Biological Variation and Quality Control of Plasma Human Immunodeficiency Virus Type 1 RNA Quantitation by Reverse Transcriptase Polymerase Chain Reaction

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Quantitation of human immunodeficiency virus type 1 (HIV-1) RNA in the plasma of seropositive individuals was performed by using an external control assay with techniques to standardize and control each measurement. Rigorous study of the variability of the assay showed that the median intraassay reproducibility was $\log_{10}$ 0.15 RNA copies per ml of plasma, while the median interassay reproducibility on replicate plasma samples was $\log_{10}$ 0.25 copies per ml. Specimen stability studies showed reproducible recovery of RNA from plasma stored at -70°C for up to 12 months. In clinically stable patients who were either untreated or taking zidovudine, the average week-to-week variation in plasma RNA levels, measured in real time, was $\log_{10}$ 0.30 RNA copies per ml. In contrast, patients either initiating or changing antiretroviral therapy showed a fall of $\log_{10}$ 0.8 to $\log_{10}$ 2.0 copies per ml in plasma RNA levels. Overall, 105 of 110 (96%) HIV-1-seropositive individuals with CD4 counts of 36 to 868 cells per mm^3 had quantifiable HIV-1 RNA over a range of $\log_{10}$ 2.70 to $\log_{10}$ 6.23 RNA copies per ml, including 81% (13 of 16) of the individuals with greater than 500 CD4 cells per mm^3 . Accurate and reproducible quantitation of plasma viremia in real time by reverse transcriptase polymerase chain reaction, particularly in asymptomatic HIV-1-infected individuals with high CD4 counts, provides a basis for the use of this virologic measure to monitor the short- and long-term effects of early intervention therapeutic strategies on viral burden.

Methods to quantitate human immunodeficiency virus type 1 (HIV-1) in infected individuals have been developed to increase understanding of the natural history of HIV-1 infection and to assess the effect of drug treatments and immunotherapies. Measurements of circulating cell-free virus by assays including those for p24 antigen and quantitative plasma cultures have been examined as risk factors for progression of clinical disease (5, 6, 9, 15, 23); however, serum p24 antigen and positive plasma cultures have been detected consistently only in symptomatic individuals with relatively advanced disease. Although p24 antigen measurements have been improved through the use of immune complex dissociation techniques, even immune complex dissociation p24 antigen assays are relatively insensitive in asymptomatic patients (17, 22). Plasma culture techniques are time-consuming and expensive and generally have limited sensitivity in asymptomatic individuals who have little plasma virus that can be measured by limiting dilution cultures (15, 23).

Detection of HIV-1 RNA sequences in plasma by polymerase chain reaction (PCR) amplification has been reported and promises to provide more sensitive methods to quantitate HIV-1 RNA in plasma. Several recent studies using PCR have demonstrated a relationship between the stage of disease as defined by clinical status, CD4 cell number, and plasma RNA levels (11, 21, 25). Furthermore, several investigators have shown a decrease in plasma RNA levels in patients starting antiretroviral therapies, suggesting that PCR methods can be used to evaluate the effect of drugs on virus load (1, 10, 12, 25).

As clinical treatment strategies address the problem of therapy for asymptomatic HIV-1 infection, sensitive and

reproducible measurements of plasma viremia are increasingly important. For most assays of HIV-1 RNA, little information has been presented on the variability of plasma RNA levels over time or the reproducibility of the assays used to measure plasma RNA. A strict evaluation of technical and biological variation in quantifying plasma HIV-1 RNA is necessary to accurately assess the effect of therapy and define changes in viremia. This report describes an improved quantitative plasma RNA assay relative to the previously described method from this laboratory (10, 12) that greatly increases the ability to accurately and reproducibly measure HIV-1 RNA in the plasma of patients across the spectrum of HIV-1 disease. Through the analysis of a large number of samples, issues such as standardization and quality control of the assay, specimen processing and storage, and biological variation of HIV-1 RNA over time were studied.

MATERIALS AND METHODS

Plasma processing. Whole blood from HIV-1-seropositive and -seronegative individuals was collected (after obtaining informed consent) into blood collection tubes containing acid-citrate dextrose anticoagulant. The tubes were centrifuged for 10 min at $400 \times g$, and the plasma was transferred to a centrifuge tube. The plasma was then centrifuged for 20 to 30 min at $400 \times g$ to remove platelets, the remaining cells, and cellular debris. The clarified plasma was then divided into 0.2- to 1.0-ml aliquots in sterile, siliconized 1.5-ml microcentrifuge tubes and centrifuged for 30 to 60 min at $125,000 \times g$ at 10°C . The supernatant was removed, the pellet was dissolved in 400 μl of 5 M guanidine thiocyanate (Solution D described in reference 4), and the samples were stored at -70°C . For some experiments, 200 μl of neat (not

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ultracentrifuged) clarified plasma was mixed with 200 μ l of 10 M Solution D and stored at -70°C .

