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Quantification of HIV-1 RNA in human plasma and molecular biological analysis of viral replication and turnover promise to provide unique insights into AIDS pathogenesis and hasten antiretroviral therapy and vaccine research efforts. We evaluated quantitative competitive polymerase chain reaction (QC-PCR) and branched DNA (bDNA) signal amplification assays for plasma viral RNA in 152 HIV-1 positive individuals at all stages of illness and in 12 patients before and after initiating zidovudine therapy. Eighty-six percent of patients had bDNA values above the 10^4 RNA Eq/ml assay sensitivity cutoff. bDNA values were significantly correlated with plasma viral RNA levels determined by QC-PCR (Spearman rank correlation, $r = 0.89$), infectious plasma virus titers ($r = 0.72$), p24 antigen ($r = 0.51$), immune complex dissociated p24 antigen ($r = 0.56$), and $CD4^+$ lymphocyte counts ($r = -0.72$; $p < 0.0001$ for all comparisons). Plasma viral RNA determinations by bDNA and QC-PCR assays were quantitatively similar in the range of 10^4 to 10^7 RNA molecules/ml (log bDNA = 0.93 + 0.80* log QC-PCR; $R^2 = 0.81$, $p < 0.0001$) and declined identically following the institution of zidovudine therapy (68-73% decrease from baseline). Further studies combined viral load measurements with molecular analyses of drug resistance development in virus populations in plasma and peripheral blood mononuclear cells (PBMCs). The magnitude and rapidity of virus turnover that we observed in plasma and PBMCs suggest that ongoing *de novo* infection of, and virus production by, a relatively short-lived (or transiently expressing) lymphoreticular cell population is primarily responsible for the rapid appearance of resistant virus and contributes substantially to the persistence of viremia observed in chronic HIV-1 infection. These findings involving novel viral load measurements and viral replication kinetics have important practical and theoretical applications to the elucidation of HIV-1 pathogenesis and the development of effective antiretroviral agents and vaccines.

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"Quantitative Assessment of HIV Replication
and Variation *In Vivo*: Relevance to Disease
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Introduction

Persistent viral replication is now recognized for its central role in human immunodeficiency virus type 1 (HIV-1) pathogenesis and natural history (1-8). Viral load determinations in plasma, peripheral blood mononuclear cells (PBMCs), and lymphoid tissue have been significantly correlated with clinical stage and $CD4^+$ lymphocyte counts, with highest viral levels occurring in primary (acute) and late stage infection and lower levels in early and intermediate stages (1-13). Certain viral measurements such as p24 antigen (p24 Ag) have been shown to have prognostic value (14, 15), but their utility as surrogate markers for clinical endpoints in therapy trials remains to be proven (16, 17).

A number of different viral markers have been pursued as potentially valuable clinical indicators of disease activity as well as for surrogate markers of clinical endpoints (reviewed in 16). These include quantitative viral cultures of PBMCs and plasma (1-3); p24 Ag and immune complex dissociated (ICD) p24 Ag (13-15, 17-21); and polymerase chain reaction (PCR) amplification of PBMC-associated viral DNA and RNA (22-26). All of these markers are directly linked to the underlying HIV disease process and thus could be expected to provide clinically relevant information. Yet, each of these assays has significant theoretical or practical limitations. For example, PBMCs comprise only a small proportion (≈2%) of total lymphoid tissue (27) and may contain transcriptionally latent or defective provirus (23-26; 28-32). When stimulated in culture, cells expressing viral proteins are targeted for destruction or suppression by autologous HIV-specific T-lymphocytes (33,34). Quantitative virus cultures of plasma are

similarly compromised as a viral load measurement by a high and variable proportion of defective virus (8) and by the effects of neutralizing antibodies (35). Furthermore, quantitative cultures are costly, time-consuming, and associated with substantial exposure to infectious virus. Another viral marker, p24 Ag, is released from cells either as a virion component or as non-virion-associated antigen (36, 37). p24 measurements thus reflect a combination of virus production, provirus expression, and even p24 Ag release from dying cells. Moreover, p24 Ag is often complexed with circulating anti-p24 antibody and is only variably detected even by the ICD-p24 Ag assay (16, 18, 19).

Recently, there has been growing recognition of plasma virion-associated RNA as a direct, sensitive, and quantifiable measure of viral load (8, 9, 23, 38, 39). In theory, plasma viral RNA should reflect virus production throughout the entire lymphoreticular system rather than the comparatively minor PBMC compartment. Viral RNA determinations are not affected by the biologically complex requirements of virus culture and propagation *in vitro* nor by the variabilities associated with p24 Ag production and measurement. A number of different PCR, T7 RNA polymerase, and branched DNA signal amplification assays for quantifying viral RNA in plasma have been developed (8, 9, 23, 38-51). Preliminary evaluation of these assay methods has generally yielded comparable data indicating that plasma viral RNA can be detected in most infected subjects, that levels are related to clinical stage and CD4 counts, and that institution of antiretroviral therapy results in declines in viral RNA.

The current contract work entitled "Quantitative Assessment of HIV Replication and Variation *In Vivo*: Relevance to Disease Pathogenesis and Response to Therapy" (DAMD17-93-C-3146) addresses the following hypotheses: (i) HIV replication and expression, tissue burden,

and genetic and biologic variability are viral parameters that are inseparably linked and which are directly related to the pathogenesis of AIDS, and (ii) Elucidation of viral pathogenesis and development of effective treatments and vaccines for HIV will be facilitated by better quantitative measures of virus replication *in vivo* and by experimental models that relate these measures of virus replication to clinically important changes in the viral envelope and reverse transcriptase genes. The specific aims that we are addressing are as follows:

1. To determine the biological and biophysical relationships between novel measures of HIV-1 replication and expression *in vivo* and to determine the potential value of these virologic parameters as surrogate markers for clinical endpoints in natural history and therapeutic interventional trials.
2. To determine, using measures of virus load and expression validated in aim #1, the simultaneous levels of HIV-1 expression in lymphoid tissue and peripheral blood in order to assess the accuracy with which the blood compartment, which contains only 2% of the body's lymphocytes, reflects the lymphoid compartment.
3. To determine the rate and molecular characteristics of genetic change in HIV-1 envelope and reverse transcriptase (RT) genes in uncultured, PCR amplified strains of HIV-1 from patients with acute and early chronic infection (CDC stages I and II) and from later stage patients who have been treated with potent non-nucleoside RT inhibitors and have developed drug resistance. The goal of this aim is to determine the relationship between rates of viral replication measured by HIV-1 RNA, DNA, p24 Ag, and infectious virus production (specific aim #1) and the accumulation of genetic changes in specific viral genes whose products are under

selective pressures *in vivo*.

