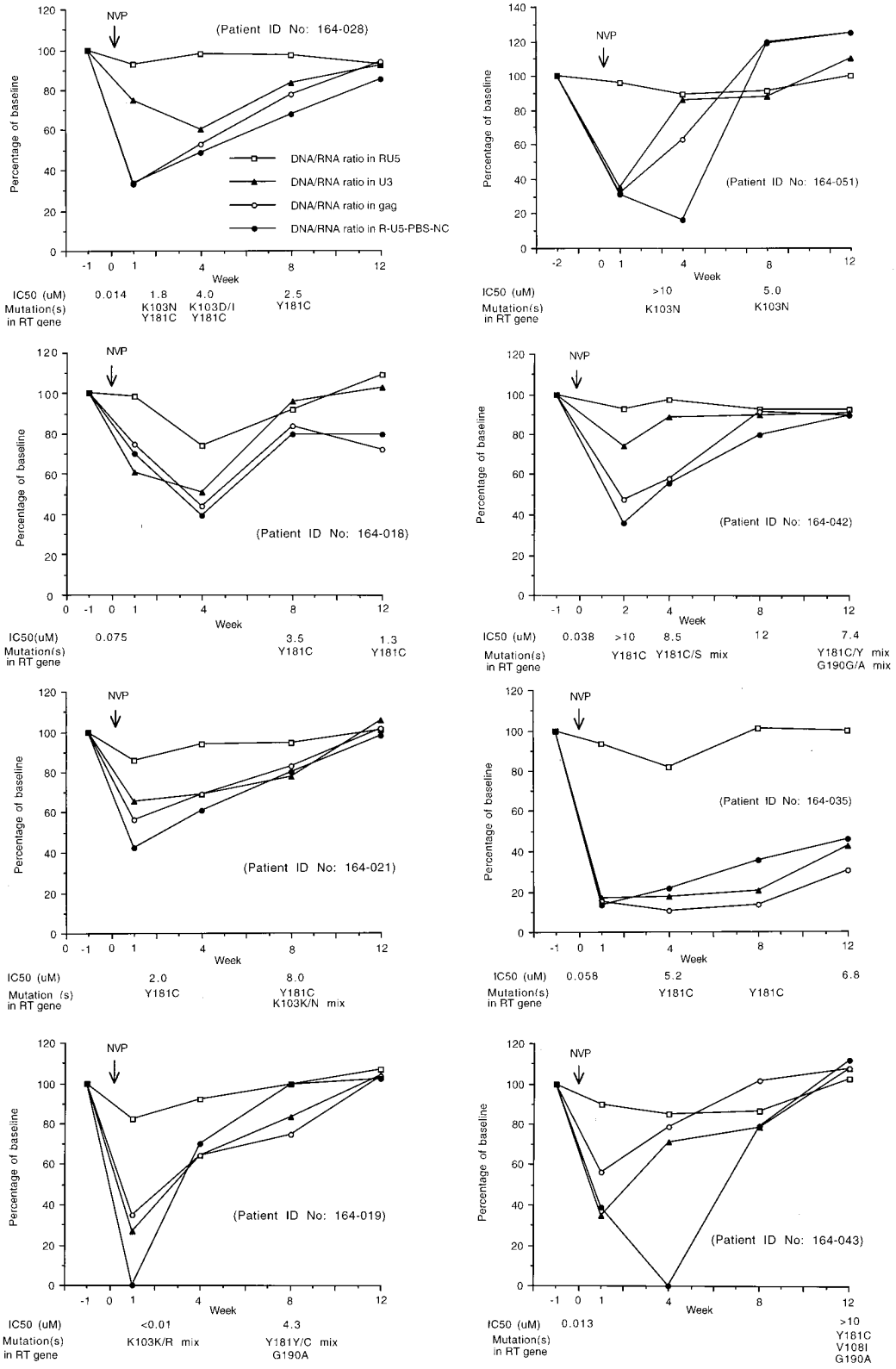

FIG. 2. Nevirapine (NVP) alters intravirion HIV-1 DNA/RNA ratios in the blood plasma of treated patients. HIV-1 virions were isolated from blood plasma collected at various time points by immunocapture methodology, and the intravirion HIV-1 DNA and RNA were detected and evaluated by quantitative HIV-1 DNA-PCR and RT-PCR, respectively. The copy numbers of DNA and RNA were determined with a PhosphorImager via comparison with their standard curves. The intravirion HIV-1 DNA/RNA ratio was then calculated and normalized to the pretreatment point as a baseline (100%). Open squares, DNA/RNA ratio in the RU5 (strong-stop DNA) region; filled triangles, DNA/RNA ratio in the U3 region; open circles, DNA/RNA ratio in the *gag* region; filled circles, DNA/RNA ratio in the RU5-PBS-5NC region. For comparison, the genotype and phenotype resistance data are also illustrated on the graphs. Each of the graphical analyses ($n = 8$) represents data obtained from at least two independent experiments performed in duplicate. IC_{50}, 50% inhibitory concentration; ID, identification.