RNA standard. Purified plasmid pDAB72, a construct containing the HIV-1 *gag* gene after the T7 promoter, was cut with *Eco*RI and RNA transcribed from the DNA template by using a MegaScript kit (Ambion, Austin, Tex.) according to the manufacturer's instructions. The transcribed RNA (cRNA) was then purified by phenol-chloroform extraction and ethanol precipitation, resuspended in diethyl pyrocarbonate-treated water, and quantitated by spectrophotometry. The number of RNA copies was calculated based on the molecular weight of the transcript, and the cRNA was diluted, divided into single-use aliquots, and stored at -70°C . Serial half-log dilutions of the cRNA were prepared immediately before inclusion into each reverse transcriptase (RT)-PCR assay.

Pooled plasma standards. Plasma assay standards were prepared and run in each assay to verify the accuracy of quantitation. Plasma was collected and pooled from 6 to 10 patients known to have measurable HIV-1 RNA. Aliquots (0.5 ml) were ultracentrifuged and processed into Solution D as described above and stored at -70°C . Eighty to one hundred aliquots were typically prepared from each batch. Duplicate samples from a pool were then quantified for HIV-1 RNA in three to five assays before being used as a running standard to control quality of each assay. Similar samples of seronegative plasma were also prepared to serve as negative controls.

RNA isolation. Duplicate plasma samples stored in Solution D were thawed, and the following reagents were added: 1 μ g of yeast tRNA in 10 μ l of diethyl pyrocarbonate-water, 26 μ l of 3 M sodium acetate (pH 4.5), 400 μ l of Tris-saturated phenol (pH 8.0), and 80 μ l of chloroform-isoamyl alcohol (49:1). The samples were vortexed thoroughly and set on ice for 20 min. The samples were then centrifuged at $12,500 \times g$ for 20 min. The aqueous upper phase was transferred to a clean 1.5-ml microcentrifuge tube, and 400 μ l of isopropanol was added. The tubes were vortexed and held at -20°C for 2 to 24 h. The samples were centrifuged for 20 min at $12,500 \times g$ at 4°C to form a pellet of the precipitated RNA. The supernatant was removed, and the pellet was dissolved with 200 μ l of 5 M Solution D and reprecipitated with 200 μ l of isopropanol. The samples were centrifuged for 20 min at $12,500 \times g$ at 4°C , the supernatant was removed, and the pellet was washed with successive additions of 1, 0.5, and 0.2 ml of 75% ethanol. After the final wash, the pellet was dried and resuspended in 10 μ l of diethyl pyrocarbonate-water. The entire sample was then subjected to RT-PCR. Plasma samples prepared by this procedure are free of contaminating DNA, as experiments showed that no amplification product was seen from samples amplified without the reverse transcription step and from seronegative plasma spiked with as much as 1 μ g of ACH2 cellular DNA prior to extraction (unpublished data). ACH2 cells (obtained from the NIH AIDS Research and Reagent Program) are subclones of CEM cells that contain one HIV-1 proviral copy per cell; thus, 1 μ g of ACH2 cellular DNA contains approximately 150,000 HIV-1 DNA copies.

RT-PCR. Extracted plasma samples were assayed by RT-PCR essentially as previously described (27). Each assay consisted of a standard curve of half-log dilutions of the cRNA standard from 300,000 to 30 copies (in duplicate), negative controls (in duplicate), and the duplicate extracted plasma samples (including pooled positive and negative calibration controls). The standard dilutions and unknowns were reverse transcribed by the addition of 10 μ l of RT

master mix containing 40 U of RNasin (Promega, Madison, Wis.), 1.25 mM deoxynucleoside triphosphates (dNTPs), 400 U of murine leukemia virus RT (Bethesda Research Laboratories, Grand Island, N.Y.), and 100 pmol of biotinylated SK39 primer in $2\times$ PCR buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM MgCl_2 , 0.1% bovine serum albumin). The reaction mixtures were incubated for 30 min at 42°C and then at 95°C for 5 min.

The reverse transcribed samples were then subjected to 33 cycles of amplification with the addition of 50 pmol of SK38 primer, 0.25 mM dNTPs, 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in 80 μ l of $1\times$ PCR buffer. The product of each reaction was quantitated in an enzyme hybridization assay using avidin-coated enzyme-linked immunosorbent assay plates (Nunc, Naperville, Ill.), a horseradish peroxidase-labeled SK19 probe (Synthetic Genetics, La Jolla, Calif.), and a Biomek 1000 Automated Workstation (Beckman Instruments, Irvine, Calif.) as previously described (14).