4. To determine the biological and clinical consequences of HIV-1 genomic variation identified in aim #3 in regard to envelope function and antigenicity. The goal of this aim is to determine the pathogenic significance of these envelope changes by characterizing their biologic properties in the genetic background of biologically relevant proviral clones representing T-cell and monocyte tropic viruses that were obtained in the current contract period.

We have made substantial progress in year 01 toward completing our objectives. The Quantitative Virology Working Group of the NIH/NIAID AIDS Clinical Trials Group (ACTG) recently conducted a blinded, multicenter evaluation of six different plasma viral RNA assays [Chiron bDNA assay (49); Roche RT-PCR assay (44); Abbott immunocapture PCR assay (50); and three noncommercial RT-PCR assays (9, unpublished data)] and concluded that several of them exhibited the requisite sensitivity, specificity, and precision to proceed with their evaluation in clinical trials (39). Despite the introduction of the bDNA assay and other commercially available assays for HIV-1 RNA quantitation into clinical trials, scant correlative information has been published describing the relation of HIV-1 RNA levels determined by these assays with respect to clinical stage, $CD4^+$ counts, other conventional virologic markers, or response to antiretroviral therapy. Moreover, no studies have been published directly comparing two independent HIV-1 RNA assays using large numbers of well-characterized clinical samples as a means to cross-validate the respective methodologies. The following work was thus designed to address our Specific Aim #1 above by: (i) evaluating the results of QC-PCR (RT-PCR) and bDNA assays in patients representing the complete clinical spectrum of HIV-1 infection; (ii)

defining the quantitative relationship between viral load measurements determined by QC-PCR and bDNA assays and those determined by quantitative plasma culture and regular and ICD-p24 Ag analysis; and (iii) determining the magnitude and kinetics of change in QC-PCR and bDNA determined viral RNA levels compared with other viral markers in patients treated with zidovudine.

Body

Materials and Methods

Patients. Peripheral blood specimens were obtained with consent from a total of 271 study subjects. At the Aaron Diamond AIDS Research Center (ADARC), blood was collected from 80 randomly selected HIV-1 seropositive individuals seen at New York University Medical Center, the New York Blood Center, and Bellevue Hospital. The subjects included patients with acute HIV-1 infection (2 cases), AIDS (3 cases), ARC (10 cases), and asymptomatic illness (65 cases). History of antiretroviral therapy in these individuals was unknown. Plasma specimens obtained from 90 HIV-1 seronegative subjects served as controls. Plasma samples (collected in heparin) were prepared from blood specimens that were centrifuged at 400 X g for 10 minutes at room temperature and were stored at -80°C until analysis. At UAB, plasma specimens for bDNA analysis were obtained from archived samples remaining from two previous clinical trials (8, 52). In the first instance, replicate frozen (-70°C) plasma samples from each of 60 subjects previously evaluated for HIV-1 plasma viral RNA by the QC-PCR method in a study of HIV-1 natural history (8) were analyzed. In the second instance, replicate plasma samples from 12 subjects representing the zidovudine (azidothymidine, AZT) control arm of a prospective, randomized, double-blinded phase I/IIA study of the non-nucleoside analogue reverse

transcriptase inhibitor L-697,661 (52, 53) were examined. In both cases, the samples culled for bDNA analysis were unselected and represented the entire sample sets for the respective studies. The 72 UAB study subjects included 6 patients with acute infection, 25 with asymptomatic infection, 24 with ARC, and 17 with AIDS. Twenty-nine control samples were obtained from healthy, uninfected volunteers. Plasma specimens at UAB were derived from blood samples that had been collected in acid citrate dextrose (ACD) and processed by sequential 15 minute centrifugations at 200 x g and 1000 x g prior to storage at -80°C in order to ensure the removal of the majority of platelets. In addition to the cross-sectional clinical samples, sequential plasma specimens were collected from two ADARC patients with primary (acute) infection and from 12 UAB patients who were begun on a six week course of zidovudine (52, 53).

p24 Antigen Assay, Quantitative Culture, and QC-PCR. Plasma p24 antigen levels were determined by commercial enzyme immunoassays (ADARC-Abbott Laboratories, Inc., N. Chicago, IL; UAB-Coulter Diagnostics, Hialeah, FL). Sensitivity cutoff values for the p24 antigen assays were 5 pg/ml for the regular assay and 15 pg/ml ICD method. The quantitation of infectious HIV-1 titers in plasma was performed using the end-point-dilution culture method as described previously (1, 3). Levels of HIV-1 RNA in plasma for all UAB specimens were determined by the QC-PCR method (8). QC-PCR, infectious plasma virus titers, and p24 antigen results for the UAB subjects have been reported previously (8, 53).

bDNA Assay. HIV-1 RNA in plasma was quantified at the ADARC and UAB study sites using the branched DNA signal amplification method (Quantiplex™ HIV-RNA Assay, Chiron Corporation, Emeryville, CA) (45, 49). All plasma specimens were coded and blinded to individuals performing the assay and recording the assay results. Virus was concentrated from

duplicate plasma specimens as recommended by the manufacturer by centrifugation in a bench top microcentrifuge (Heraeus Centrifuge Model 17RS, rotor 3753) at 23,500 g for 1 hr. Because of limited sample volumes, 0.25-1.0 ml specimens were initially used, adjusting their total volume as necessary to 1.0 ml with normal human plasma, and correcting the final bDNA readout by the corresponding dilution factor. This approach was shown experimentally not to affect the quantitative results of the bDNA assay except for its threshold sensitivity (unpublished data). For specimens yielding a negative bDNA result and for which less than the recommended duplicate 1.0 ml plasma volumes were initially tested, the assay was repeated using 1.0 ml samples. The virus pellet was extracted in a buffer containing proteinase K, lithium laurel sulfate, and target probes complementary to the HIV-1 *pol* gene. This was followed by a 15-minute centrifugation step at 23,500 g, after which the supernatant was transferred to microwells (in a 96 well plate) coated with capture probes. By this means, the RNA-target probe complexes were captured onto the surface of microwells during an overnight incubation. The wells were washed and bDNA amplifier molecules were hybridized to the immobilized target-probe complexes, followed by hybridization of multiple alkaline-phosphatase-labeled probes to each bDNA molecule. The complexes were then incubated with a chemiluminescent substrate (dioxetane), and light emission was measured in a luminometer. The light emission was directly proportional to the amount of HIV-1 RNA present in the plasma specimen. The concentration of RNA in a specimen, expressed as HIV RNA equivalents (Eq) per ml, was determined from a standard curve with a dynamic range of 10^4 to 1.6×10^6 RNA Eq/ml of plasma. Samples containing less than 10^4 RNA Eq/ml were recorded as negative and those exceeding 1.6×10^6 were diluted 1:10 or 1:100 in negative plasma and re-assayed.