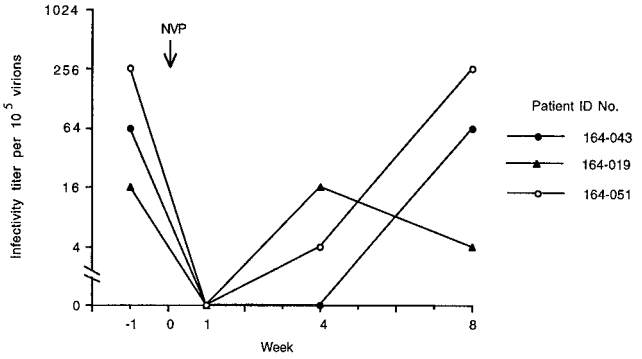

FIG. 3. Nevirapine (NVP) alters the infectivity of HIV-1 virions in the blood plasma of HIV-1-infected-individuals. Infectious HIV-1 virion titers in blood plasma were determined at each time point prior to and after nevirapine treatment by a quantitative culture assay as described elsewhere (13). The viral input was normalized to virion particle numbers, which were determined by quantitative RT-PCR. The HIV-1 virions (2×10^5 to 8×10^5 RNA equivalents) were serially diluted (1:4) and were used to infect phytohemagglutinin- and interleukin-2-activated peripheral blood mononuclear cells for 24 h. After unbound virions were washed off, the cells were cultured for 28 days. The infectivity titer of each sample was then determined. The data are given as infectivity titers per 10^5 virion particles for each time point. The data represent duplicate experiments of three representative patients, with standard deviations of less than 10%. ID, identification.

tion may play a role in vivo in interhost and intrahost retrovirus transmission and spread.

The present studies demonstrate that intravirion HIV-1 reverse transcription is a dynamic process in vivo which can be inhibited by nevirapine. Moreover, when resistant viral mutants develop, intravirion HIV-1 reverse transcript levels return to pretreatment levels. Compared with the potent inhibition of HIV-1 intravirion RT elongation by nevirapine, the initiation of reverse transcription is less affected, as demonstrated by the rather small changes in strong-stop (RU5) viral DNA. This may be secondary to the increased chance for inhibition of RT by nevirapine, since more HIV-1 DNA is synthesized. It is also possible that the HIV-1 RT enzyme's conformation for strong-stop DNA synthesis is slightly different from that for the rest of viral DNA synthesis, such that nevirapine does not bind to the enzyme as tightly prior to the first template switch during reverse transcription.

In contrast to nucleoside analog inhibitors of RT (such as AZT), it is not necessary for triphosphorylation of nevirapine to occur within host cells for activation of the compound (13, 20, 21). Nevirapine may diffuse into the HIV-1 virions and bind to the mature RT enzyme and, therefore, directly inhibit intravirion reverse transcription. It is unlikely that nevirapine can bind to the precursor of the RT enzyme (Gag-Pol fusion protein [p160]) in the virus-producing cells. Only after the mature RT enzyme is released by the HIV-1 protease from its precursor, during or after virion budding, could nevirapine bind to the hydrophobic pocket structure formed in the mature p66/p51 heteroduplex dimer of HIV-1 RT (12).

The initiation of reverse transcription takes place in a relatively small portion of HIV-1 virions in vivo. This is possibly due to limited substrate (e.g., deoxyribonucleoside triphosphates) or time-consuming release of mature RT enzyme from its precursor (reference 28 and unpublished data). However, in vitro experiments indicated that these levels of intravirion reverse transcripts are important, even though not absolutely essential, for efficient virion infectivity (27–29; unpublished data). These findings may be due to several molecular mechanisms, including more-rapid kinetics of HIV-1 replication upon infection of certain cell types (26, 28, 29). Inhibition of intravirion HIV-1 reverse transcription will decrease the chance for the target cells, especially quiescent cells, to become productively infected by HIV-1 viruses (27). In vivo data now also support these in vitro findings (Fig. 3). These data may have clinical significance for certain critical situations. For example, the administration of AZT to pregnant women dramatically decreases the incidence of mother-to-fetus HIV-1 transmission (5). Although this may be secondary to a total decrease in HIV-1 viremia, the effect may also be attributed at least in part to inhibition of intravirion reverse transcription by AZT-triphosphate. Consequently, the macrophages (Hofbauer cells) or microvascular endothelial cells in the placenta, which appear to be in a stationary phase and should not efficiently triphosphorylate AZT (17), may be less susceptible to the infection by HIV-1 virions lacking intravirion reverse transcripts (28; unpublished data). Similar consequences may occur for HIV-1 infection of macrophage/microglia and microvascular endothelial cells along the blood-brain barrier in the central nervous system (16), as well as for monocytes/macrophages (1, 22) and quiescent T lymphocytes in the periphery (6).