Copy number determination. A line was generated by plotting the input copy number of each cRNA point versus the mean $\log_{10}$ optical density (OD) of the duplicate wells for that standard. A best-fit line was generated through the linear portion of the curve by using computer software. The equation that describes the line was used to calculate the $\log_{10}$ copy number of the unknown specimens. For unknown samples, only $\log_{10}$ OD values that fell between the highest and lowest measured values on the cRNA standard curve for each particular assay were accepted. Results of each determination are expressed as the mean $\log_{10}$ copy number per milliliter of the duplicate samples, in which the individual ODs for samples of the duplicates were calculated to $\log_{10}$ copy number per milliliter first and then averaged. Samples whose OD values fell below the standard curve values were expressed as "less than" the detection limit for that assay. Samples that were above the upper limit of the standard curve were reassayed with aliquots processed with a smaller amount of plasma, when available, or by assaying only a portion of the extracted 1-ml aliquot by RT-PCR. In these cases, the $\log_{10}$ copy number value for that sample was corrected for volume to $\log_{10}$ copies per milliliter.

Statistical methods. Results from groups of specimens were reported as the mean $\pm$ the standard error of the mean, and statistical comparisons of the means were performed with Student's *t* test. Significance tests of the relationship between CD4 cell counts and RNA levels were performed by standard linear regression methods.

The significance of changes in RNA levels was assessed by determining a confidence interval based on week-to-week variation in RNA levels of individuals. The standard deviation of the mean difference between weekly RNA evaluations performed in real time (which thus includes assay and biological variations) was calculated to be $\log_{10}$ 0.215 copies per ml on the basis of 62 evaluations of 16 patients. The difference between the pretherapy and posttherapy RNA levels was divided by the error estimate ($\sqrt{2}$ [standard deviation]) to determine a *t* statistic. When the above-defined calculated standard deviation from weekly evaluations was used, differences between two evaluations of at least $\log_{10}$ 0.7 copies per ml were significant at *P* of <0.05 , differences between evaluations of at least $\log_{10}$ 0.8 copies per ml are significant at *P* of <0.02 , and differences between evaluations of at least $\log_{10}$ 0.9 copies per ml are significant at *P* of <0.01 .

RESULTS

Assay standardization and quality control. The plate-based quantitation of PCR product detection used in our studies produces highly sensitive and reproducible measurements of RNA levels. The dynamic range of the ODs obtained in a typical standard curve varied from 0.002 to 0.012 for negative samples to 2.500 to 2.950 for samples greater than $\log_{10}$ 5.48 copies per ml. This resulted in a typical linear range of a cRNA standard curve amplified for 33 cycles from $\log_{10}$ 5.48 to $\log_{10}$ 2.47 copies per ml. Over a 7-month period of performing 52 RT-PCR assays, the mean slope of the cRNA standard curve was 0.734 ± 0.016 , and the mean regression coefficient of these standard curves was 0.992 ± 0.005 .

The pooled positive and negative plasma standards that were included in each assay were used throughout our studies to monitor the validity of each test. The pooled positive plasma standards were initially tested three to five times to obtain a mean $\log_{10}$ copy number per milliliter. The value obtained for this pool was then examined in each subsequent assay to determine whether to accept or reject the associated assay results. The decision to reject an assay was made when the pooled positive standard differed by $\log_{10}$ 0.50 copies per ml from the typical mean value. Over a 7-month period, 4 assays in 52 were rejected by this criterion. The mean $\log_{10}$ copy number value of pool A was $\log_{10}$ 4.25 $\pm$ 0.12 copies per ml ($n = 12$ assays), and that of pool B was 4.77 ± 0.05 copies per ml ($n = 40$ assays).

The negative pooled standard was also used to validate each assay and to monitor for template or product contamination. Assays were rejected when the pooled negative standard produced an OD higher than the "no-template" control in the assay. None of the assays run over the 7 months were rejected because the negative standard tested positive.

Only plasma samples with a mean OD of greater than 0.100 were accepted to ensure reliable quantitation of low positives. Samples below this cutoff (and the associated standard curve) were amplified for an additional 3 cycles and requantified in the enzyme hybridization assay. After the additional amplification, 16 of 21 samples originally below this cutoff subsequently had ODs greater than 0.100. Since the standard curve originally run with the samples was also amplified for an additional 3 cycles, the boosted standard curve was used to accurately quantitate the $\log_{10}$ copy number values of the unknowns. The accuracy of this approach was verified in preliminary experiments by boosting 15 samples that were relatively low positives at 33 cycles (OD range, 0.105 to 0.547) to 36 cycles. The mean difference in $\log_{10}$ copy number between samples assayed at 33 and at 36 cycles was $\log_{10}$ 0.02 $\pm$ 0.04 RNA copies per ml. Additional cycles of amplification for the samples remaining negative were not performed. Quantitation at a very high cycle number is unreliable, as the background signal (seen in negative controls) increases appreciably and the range of linearity of the standard curve is extremely small (unpublished observations).