Statistical Analysis. Descriptive statistics as well as nonparametric analyses were used to evaluate the data (54, 55). Spearman rank correlations and Pearson linear correlations were performed. Linear and non-linear regression analyses were employed to define quantitative relationships between variables. The Chi square test was used to compare detection frequencies of various virologic assay measurements in relation to patient groups exhibiting $CD4^+$ cell ranges of <200, 200-500, and >500/mm^3. The Kruskal-Wallis test was used to compare viral marker levels among the different groups, and the Wilcoxon signed rank test was used for one-sample or paired two-sample data analysis to assess the significance of treatment-related viral load changes.

Results

Branched DNA signal amplification of plasma viral RNA, quantitative plasma virus cultures, and regular p24 Ag assays were performed on plasma specimens from a total of 152 HIV-1 seropositive patients at the two study sites. Represented in this group of patients were 8 subjects with acute (primary) infection and 144 subjects with chronic infection. Eighty-six percent (131 of 152) of all patients, including those with acute infection, had detectable bDNA values above the 10,000 RNA Eq/ml assay sensitivity cutoff. bDNA values for plasma viral RNA ranged from $<10^4$ Eq/ml to 9×10^7 Eq/ml. Fifty-eight percent (88 of 152) of patients had culturable plasma virus and 46% (70 of 152) had measurable p24 Ag. QC-PCR analysis of plasma viral RNA and ICD-p24 Ag testing were performed on specimens from the 72 patients at the UAB site (6 with acute infection and 66 with chronic infection). The sensitivities of QC-PCR and ICD-p24 Ag assays were 100% (72 of 72) and 61% (44 of 72), respectively. QC-PCR determined RNA values ranged from 10^2 to 2×10^7 molecules/ml. None of 119 HIV

seronegative control subjects had positive marker results by any of the assays used.

Table 1 shows the relative sensitivities of the bDNA assay compared with the other assay methods as a function of $CD4^+$ lymphocyte counts in patients with chronic infection. For this analysis, the 8 subjects with acute illness were excluded since such patients are uniformly viremic and have viral load measurements that bear no consistent relationship to CD4 counts (8, 12, 13). Eighty-five percent of patients had viral RNA detectable by bDNA compared with 100% by QC-PCR and 56% by viral culture. Forty-three percent of subjects had detectable p24 Ag and this was increased to 58% by use of the ICD-p24 Ag assay. Except for QC-PCR, which was positive in all subjects tested, there was an obvious, highly significant inverse correlation between CD4 counts and the frequency of detection of each virologic marker ($p < 0.001$ for all). The mean bDNA values for plasma viral RNA in patients with $CD4^+$ cell counts of $<200/mm^3$, 200-$500/mm^3$, and $>500/mm^3$ were 583×10^3, 71×10^3, and 45×10^3 Eq/ml, respectively. bDNA values for patients with $CD4^+$ cells $<200/mm^3$ were significantly greater than for patients with higher CD4 counts ($p<0.0001$). Of the 123 bDNA positive patients with chronic infection (Table 1), one had a value between 10^7 and 10^8 RNA Eq/ml, five had values between 10^6 and 10^7, 56 had values between 10^5 and 10^6, 61 had values between 10^4 and 10^5 (including 13 patients with bDNA values less than 25,000 RNA Eq/ml). There were no statistically significant differences in detection rates for any of the viral markers between the two study sites except for plasma virus cultures which were more commonly positive at ADARC (65%) than at UAB (44%) ($p < 0.05$).

The availability of replicate plasma samples from the UAB study site for which bDNA, QC-PCR, p24 Ag, ICD-p24 Ag, and quantitative viral culture results were all determined enabled us to analyze directly the quantitative relationship between bDNA values and these other viral

markers. Figure 1 depicts these data, showing positive correlations between bDNA and QC-PCR, bDNA and ICD-p24 Ag, and bDNA and culture results. Table 2 summarizes the Spearman rank correlation coefficients among these viral load markers and between each of them and CD4 levels. bDNA and QC-PCR correlated most strongly with $CD4^+$ cells (r=-0.72 and -0.75, $p < 0.0001$ for both). Among the viral load markers, bDNA and QC-PCR correlated with each other most strongly (r=0.89, $p<0.0001$). Pearson correlation analysis, which evaluates linear relationships, revealed a similarly strong correlation between $\log_{10}$ bDNA and $\log_{10}$ QC-PCR (r=0.89, $p<0.0001$). In order to evaluate further the quantitative relationship between plasma viral RNA levels determined by bDNA and those determined by QC-PCR, a linear regression analysis was performed of the data shown in Figure 1A. The best fit line is described by the equation log bDNA=0.93+0.80 * log QC-PCR (R^2=0.81, $p<0.0001$).

Inter-assay reproducibility of the bDNA assay was determined by quantifying one positive specimen and one negative specimen, in duplicate, over the course of 17 assay runs at ADARC and UAB. The overall (inter-laboratory and inter-assay) mean and standard deviation for quantitation of the positive specimen were 66.6×10^3 and 13.7×10^3 RNA Eq/ml, respectively, resulting in a 21% coefficient of variation. In all of the 17 assay runs, the negative control quantitated below the assay cutoff of 10^4 RNA Eq/ml.