Clearly, RT is an important target for the treatment of HIV-1 infection. However, the emergence of resistant HIV-1 mutants is an immense problem for the continuation of any anti-RT treatment (18). The resistant HIV-1 viral mutants usually are detected and examined from both plasma and infected cells by tissue culture (phenotype) or by mapping the RT gene (genotype) (18). However, both phenotypic and genotypic analyses are time-consuming processes. There are also several other problems involved with genotypic detection of HIV-1 resistance. If several mutations coexist or the pattern of the mutations has shifted with or without coadministration of other anti-RT agents, it will be difficult to detect all of the critical mutations. Furthermore, it is often uncertain whether a specific mutation(s) in other regions of the HIV-1 RT gene is responsible for the functional viral resistance. The present data suggest an alternative assay to monitor resistant mutations to anti-RT agents: analysis of the intravirion HIV-1 DNA/RNA ratios in the blood plasma. This DNA/RNA ratio directly reflects the functional efficiency of the HIV-1 RT enzyme in vivo. As indicated in Table 3 and Fig. 2, the intravirion HIV-1 DNA/RNA ratio is quite consistent with genotypic and phenotypic HIV-1 resistance analyses. These analyses, which require quantitative DNA and RT-PCR techniques, may be in certain respects somewhat quicker than and complementary to previously reported resistance assays. Moreover, the results reflect the development and function of totally resistant viral mutant(s) in the whole viral population within the blood plasma.

In summary, intravirion HIV-1 reverse transcription is a dynamic process in vivo which also reflects the emergence of drug-resistant enzymes with treatment. These findings support the potential utility of analysis of intravirion HIV-1 reverse transcripts as a direct, functional assessment of viral resistance to anti-RT compounds.