Intra- and interassay reproducibility. Duplicate aliquots of plasma collected at each time point were assayed to determine the $\log_{10}$ RNA copies per milliliter. The intraassay variation in the duplicates measured within a given assay is presented in Fig. 1A. Comparisons of 363 sets of duplicate samples resulted in a median difference between duplicate plasma aliquots of $\log_{10}$ 0.15 copies per ml. Among these comparisons, 95% of the differences between the duplicate samples were less than $\log_{10}$ 0.55 copies per ml. Typically,

FIG. 1. Intra- and interassay reproducibility of plasma RNA determinations. (A) Intraassay reproducibility. Duplicate samples of ultracentrifuged plasma were measured for RNA in the same assay, and the $\log_{10}$ RNA copy number for each sample was determined. Data are presented as the number of samples having a given difference between the $\log_{10}$ copy number value for each individual aliquot of the pairs run in each assay ($n = 363$ pairs). (B) Interassay reproducibility. Plasma from 34 different patients was collected, stored, and assayed in 2 to 40 different assays. Data are presented as the number of determinations having a given difference between the $\log_{10}$ copy number value obtained from the replicate samples quantitated in different assays ($n = 756$ comparisons).

samples with a difference between the duplicates of greater than $\log_{10}$ 0.55 copies per ml were retested in a different assay.

The interassay variation of RNA quantitation achieved by our method was examined by assaying plasma samples in different assays. In these studies, multiple aliquots of patient plasma were processed and stored at -70°C , and duplicate samples were tested in 2 to 40 assays. A summary of the variability between different assays is shown in Fig. 1B. A total of 756 comparisons between samples quantitated in different assays were obtained from plasma specimens from 34 patients. The median difference in plasma RNA measurements between replicate samples containing quantifiable

RNA measured in different assays was $\log_{10}$ 0.25 copies per ml. Of these evaluations, 95% of the differences between replicate values obtained in different assays were less than $\log_{10}$ 0.71 copies per ml.

Effect of sample processing and storage. The effect of ultracentrifugation and Solution D on the recovery of RNA from fresh and frozen plasma was examined. Plasma samples (200 μ l) were placed directly in 200 μ l of 10 M Solution D as previously described (10) or first ultracentrifuged (100,000 $\times g$ for 30 min), and the pellet was resuspended in 400 μ l of 5 M Solution D. Both sets of samples were then tested for plasma RNA in the same assay. A consistently higher recovery of RNA was obtained from plasma in which the viral particles were pelleted than from plasma that was directly extracted (mean difference of $\log_{10}$ 0.75 $\pm$ 0.080 copies per ml; n = 6). A similar difference was seen from plasma that was initially stored neat at -70°C for 1 to 2 months and then processed as described above (mean difference of $\log_{10}$ 0.63 $\pm$ 0.039 copies per ml; n = 4).

Conditions for efficiently pelleting HIV-1 from plasma were studied by subjecting plasma to different centrifugation parameters and assaying the pellets for RNA. Plasma samples (1 ml each) were ultracentrifuged at 100,000 $\times g$ for 10 to 60 min at 10°C or centrifuged in a standard tabletop microcentrifuge at 14,000 $\times g$ for 30 min at 10°C . Results showed that there was no detectable difference in the recovery of RNA from plasma ultracentrifuged at 100,000 $\times g$ (mean $\log_{10}$ 4.56 $\pm$ 0.281 copies per ml; n = 8) or centrifuged at 14,000 $\times g$ for 30 min (mean $\log_{10}$ 4.46 $\pm$ 0.368 copies per ml; n = 6; P > 0.1).

The requirement for guanidine thiocyanate to stabilize RNA during storage at -70°C was examined by comparing the amount of RNA recovered from pairs of plasma samples stored in Solution D at the time of collection or stored neat. Samples were stored as 200 μ l of fresh plasma in 200 μ l of 10 M Solution D or as neat plasma from which 200- μ l thawed aliquots were mixed with 200 μ l of 10 M Solution D at the time of assay. Results of these experiments showed that the amount of RNA measured in plasma stored with Solution D was not different from that in plasma stored neat. Fourteen samples stored for 6 months in Solution D showed a mean RNA level of $\log_{10}$ 4.63 $\pm$ 0.139 copies per ml, while duplicate samples stored neat showed a mean RNA level of $\log_{10}$ 4.36 $\pm$ 0.135 copies per ml (P > 0.5). Thirteen samples stored for 12 months in Solution D showed a mean RNA level of $\log_{10}$ 4.41 $\pm$ 0.199 copies per ml, while duplicate samples stored neat showed a mean RNA level of $\log_{10}$ 4.44 $\pm$ 0.195 copies per ml (P > 0.5).

An additional group of fresh plasma samples was stored at -70°C either as neat plasma or as ultracentrifuged pellets dissolved in Solution D. Aliquots were assayed immediately, and after 4 and 6 months of storage, neat plasma samples were thawed, ultracentrifuged, resuspended in Solution D, and assayed along with plasma aliquots that were ultracentrifuged and stored in Solution D in real time. Table 1 shows that the amount of RNA measured in these samples did not change over time, regardless of whether the plasma was stored neat or in Solution D.