Having characterized in this patient population the bDNA assay's sensitivity (85%), specificity (100%), reproducibility (coefficient of variation 21%), quantitative range (10^4-10^8 RNA Eq/ml), and correlation with $CD4^+$ cell counts (r= -0.72, $p<0.0001$) and four direct virologic markers (r=0.51 to 0.89, $p<0.0001$ for all), we next sought to evaluate the assay's dynamic response in the clinical setting of primary (acute) HIV-1 infection and following the institution

of antiretroviral therapy. Acute HIV-1 infection is characterized by high levels of viral replication with infectious virus titers, virion-associated RNA (determined by PCR methods), and plasma p24 Ag all reported to be high prior to antibody seroconversion (8, 12, 13). We thus determined plasma viral RNA levels by the bDNA method, along with infectious virus titers and p24 Ag levels, through the period of acute infection and seroconversion in two ADARC patients (Figure 2). Peak levels of plasma virion-associated RNA ranged from 4×10^6 to 9×10^7 Eq/ml and fell by 100-to 10,000 fold following antibody seroconversion. Infectious virus titers and p24 Ag levels paralleled those of bDNA. Plasma virus assayed by the bDNA method, but not by culture or p24 Ag assay, remained detectable throughout the 1-2 years of clinical follow-up. bDNA assays were also performed on plasma specimens from 6 patients at UAB who had acute HIV-1 infection. These determinations were made on single plasma specimens corresponding to time points of peak viremia based on QC-PCR and p24 Ag measurements (8). bDNA values for these patients ranged from 7×10^5 to 2×10^7 RNA Eq/ml while QC-PCR measurements of replicates of the same plasma specimens ranged from 4×10^5 to 2×10^7 RNA molecules/ml. RNA results determined by the two assay methods for these six patients were highly correlated ($r=0.87$, $p < 0.03$).

Finally, sequential plasma specimens from 12 patients beginning zidovudine therapy were analyzed by bDNA, QC-PCR, p24 Ag, and ICD-p24 Ag assays before, during, and one week after a 6 week course of drug therapy (figure 3). Of note, all four assays were performed on batched samples so as to minimize inter-assay variability. Figure 3A shows that initiation of zidovudine therapy resulted in an immediate and generally sustained fall in viral RNA as measured by the bDNA assay. Discontinuation of zidovudine after week 6 led to an immediate

increase and return to baseline of viral RNA. The viral RNA response to zidovudine therapy measured by bDNA and QC-PCR were virtually identical in kinetics and magnitude (figure 3b), resulting in a statistically significant drop of both markers at week one to 27-32% of their baseline values ($p < 0.001$); changes in p24 Ag and ICD-p24 Ag paralleled those for viral RNA although they were of lesser magnitude, declining at week one to 55% of pretreatment levels. After discontinuing zidovudine at week six, viral RNA levels returned promptly to baseline, a change paralleled to a lesser degree by p24 Ag and ICD-p24 Ag.

Discussion

A number of investigative groups have independently developed and reported novel assay methods for quantifying HIV-1 RNA in plasma (8, 9, 23, 38, 40, 44, 45, 47-51). In the early stages of these assays' development, discordant results were frequently reported regarding the frequency of detection, magnitude, and stability of HIV-1 RNA in plasma (8, 9, 23, 24, 38, 40-51). More recently, primarily because of technical improvements, consistent and reproducible findings linking plasma viral RNA to HIV-1 pathogenesis and natural history have been reported (8, 9, 23, 38, 56). Using an internally controlled quantitative competitive RT-PCR method (QC-PCR), Piatak et al. (8) have demonstrated conclusively that: (i) virion-associated HIV-1 RNA can be detected in virtually all seropositive individuals regardless of disease stage; (ii) viral RNA levels generally range from 10^2 to 10^7 molecules per milliliter of plasma, (iii) viral RNA levels correlate significantly with other virologic load markers such as infectious plasma virus and p24 Ag, with advanced clinical stages, and with low $CD4^+$ cell counts; and (iv) viral RNA levels fall significantly in association with seroconversion and following the institution of antiretroviral therapy.

Using a different RT-PCR assay procedure, Winters *et al.* (9) reported findings similar to those of Piatak, detecting HIV-1 RNA levels in the range of 10^2 to 10^6 molecules per ml in >95% of infected subjects. Furthermore, these investigators performed rigorous analyses of the natural biological variation of HIV-1 RNA in plasma, of assay reproducibility, and of the stability of plasma virus with long-term storage. They found a median intraassay reproducibility of $\log_{10}$ 0.15 RNA molecules per ml, median interassay reproducibility of $\log_{10}$ 0.25 molecules per ml, biological variation in viral RNA levels of $\log_{10}$ 0.30 molecules per ml in patients on no therapy or unchanged therapy, and stability of virion-associated RNA in plasma stored at -70°C for up to one year. Coombs, *et al.* (46), and Aoki-Sei *et al.* (38), using still other methods for quantifying plasma virus, also demonstrated consistent and reproducible virus quantitation in fresh and stored human plasma.

Based on these reports and others, there has been increasing interest and concentration on the part of clinical investigators, clinicians, and patients alike to explore the use of plasma viral load measurements to assess drug activity in clinical trials and in the setting of individual patient management. In this context, however, it is important to emphasize that only the more technically demanding research based assays described above have been evaluated clinically. While there is the expectation that commercial assays designed for use in clinical or clinical research laboratories will yield data similar to those of basic research assays, it is essential to evaluate this question formally. Lin *et al.* (39) have provided important data in this regard demonstrating that six different HIV-1 RNA assay methods, including two commercial tests (Chiron and Roche), were able to discriminate and accurately rank a constructed 10-fold dilution series of cultured HIV-1 virus spiked into normal human plasma. Furthermore, these assays

could discriminate between 19 positive or negative clinical samples and between positive samples with high versus low virus loads, although method-specific differences in the quantitative results for individual patient specimens were as high as 100-fold. Nonetheless, the reproducibility of certain of the assays, including the Chiron bDNA assay, was such that an empirical fourfold difference in RNA levels could be viewed as significant and it was recommended that they be advanced to clinical trial evaluation (39).