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REFERENCES

1. **Arner, E. S., A. Valentin, and S. Eriksson.** 1992. Thymidine and 3′-azido-3′-deoxythymidine metabolism in human peripheral blood lymphocytes and monocyte-derived macrophages. J. Biol. Chem. **267:**10968–10975.
2. **Bagnarelli, P., S. Menzo, A. Valenza, A. Manzin, M. Giacca, F. Ancarani, G. Scalise, P. E. Varaldo, and M. Clementi.** 1992. Molecular profile of human immunodeficiency virus type 1 infection in symptomless patients and in patients with AIDS. J. Virol. **66:**7328–7335.
3. **Cheeseman, S. H., D. Havlir, M. M. McLaughlin, T. C. Greenough, J. L. Sullivan, D. Hall, S. E. Hattox, S. A. Spector, D. S. Stein, M. Myers, and D. D. Richman.** 1995. Phase I/II evaluation of nevirapine alone and in combination with zidovudine for infection with human immunodeficiency virus. J. Acquired Immune Defic. Syndr. **8:**141–151.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Connor, E. M., R. S. Sperling, R. Gelber, P. Kiselev, G. Scott, M. J. O'Sullivan, R. Van Dyke, M. Bey, W. Shearer, R. L. Jacobson, E. Jimenez, E. O'Neill, B. Bazin, J.-F. Delfraissy, M. Culnane, R. Coombs, M. Elkins, J. Moye, P. Stratton, and J. Balsley.** For the pediatric AIDS clinical trials group protocol 076 study group. 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. N. Engl. J. Med. **331:**1173–1180.
6. **Gao, W., A. Cara, R. C. Gallo, and F. Lori.** 1993. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. USA **90:**8925–8928.
7. **Havlir, D., S. H. Cheeseman, M. McLaughlin, R. Murphy, A. Erice, S. A. Spector, T. C. Greenough, J. L. Sullivan, D. Hall, M. Myers, M. Lamson, and D. D. Richman.** 1995. High-dose nevirapine: safety, pharmacokinetics, and antiviral effect in patients with human immunodeficiency virus infection. J. Infect. Dis. **171:**537–545.
8. **Henrard, D. R., W. F. Mehaffey, and J. P. Allain.** 1992. A sensitive viral capture assay for detection of plasma viremia in HIV-infected individuals. AIDS Res. Hum. Retroviruses **8:**47–52.
9. **Ho, D. D., T. Moudgil, and M. Alam.** 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J. Med. **321:**621–625.
10. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
11. **Holodniy, M., D. A. Katzenstein, D. M. Israelski, and T. C. Merigan.** 1991. Reduction in plasma human immunodeficiency virus ribonucleic acid after dideoxynucleoside therapy as determined by the polymerase chain reaction. J. Clin. Invest. **88:**1755–1759.
12. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256:**1783–1790.
13. **Koup, R. A., and D. D. Ho.** 1990. Quantitative culture assay for HIV-1 in peripheral blood. *In* A. Aldovini and B. D. Walker (ed.) Techniques in HIV research, p. 107–112. Stockton Press, New York.
14. **Lori, F., F. M. Veronese, A. L. DeVico, P. Lusso, M. S. Reitz, and R. C. Gallo.** 1992. Viral DNA carried by HIV-1 virions. J. Virol. **66:**5067–5074.
15. **Merluzzi, V. J., K. D. Hargrave, M. Labadia, K. Grozinger, M. Skoog, J. C. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosehthal, R. Faanes, R. J. Eckner, R. A. Koup, and J. L. Sullivan.** 1990. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science **250:**1411–1413.
16. **Moses, A. V., F. E. Bloom, C. D. Pauza, and J. A. Nelson.** 1993. Human immunodeficiency virus infection of human brain capillary endothelial cells occurs via a CD4/galactosylceramide-independent mechanism. Proc. Natl. Acad. Sci. USA **90:**10474–10478.
17. **Qian, M., T. Bui, R. J. Ho, and J. D. Unakat.** 1994. Metabolism of 3′-azido-3′-deoxythymidine (AZT) in human placental trophoblasts and Hofbauer cells. Biochem. Pharmacol. **48:**383–389.
18. **Richman, D. D.** 1994. Viral resistance to antiretroviral therapy. *In* S. Broder, T. C. Merigan, and D. Bolognesi (ed.), Textbook of AIDS medicine, p. 795–805. Williams & Wilkins, Baltimore.
19. **Richman, D. D., D. Havlir, J. Corbeil, D. Looney, C. Ignancio, S. A. Spector, J. Sullivan, S. Cheeseman, K. Baringer, D. Pauletti, C.-K. Shih, M. Myers, and J. Griffin.** 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. **68:**1660–1666.
20. **Richman, D. D., C.-K. Shih, I. Lowy, J. Rose, P. Prodanovich, S. Goff, and J. Griffin.** 1991. Human immunodeficiency virus type 1 mutants resistant to non-nucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. USA **88:**11241–11245.
21. **Spence, R. A., W. M. Kati, K. S. Anderson, and K. A. Johnson.** 1995. Mechanisms of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. Science **267:**988–993.
22. **Tornevik, Y., B. Jacobsson, S. Britton, and S. Eriksson.** 1991. Intracellular metabolism of 3′-azidothymidine in isolated human peripheral blood mononuclear cells. AIDS Res. Hum. Retroviruses **7:**751–759.
23. **Trono, D.** 1992. Partial reverse transcripts in virions from HIV and MLV. J. Virol. **66:**4893–4900.
24. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.
25. **Winters, M. A., L. B. Tan, D. A. Katzenstein, and T. C. Merigan.** 1993. Biological variation and quality control of plasma human immunodeficiency virus type 1 RNA quantitation by reverse transcriptase polymerase chain reaction. J. Virol. **31:**2960–2966.
26. **Zhang, H., O. Bagasra, M. Niikura, B. J. Poiesz, and R. J. Pomerantz.** 1994. Intravirion reverse transcripts in the peripheral blood plasma of HIV-1-infected individuals. J. Virol. **68:**7591–7597.
27. **Zhang, H., G. Dornadula, M. Niikura, O. Bagasra, B. J. Poiesz, and R. J. Pomerantz.** 1995. Natural endogenous reverse transcription (NERT) of human immunodeficiency virus type 1 (HIV-1). J. Cell. Biochem. Suppl. **21B:** 200. (Abstract.)
28. **Zhang, H., L. Duan, G. Dornadula, and R. J. Pomerantz.** 1995. Increasing transduction efficiency of recombinant murine retroviral vectors by initiation of endogenous reverse transcription: potential utility for genetic therapies. J. Virol. **69:**3929–3932.
29. **Zhang, H., Y. Zhang, T. P. Spicer, L. Z. Abbott, M. Abbott, and B. J. Poiesz.** 1993. Reverse transcription takes place within extracellular HIV-1 virions: Potential biological significance. AIDS Res. Hum. Retroviruses **9:**1287–1296.