Biological variation of plasma RNA levels and effect of therapy. The variability of plasma RNA levels in patients over time was evaluated in untreated asymptomatic individuals and in patients who had received zidovudine (AZT) for longer than 12 months and were clinically stable. Plasma was collected weekly and assayed for HIV-1 RNA levels in real time (i.e., the week the sample was collected). Figure 2 shows that plasma samples from patients collected and

TABLE 1. Effect of storage at -70°C on quantitation of plasma HIV-1 RNA

Patient	Storage ^a	Log ₁₀ RNA copies/ml at:		
		Real time	4 mo	6 mo
A	In D	4.53	4.75	4.57
	Neat	NT ^b	4.45	4.55
B	In D	3.53	3.86	3.76
	Neat	NT	3.46	3.44
C	In D	4.15	3.97	4.48
	Neat	NT	4.04	4.52
D	In D	4.15	3.78	4.79
	Neat	NT	3.77	4.48
E	In D	4.35	4.47	5.10
	Neat	NT	4.41	5.02
F	In D	4.39	NT	4.08
	Neat	NT	NT	4.07
G	In D	5.76	NT	5.41
	Neat	NT	NT	5.20

^a Neat, freshly isolated plasma stored directly at -70°C , with 1-ml aliquots ultracentrifuged immediately prior to assay and the pellets dissolved in 5 M Solution D. In D, 1-ml aliquots of freshly isolated plasma ultracentrifuged, with the pellets dissolved in 5 M Solution D and stored at -70°C until assay.

^b NT, not tested.

assayed on different weeks had consistent levels of plasma HIV-1 RNA over the time tested for both AZT-treated (panel A) and untreated (panel B) groups. The mean variation between the three weekly time points for AZT-treated patients was $\log_{10}$ 0.35 $\pm$ 0.048 copies per ml, and the mean variation between the weekly time points for the untreated individuals was $\log_{10}$ 0.19 $\pm$ 0.026 copies per ml. Figure 2C shows the results of antiretroviral therapy on plasma RNA levels. Three patients receiving AZT were switched to ddI at day zero, and one patient who was previously untreated received AZT beginning on day zero. Plasma samples were collected and assayed in real time. Significant falls ($\log_{10}$ 0.8 to 2.0 copies per ml; P < 0.02) in plasma RNA levels compared with the three baseline determinations were seen for all patients in the weeks following initiation or change of therapy.

Relationship to CD4 count. Quantitation of HIV-1 RNA in the plasma of individuals during different stages of HIV-1 infection was performed. Results from RNA evaluations of AZT-treated and untreated patients with CD4 counts ranging from 12 to 868 cells per mm^3 are shown in Fig. 3. Ninety-six percent (105 of 110) of all patients tested had quantifiable plasma RNA, including 13 of 16 (81%) patients with more than 500 CD4 cells per mm^3 . A significant linear relationship between the CD4 count and $\log_{10}$ RNA levels was seen for the entire group of patients (r = 0.529; n = 110; P < 0.001). This relationship existed for both the AZT-treated group (r = 0.411; n = 52, P < 0.001) and the untreated group (r = 0.529; n = 58; P < 0.001). Patients with fewer than 400 CD4 cells per mm^3 (the 43 AZT-treated patients and the 36 untreated patients) had similar CD4 counts (204 $\pm$ 14 versus 247 $\pm$ 20 cells per mm^3 ; P > 0.1) and had levels of plasma RNA that were not significantly different ($\log_{10}$ 5.03 $\pm$ 0.072 copies per ml for AZT-treated patients versus $\log_{10}$ 4.85 $\pm$ 0.096 for untreated patients; P > 0.1).

FIG. 2. Biological variation and effect of therapy on plasma RNA levels. Plasma samples were obtained from patients and assayed for plasma RNA on different weeks. Results are expressed as $\log_{10}$ RNA copies per milliliter of plasma for patients who were clinically stable on AZT for 16 to 29 months (A), patients receiving no therapy (B), and patients receiving AZT who were switched to ddI at day zero (open symbols), and one previously untreated patient initiating AZT therapy at day zero (closed symbol) (C). Different symbols represent individual patients.

DISCUSSION

Rapid and reproducible techniques for quantitating HIV-1 RNA may be important to accurately identify the stage of disease and to assess the response of circulating levels of

FIG. 3. Relationship between CD4 count, treatment status, and plasma RNA level. Plasma was collected and quantitated for RNA from 52 patients who received AZT for longer than 16 months (mean, 34 ± 10 months) (closed symbols) and in 58 patients who had never received antiretroviral therapy (open symbols). Results are expressed as $\log_{10}$ RNA copies per milliliter of plasma at each patient's CD4 count (cells per cubic millimeter). Plasma samples containing fewer than $\log_{10}$ 2.7 copies per ml were considered negative.

virus to antiviral and immunomodulatory therapies. Although a few studies have demonstrated that HIV-1 RNA in plasma can be detected in most HIV-1-seropositive individuals at all stages of infection (11, 24, 25), they have not specifically addressed issues of quality control of specimen processing, storage, extraction, or assay reproducibility and standardization. As an increasing number of quantitative HIV-1 RNA assays using different molecular techniques are developed, each of these issues will assume increasing importance in the quantitation of plasma HIV-1 RNA.