The present study represents one of the first large clinical research evaluations of the Chiron branched DNA signal amplification assay and it is unique in having a combination of other viral load measurements, including QC-PCR determinations of viral RNA, available for direct comparison. In this study, we determined bDNA values in 152 patients at all stages of infection and in 119 HIV negative controls and we related this information to clinical stage, $CD4^+$ cell counts, results of other viral load measurements, clinical course following acute infection, and response to therapy. The sensitivity of the bDNA assay in this relatively advanced patient population (83 patients with $CD4^+$ counts $<200/mm^3$, 37 with counts from $200\text{-}500/mm^3$, and 24 with counts $>500/mm^3$) was 85%. Its specificity was 100%, reproducibility 21% (coefficient of variation), working range for clinical samples $10^4\text{-}10^8$ RNA Eq/ml, correlation (r value) with $CD4^+$ counts -0.72 ($p<0.0001$), and correlation with four other viral markers ranging from 0.51 to 0.89 ($p<0.0001$ for all). Of note, the rank correlation coefficients (Table 2) were strongest between bDNA and QC-PCR (0.89), followed by bDNA and culturable virus (0.72), and then by bDNA and ICD-p24 Ag (0.56) and p24 Ag (0.51). In comparison, p24 Ag and ICD-p24 Ag were correlated at the 0.90 level. These empiric findings are consistent with expectations based on the viral components targeted by the respective assays: bDNA and QC-PCR detect total virion-

associated RNA, plasma cultures detect infectious virus, and p24 Ag and ICD-p24 Ag detect the various forms of virion- and non-virion-associated core antigen.

Theoretically, results of the bDNA and QC-PCR assays should be very similar or identical since they both measure viral RNA from pelleted virus. In fact, results of the two assays were highly correlated (Spearman rank and Pearson correlation coefficients of 0.89, $p<0.0001$ for both) over a broad range of values from 10^4 to 2×10^7 viral RNA molecules/ml (Table 2 and Figure 1). In a total of 72 patients for whom bDNA and QC-PCR data were available (Table 1 plus six patients with primary infection), 75% had bDNA and QC-PCR results that differed by less than 0.5 $\log_{10}$; 99% of patients had bDNA and QC-PCR results that differed by less than 1.0 $\log_{10}$. Regression analyses revealed highly significant correlations between bDNA and QC-PCR results, indicating a nearly one to one relationship between bDNA and QC-PCR values over a 3 $\log_{10}$ range. Further analysis revealed a small but statistically significant method-associated trend for QC-PCR results to exceed bDNA results by an average of 0.168 $\log_{10}$ ($p < 0.001$). The fact that two independent viral RNA assays, based on completely different amplification strategies and having differently prepared quantitative standards, yielded nearly the same quantitative results for clinical samples over a 3 $\log_{10}$ dynamic range is important. Such data provide independent and mutual confirmation of the quantitative values for plasma viral RNA recorded by these assays. Other reports (24, 44) suggesting that plasma viral RNA levels are generally 10 to 100 fold lower than those we determined by the bDNA and QC-PCR assays likely reflect the use of different assay methods, different quantitative standards, or less well preserved clinical specimens. The technically demanding nature of target (PCR) and signal (bDNA) amplification assays will require the use of common assay standards by laboratories performing these assays in clinical

trial settings and planning to analyze data collectively.

Overall reproducibility of the bDNA results reported in this study was estimated by quantifying replicates of a single HIV-1 positive specimen and a single negative specimen over the course of 17 assay runs at ADARC and UAB. The overall inter-laboratory and inter-assay mean and standard deviation for the positive specimen was $66.6 \times 10^3 \pm 13.7 \times 10^3$ RNA Eq/ml, resulting in a coefficient of variation of 21%. In three other studies of bDNA assay reproducibility (39, 45, 57) in which as many as 12 different operators performed the test, overall coefficients of variation ranged from 18% to 23%. In the ACTG Virology Working Group study (39), assay reproducibility was determined by pooled standard deviations of results on pairs of blinded patient samples. In that study, the Chiron bDNA assay exhibited the smallest pooled standard deviation, and thus the greatest reproducibility, of all tests analyzed. Taken together, the results of the five studies indicate that differences in viral RNA results of as little as twofold, within or between assay runs, would be expected to be significant at the $p<0.05$ level.

An important question to investigators involved in many different types of clinical HIV-1 research (including antiretroviral chemotherapy, immunotherapy, natural history, pathogenesis and vaccine efforts) is which plasma viral RNA assays are most useful for quantifying virus load *in vivo*. In our view, a qualified answer is necessary depending on the patient population under study, baseline ranges in viral load, sensitivity and accuracy of measurements required to answer the questions posed, as well as feasibility issues such as commercial availability and ease of use of an assay method. The current study provides data for the Chiron bDNA assay relating to each of these issues. Adults at all clinical stages were evaluated, and even those with greater than 500 $CD4^+$ cells/mm^3 generally had detectable bDNA values, albeit at lower levels ($45 \pm 40 \times 10^3$

RNA Eq/ml, mean ± 1 S.D.). Enhancing the sensitivity of the bDNA assay from a lower cutoff limit of 10^4 RNA Eq/ml to 5 X 10^3 RNA Eq/ml, as has been done recently (unpublished data), will further increase the proportion of HIV-1 infected individuals with detectable viral RNA and will expand by twofold the range over which changes in viral load can be quantified. Sample volume requirements (duplicate 1 ml plasma specimens) specified for the bDNA assay can be problematic for pediatric studies or certain other applications; we successfully used sample volumes as low as 0.25 ml. Accuracy of the bDNA assay over a 3 $\log_{10}$ range (10^4 to 2 X 10^7 RNA Eq/ml) was independently validated by direct comparison of assay results with QC-PCR determined values using replicate plasma samples. Facility of use of the bDNA assay was confirmed by test performance at two clinical sites (ADARC and UAB).

Plasma viral RNA determinations by the Genelabs QC-PCR assay were employed for comparison with bDNA results in this study. Whereas the bDNA assay was able to detect and quantify HIV-1 RNA in the plasma of 62 of 72 UAB subjects (Table 1 plus six patients with acute infection), the QC-PCR assay was positive in all 72. The mean QC-PCR level in the ten subjects with negative (<10^4 RNA Eq/ml) bDNA results was $10^{4.10}$ RNA molecules/ml with a standard deviation of $\log_{10}$ 0.83. The accuracy of the QC-PCR assay has been determined experimentally by measuring recombinant HIV-1 RNA and DNA standards of known concentration, by quantifying viral RNA in culture supernatants for which virion particle counts were independently determined, and now by direct comparison with bDNA results on clinical samples. The precision and reproducibility of the QC-PCR has been determined to be approximately 20-25% (8). Because of the routine sensitivity of the QC-PCR assay of approximately 2,000 RNA molecules/ml, and its ability to detect and quantify plasma viral RNA

at levels as low as 100 molecules per ml on an as needed basis, this assay has proven to be particularly useful in studies where accurate quantitation of viral RNA in the range of 100 to 50,000 molecules per ml is important.