Methods for quantitating HIV-1 RNA by RT-PCR by using external (1, 11) and internal (20, 24) standards have been described. Internal standards, competitive templates with genetically engineered characteristics to distinguish their product from the natural target, may control for the presence of inhibitors or sample-to-sample variations in RT and amplification efficiency between samples run in the same assay. However, other than studies on inhibition by heparin (3, 13), no data have been presented indicating that inhibitors of RT and *Taq* polymerase can be found in plasma samples. Typical competitive RT-PCR assays require the use of several dilutions of plasma samples with the internal control and gel electrophoresis and band size quantitation to obtain a final result. These factors may greatly increase the cost of quantitating HIV-1 RNA in each specimen and reduce the number of samples that can be assayed. External controls allow the application of a single standard curve to many plasma samples. This type of assay, however, may be subject to wide variation between assays due to variability in RT and PCR efficiencies, and may be affected over time by the stability of external RNA standards during storage. The data presented in this report, however, suggest that tube-to-tube variability of the amplification of cRNA control templates and plasma samples is not a significant source of error for this external control-based assay. Evaluation of a large number of specimens showed a low level of variability between duplicate plasma samples quantitated in the same assay (Fig. 1A).

The between-assay variation provides a slightly larger source of error in quantitating plasma RNA (Fig. 1B); however, the overall variability between samples is relatively small (median $\log_{10}$ 0.25 copies per ml) with respect to the dynamic range of RNA measurements (greater than 3 orders of magnitude). Changes in overall efficiency of the RT-PCR reaction did not significantly affect accuracy, since the same plasma samples quantitated in high-efficiency and low-efficiency assays (based on total PCR product generation) yielded the sample $\log_{10}$ RNA copy number when transformed from that assay's respective standard curve (unpublished observations). Thus, by performing and analyzing numerous assays, standardization limits have been developed for this assay system, allowing rejection criteria to be implemented for each run. This quality control allows reliable comparison of datum points between assays, allows the analysis of storage and processing factors, and permits the continuous monitoring of patient RNA levels on a real-time basis.

Modification of the specimen-processing procedure significantly enhanced the sensitivity and reproducibility of the RNA determinations. Compared with direct extraction of plasma, the ultracentrifugation to pellet cell-free virus and consequent removal of most of the plasma proteins prior to extraction provided consistent enhancement of RNA recovery from the same volume of plasma. In addition, ultracentrifugation allows the concentration of virus from a large volume of plasma for a simple small-volume organic extraction procedure. The overall recovery of HIV-1 RNA from ultracentrifuged plasma permitted quantitation of RNA in most HIV-1-seropositive individuals (Fig. 3).

The effect of storage of frozen plasma on the recovery of RNA and accuracy of quantitation may be important when analyzing specimens retrospectively. Conflicting information regarding the stability of RNA in frozen plasma has been presented. Poor recovery of RNA from plasma stored frozen for an unknown duration (18) and good recovery from serum (28) or plasma stored for 4 to 6 months (1) have been reported. Published data on six plasma specimens indicated that one cycle of freeze-thawing (of unknown duration) had no effect on the recovery of RNA (1). The use of a chaetropo to protect HIV-1 RNA stored at -70°C has also been suggested (10). However, the experiments presented here suggest that the storage of plasma in the chaetropo guanidine thiocyanate does not appear to be necessary. There was, on average, no significant difference in the amount of HIV-1 RNA measured in pairs of plasma aliquots stored with or without guanidine thiocyanate for as long as 12 months. Separate experiments showed that the total amounts of RNA measured in plasma samples stored for up to 6 months at -70°C with or without guanidine thiocyanate were not different from those of duplicate plasma samples assayed immediately after collection (Table 1). Studies are currently under way to examine the stability of HIV-1 RNA in plasma stored for longer than 1 year.

While several studies have examined plasma from individuals initiating antiretroviral therapies for changes in HIV-1 RNA levels (1, 10, 12, 25), very little information has been presented on the stability of plasma RNA levels in patients over time. Only two studies examining the effect of therapy on RNA levels have presented data describing more than one pretherapy time point (10, 12). Results presented here show that the amount of RNA measured in the plasma of clinically stable patients over a period of several weeks was relatively stable, regardless of whether patients were receiving AZT (Fig. 2A) or were untreated (Fig. 2B). This stability

of RNA levels provides a basis for measuring the effect of therapy or the progression of disease. The weekly variation in plasma RNA levels seen in clinically stable AZT-treated or untreated patients was approximately $\log_{10}$ 0.3 copies per ml, while patients that initiated or changed their antiretroviral therapy showed a fall of $\log_{10}$ 0.8 to $\log_{10}$ 2.0 copies per ml in RNA levels (Fig. 2C).

Several reports on qualitative assays have indicated that nearly all HIV-1-seropositive individuals have detectable HIV-1 RNA in plasma or serum (2, 8, 18, 25). The methodologies used in these studies were very sensitive in detecting HIV-1 RNA but are not easily applicable to quantifying the amount of RNA present. Several quantitative assays have reported rates of detection of HIV-1 RNA in relatively small numbers of patients across the spectrum of HIV-1 infection from 69 to 96% (1, 10, 11, 29). Results presented here on assays using 1-ml aliquots of ultracentrifuged plasma showed that 105 of 110 (96%) patients with CD4 counts ranging from 12 to 868 cells per mm^3 had quantifiable plasma RNA. With the greater focus on early intervention in HIV-1 disease and the development and testing of immune-based therapies for HIV-1, the need to monitor patients with higher CD4 counts has increased. Results presented here (Fig. 3) show that 13 of 16 (81%) individuals with more than 500 CD4 cells per mm^3 had quantifiable plasma HIV-1 RNA. These results are similar to those in a recent study that showed 19 of 20 (95%) patients with more than 500 CD4 cells per mm^3 to have quantifiable HIV-1 RNA (21). Two of the patients in our study that were negative by the quantitative RNA assay had detectable RNA when an RT-nested PCR reaction was used (unpublished observation). This evidence supports recent observations (16, 19, 21) that HIV-1 continues to actively replicate at all times during the course of infection.

While the acute effect of AZT on virologic markers has been well established, the effect of chronic AZT treatment on plasma RNA levels has not been examined. Results presented here show that in individuals with fewer than 400 CD4 cells per mm^3 , those treated with AZT for longer than 16 months had the same levels of plasma RNA as a similar group of patients who never received antiretroviral therapy. These results suggest that the initial decline in RNA levels seen after initiation of AZT therapy is temporary, and that RNA levels increase after approximately 16 months. These virologic data parallel the results of clinical studies that showed that AZT provides only a transient clinical benefit and that clinical and immunological decline resumes after approximately 1 year of therapy (7, 26).

In conclusion, this report describes an improved quantitative plasma RNA assay, compared with the previously described assay from this laboratory (10, 12), that can accurately and reproducibly measure HIV-1 RNA in the plasma of patients across the spectrum of HIV-1 disease. The consistent use of external standards and strict monitoring of quantified positive and negative controls provided validation criteria for each assay and minimized interassay variation. The ability to accurately compare results between assays allowed biological variation and the effect of therapy to be monitored in real time. Comparison of fresh and frozen specimens showed that RNA can be accurately measured in either setting. Quantitation of HIV-1 RNA of patients across the spectrum of disease, especially those with high CD4 counts, enhances the potential of this virologic marker for monitoring the effect of drugs, vaccines, and other early intervention therapies on viral burden.