A Roche Molecular Systems RT-PCR assay for quantifying plasma viral RNA has been described and its performance characteristics for detecting and quantifying HIV-1 RNA expressed from plasmid vectors and in culture supernatants reported in detail (44). More limited data is available regarding the performance of this assay on clinical specimens (39, 44). If the sensitivity (200 HIV-1 RNA copies per ml plasma), low sample volume requirements (200 µl plasma), accuracy, and reproducibility reported for the research based assay (44) are maintained in a commercially available assay, then it will represent an important experimental tool for clinical AIDS research.

In summary, there are currently three commercial (8, 44, 45) and a number of non-commercial (9, 24, 38, 39, 47, 50, 51) assays available for assessing HIV-1 plasma viral load in clinical specimens. The findings reported here and elsewhere (8, 9, 39, 44, 45) suggest that at least some of these assay methods detect and accurately quantify the same viral RNA target molecule which exists in plasma in virion-associated form throughout all stages of clinical infection (8, 9, 23, 38, 51). The results of the present study in particular demonstrate for the first time that two viral RNA quantitation methods, which evolved out of basic molecular virologic research, provide nearly identical quantitative results when applied to clinical specimens. This represents a significant contribution to the field of antiretroviral and vaccine research since these new RNA assays are substantially more sensitive than earlier virologic load measurements. In fact, very recent studies by the ACTG Virology RNA Validation Group have, for the first time,

provided evidence that therapy-induced changes in HIV-1 viral RNA load may be significantly associated with clinical outcome (R. Coombs, personal communication). These latter findings, along with the clinical evaluation of bDNA and QC-PCR assays described here, suggest that plasma viral RNA determinations may indeed fulfill the requirements and promise of a clinically useful surrogate marker for evaluating the clinical benefits of new antiretroviral therapies (58, 59).

Our overall hypothesis is that load, replication, and variation are inseparably linked and are fundamentally involved in HIV-1 pathogenesis. We have thus also addressed Specific Aims #3 and #4 in year 01 of the contract in addition to specific aim #1. The addition of Nevirapine (NVP) to existing therapy with ZDV and ddI (Protocol BIPI1009) results in a rapid initial decline in HIV-1 markers that generally returns to baseline within 6-24 weeks (Fig 4). These changes in viral load are mirrored by reciprocal increases in $CD4^+$ T-lymphocyte counts and by the development of phenotypic resistance to NVP. We determined the genetic basis for viral resistance in such patients and examined the dynamics of virus turnover in plasma and PBMCs. 22 full-length RT genes from *cultured* PBMCs, *uncultured* PBMCs, and *uncultured* plasma of four patients before and 18-20 weeks after beginning NVP therapy were amplified by PCR/RT-PCR, cloned, tested for function and drug susceptibility, and sequenced in their entirety (Fig 5-7). To evaluate the kinetics of virus turnover, direct automated population sequencing of HIV-1 RNA and DNA from *uncultured* plasma and *uncultured* PBMCs, respectively, was performed for RT codons 0-250 before and at 14, 28, 42, 84, and 140 days after initiation of NVP therapy (Fig 8-13). NVP resistance-associated mutations were identified in 11 phenotypically resistant clones at positions F188L (NVP+ddI treated patient) or Y181C, G190A/S, and Y181C + G190A

(NVP+ddI+ZDV treated patients) (Fig 7). Additional codon changes associated with nucleoside analogue resistance were observed at positions 41, 67, 69, 70, 210, 215, and 219. Before NVP therapy, direct sequencing of plasma viral RNA and PBMC DNA revealed that 100% of viral sequences were wild-type at RT codons 181, 188, and 190. Fourteen to 28 days after starting NVP, 75-100% of viral RNA in plasma exhibited resistance-associated mutations at these positions (Fig 8-11). Acquisition of NVP mutations in PBMCs followed those in plasma by an additional 14-28 days; in some instances, virus turnover in PBMCs was less complete than in plasma (Fig 12,13). From these studies, we conclude that in nucleoside analogue experienced patients, the addition of NVP to ZDV/ddI selects for NVP resistant virus that replicates readily *in vivo*. NVP associated resistance changes are found preferentially at RT codons 181, 188, and 190. The magnitude and rapidity of virus turnover in plasma and PBMCs suggest that ongoing *de novo* infection of, and virus production by, a relatively short-lived (or transiently expressing) lymphoreticular cell population is primarily responsible for the rapid appearance of resistant virus and contributes substantially to the persistence of viremia observed in chronic HIV-1 infection.

Conclusions

The work described above directly addresses the stated hypotheses and specific aims of this contract (DAMD17-93-C-3146). Although we have only just completed year 01 of our studies, we are already heavily engaged in three of the four specific aims (#1, 3, 4) and have produced data relating to viral load and viral kinetic measurements that impact significantly on both practical and theoretical aspects of AIDS research. We are currently preparing five manuscripts describing the above findings for submission to scientific journals. In year 02, we will continue the studies described above and will initiate work relating to aim #2. Altogether,

the work supported by the DOD in our laboratory promises to provide important new insights into HIV-1 pathogenesis and hasten the development of effective treatments and vaccines for AIDS.

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Table 1: Comparison of Virologic Assay Detection Rates in Different Stages of HIV-1 Infection*

Site	CD4 Count	Number Tested	Plasma Culture	p24 Ag		Plasma Viral RNA	
				Regular	ICD	QC-PCR	bDNA
ADARC	<200	52	45(87)▲	26(50)	ND	ND	47(90)
	200-500	18	6(33)	3(17)	ND	ND	14(78)
	>500	8	0(0)	0(0)	ND	ND	6(75)
	Subtotal	78	51(65)	29(37)	ND	ND	67(86)
UAB	<200	31	24(77)	26(84)	27(87)	31(100)	30(97)
	200-500	19	5(26)	6(32)	8(42)	19(100)	15(79)
	>500	16	0(0)	1(6)	3(19)	16(100)	11(69)
	Subtotal	66	29(44)	33(50)	38(58)	66(100)	56(85)
All Sites	Total	144	80(56)	62(43)	38(58)	66(100)	123(85)

* Patients with primary (acute) HIV-1 infection were excluded from this analysis.

▲ Number and (percentage) of subjects with positive assay results.

Table 2: Spearman Rank Correlations Among Viral Load Measurements and CD4+ Lymphocyte Counts*

	CD4+ Cells	p24 Ag	ICD p24 Ag	Plasma Culture	QC-PCR	bDNA
CD4+ Cells	–	-.58	-.61	-.67	-.75	-.72
p24 Ag		–	.90	.62	.52	.51
ICD-p24 Ag			–	.58	.54	.56
Plasma Culture				–	.76	.72
QC-PCR					–	.89
bDNA						–

*Comparisons performed on viral load data depicted in Figure 1 along with corresponding p24 Ag data and CD4+ lymphocyte counts. Patients with primary (acute) infection and those with negative bDNA values were excluded.

All correlations shown are significant at the $p < 0.0001$ level.

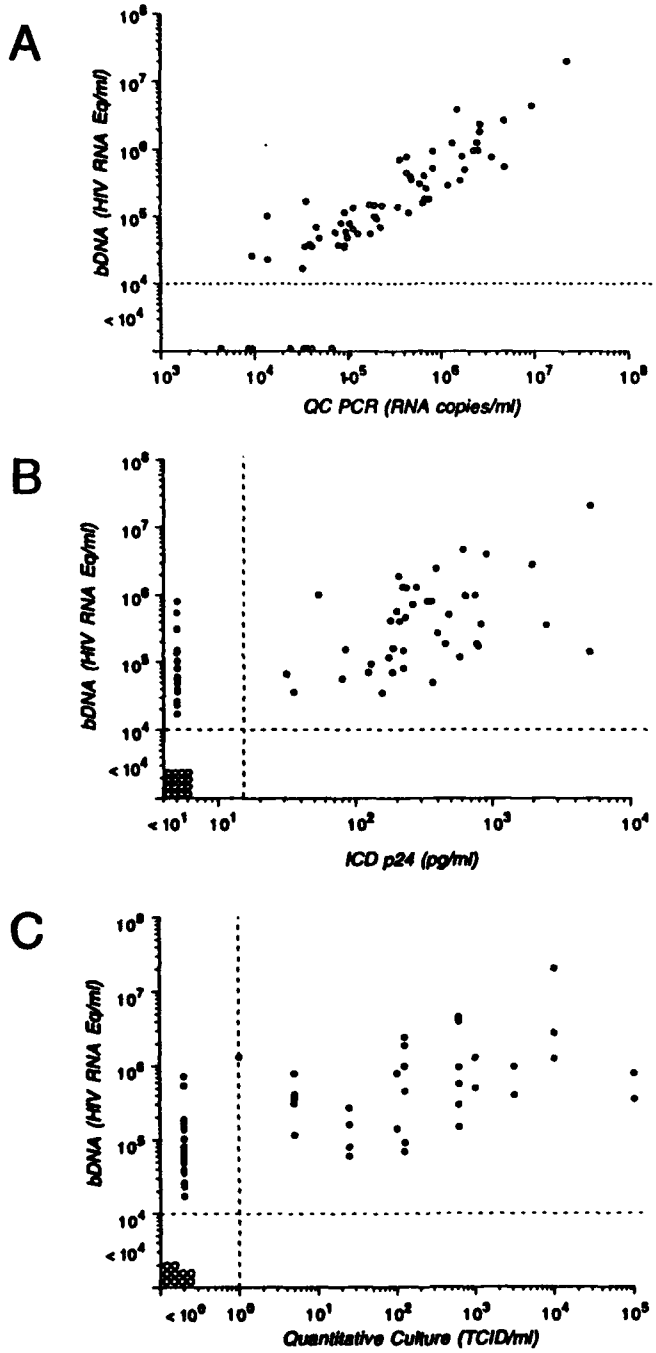

Figure 1: Plasma viral RNA levels determined by bDNA assay compared with plasma viral RNA assayed by QC-PCR (Panel A), plasma viral p24 Ag determined by the immune complex dissociation (ICD) assay (panel B), and infectious plasma virus determined by endpoint culture (panel C). Sensitivity cutoff levels for the respective assays are represented by dashed lines.