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REFERENCES

- Aoki-Sei, S., R. Yarchoan, S. Kageyama, D. T. Hoekzema, J. M. Pluda, K. M. Wyvill, S. Broder, and H. Mitsuya. 1992. Plasma HIV-1 viremia in HIV-1 infected individuals assessed by polymerase chain reaction. *AIDS Res. Hum. Retroviruses* 8:1263-1270.
- Bagnarelli, P., S. Menzo, A. Manzin, M. Giacca, P. E. Varaldo, and M. Clementi. 1991. Detection of human immunodeficiency virus type 1 genomic RNA in plasma samples by reverse transcription polymerase chain reaction. *J. Med. Virol.* 34:89-95.
- Beutler, E., T. Gelbart, and W. Kuhl. 1990. Interference of heparin with polymerase chain reaction. *BioTechniques* 90:166-167.
- Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Coombs, R. W., A. C. Collier, J. Allain, B. Nikora, M. Leither, G. F. Gjerset, and L. Corey. 1989. Plasma viremia in human immunodeficiency virus infection. *N. Engl. J. Med.* 321:1626-1631.
- Ehrnst, A., A. Sonnerborg, S. Berdahl, and O. Strannegard. 1988. Efficient isolation of HIV-1 from plasma during different stages of HIV-1 infection. *J. Med. Virol.* 26:23-32.
- Fischl, M. A., D. D. Richman, M. H. Greico, et al. 1987. The efficacy of azidothymidine in the treatment of patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. *N. Engl. J. Med.* 317:185-191.
- Goswami, K. K., R. F. Miller, M. J. Harrison, D. J. Hamel, R. S. Daniels, and R. S. Tedder. 1991. Expression of HIV-1 in the cerebrospinal fluid detected by the polymerase chain reaction and its correlation with central nervous system disease. *AIDS* 5:797-803.
- 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.* 321:1621-1631.
- 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.
- Holodniy, M., D. A. Katzenstein, S. Sengupta, A. M. Wang, C. Casipit, D. H. Schwartz, M. Konrad, E. Groves, and T. C. Merigan. 1991. Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. *J. Infect. Dis.* 163:862-866.
- Holodniy, M., D. A. Katzenstein, M. A. Winters, J. Montoya, R. Shafer, M. Kozal, M. Ragni, and T. C. Merigan. 1993. Gene amplification techniques to measure virus load and genotypic resistance in asymptomatic subjects treated with combination therapy. *J. Acquired Immune Defic. Syndr.* 6:366-369.
- Holodniy, M., S. Kim, D. A. Katzenstein, M. Konrad, R. Groves, and T. C. Merigan. 1991. Inhibition of human immunodeficiency virus gene amplification by heparin. *J. Clin. Microbiol.* 29:676-679.
- Holodniy, M., M. A. Winters, and T. C. Merigan. 1992. Detection and quantification of gene amplification products by a nonisotopic automated system. *BioTechniques* 12:37-39.
- Katzenstein, D. A., M. Holodniy, D. M. Israelski, S. Sengupta, and T. C. Merigan. 1992. Plasma viremia in human immunodeficiency virus infection: relationship to stage of disease and anti-viral treatment. *J. Acquired. Immune Defic. Syndr.* 5:107-112.
- Michael, N. L., M. Vahey, D. S. Burke, and R. R. Redfield. 1992. Viral RNA and mRNA expression correlate with the stage of human immunodeficiency virus (HIV) type 1 infection in humans: evidence for viral replication in all stages of HIV disease. *J. Virol.* 66:310-316.
- Nishanian, P., K. R. Huskins, S. Stehn, R. Detels, and J. L. Fahey. 1990. A simple method for an improved assay that demonstrates that HIV-1 p24 antigen is present as immune complexes in most sera from HIV-1-infected individuals. *J. Infect. Dis.* 162:21-28.
- Ottmann, M., P. Innocenti, M. Thenadey, M. Micoud, F. Pelloquin, and J. Seignurin. 1991. The polymerase chain reaction for the detection of HIV-1 genomic RNA in plasma from infected individuals. *J. Virol. Methods* 31:273-284.
- Pan, L.-Z., A. Werner, and J. A. Levy. 1993. Detection of plasma viremia in human immunodeficiency virus-infected individuals at all clinical stages. *J. Clin. Microbiol.* 31:283-288.
- Piatak, M., Jr., D. C. Luk, B. Williams, and J. D. Lifson. 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV-1 DNA and RNA species. *BioTechniques* 14:70-80.
- Piatak, M., Jr., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K.-C. Luk, B. H. Hahn, J. M. Shaw, and J. D. Lifson. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259:1749-1754.
- Reddy, M. M., E. E. Winger, D. Hargrove, T. McHugh, G. F. McKinley, and M. H. Grieco. 1992. An improved method for monitoring efficacy of anti-retroviral therapy in HIV-1-infected individuals: a highly sensitive HIV-1 p24 antigen assay. *J. Clin. Lab. Anal.* 6:125-129.
- Saag, M. S., M. J. Crain, W. D. Decker, S. Campbell-Hill, S. Robinson, W. E. Brown, M. Leuther, R. J. Whitley, B. H. Hahn, and G. M. Shaw. 1991. High-level viremia in adults and children infected with human immunodeficiency virus: relation to disease stage and CD4+ lymphocyte levels. *J. Infect. Dis.* 164:72-80.
- Scadden, D. T., Z. Wang, and J. E. Groopman. 1992. Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction. *J. Infect. Dis.* 165:1119-1123.
- Semple, M., C. Loveday, I. Weller, and R. Tedder. 1991. Direct measurement of viraemia in patients infected with HIV-1 and its relationship to disease progression and zidovudine therapy. *J. Med. Virol.* 35:38-45.
- Volberding, P. A., S. W. Lagakos, M. A. Koch, et al. 1990. Zidovudine in asymptomatic human immunodeficiency virus infection. *N. Engl. J. Med.* 322:941-949.
- Winters, M. A., M. Holodniy, D. A. Katzenstein, and T. C. Merigan. 1992. Quantitative RNA and DNA gene amplification can rapidly monitor HIV-1 infection and antiviral activity in cell cultures. *PCR Methods Appl.* 1:257-262.
- Yerly, S., E. Chamot, B. Hirschel, and L. H. Perrin. 1992. Quantitation of human immunodeficiency virus provirus and circulating virus: relationship with immunologic parameters. *J. Infect. Dis.* 166:269-276.
- Zhang, L. Q., P. Simmonds, C. A. Ludlam, and A. J. L. Brown. 1991. Detection, quantification, and sequencing of HIV-1 from the plasma of seropositive individuals and from factor VIII concentrates. *AIDS* 5:675-681.