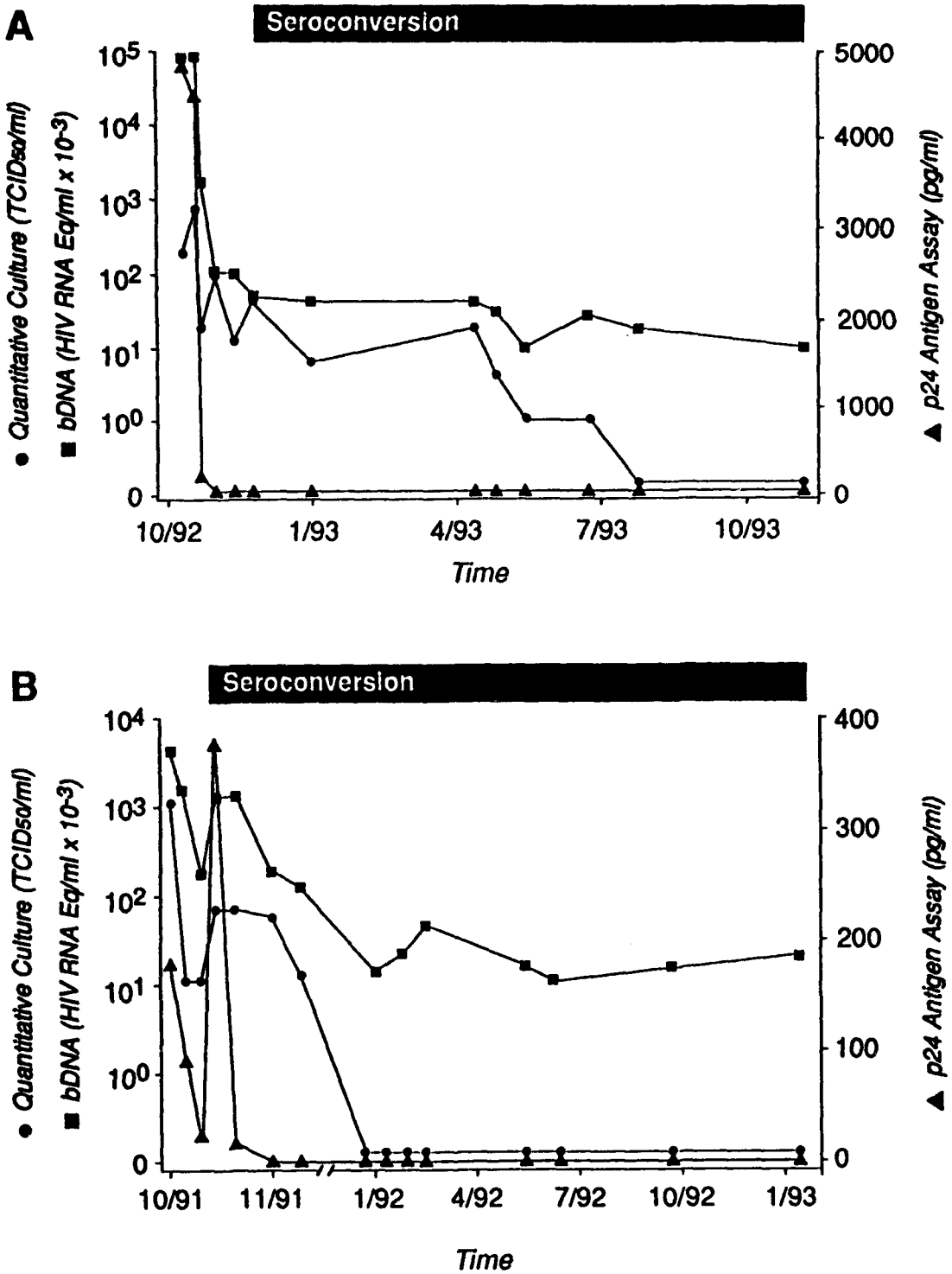

Figure 2: Plasma viral load measurements by bDNA, p24 Ag, and endpoint culture assays in two patients (panels A and B) with primary (acute) HIV-1 infection. The timing of first antibody detection and seroconversion are indicated by the bar labelled seroconversion. Note that bDNA values as plotted have been multiplied by 10^{-3}, so that a value plotted at 10^5 actually represents 10^6 HIV-1 RNA Eq/ml.

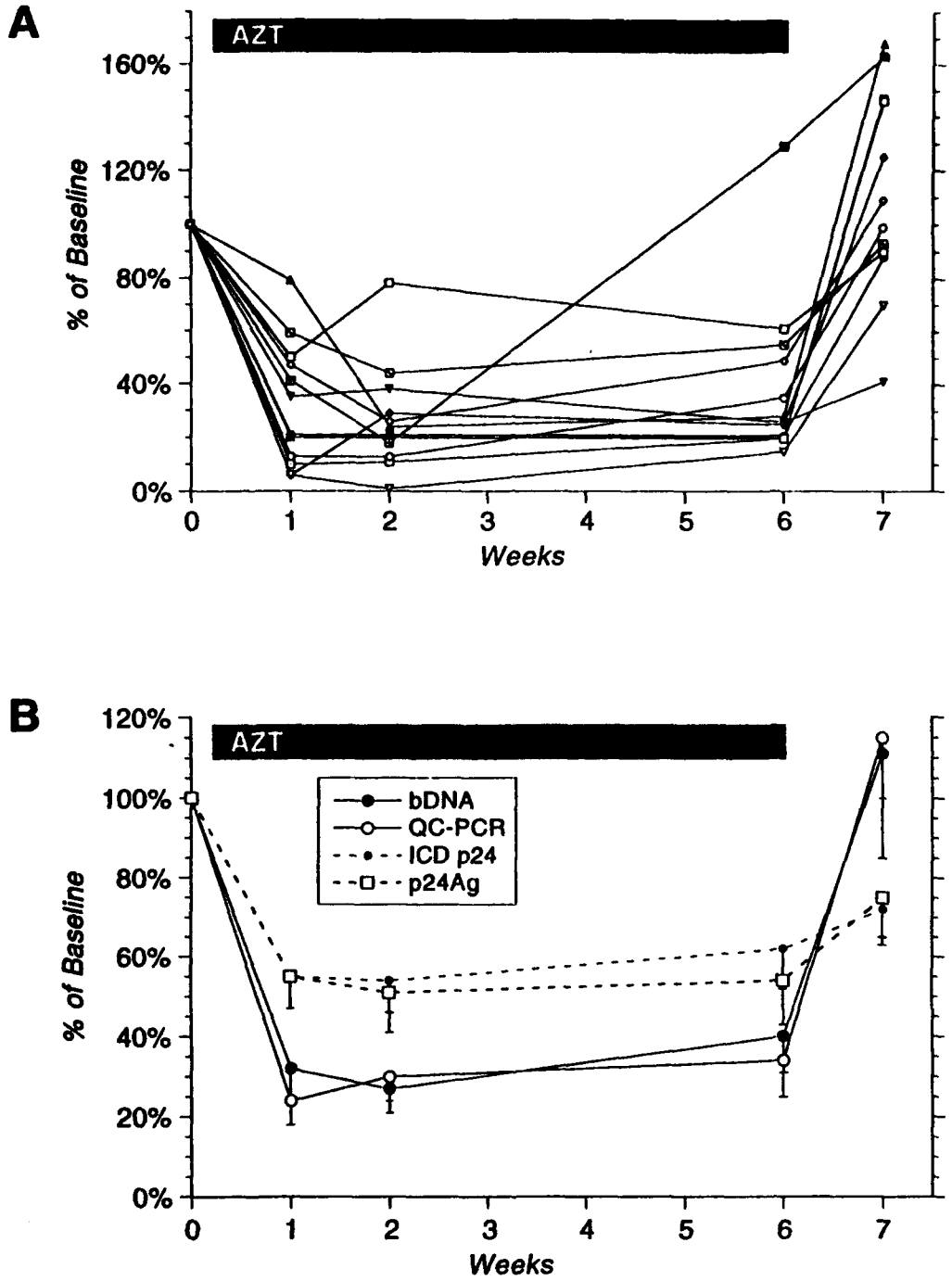

Figure 3: Changes in plasma viral RNA determined by bDNA and QC-PCR assays and plasma viral core antigen determined by regular and ICD-p24 Ag assays following the institution of a six week course of zidovudine (AZT) at 500 mg qd. (52). Therapy was discontinued after week six. Viral load measurements are plotted as percent of baseline (mean ± 1 S.E.M.). Individual patient data for the bDNA assay are shown in panel A (n=12) and combined data (mean ± 1 S.E.M.) are shown in panel B. Baseline values for viral RNA determined by bDNA assay ranged from 4.8 X 10^4 to 7.9 x 10^5 Eq/ml, and by QC-PCR assay, from 3.5 X 10^4 to 1.2 x 10^6 RNA molecules/ml. Baseline p24 Ag ranged from 0-226 pg/ml and ICD-p24 Ag from 0-783 pg/ml.

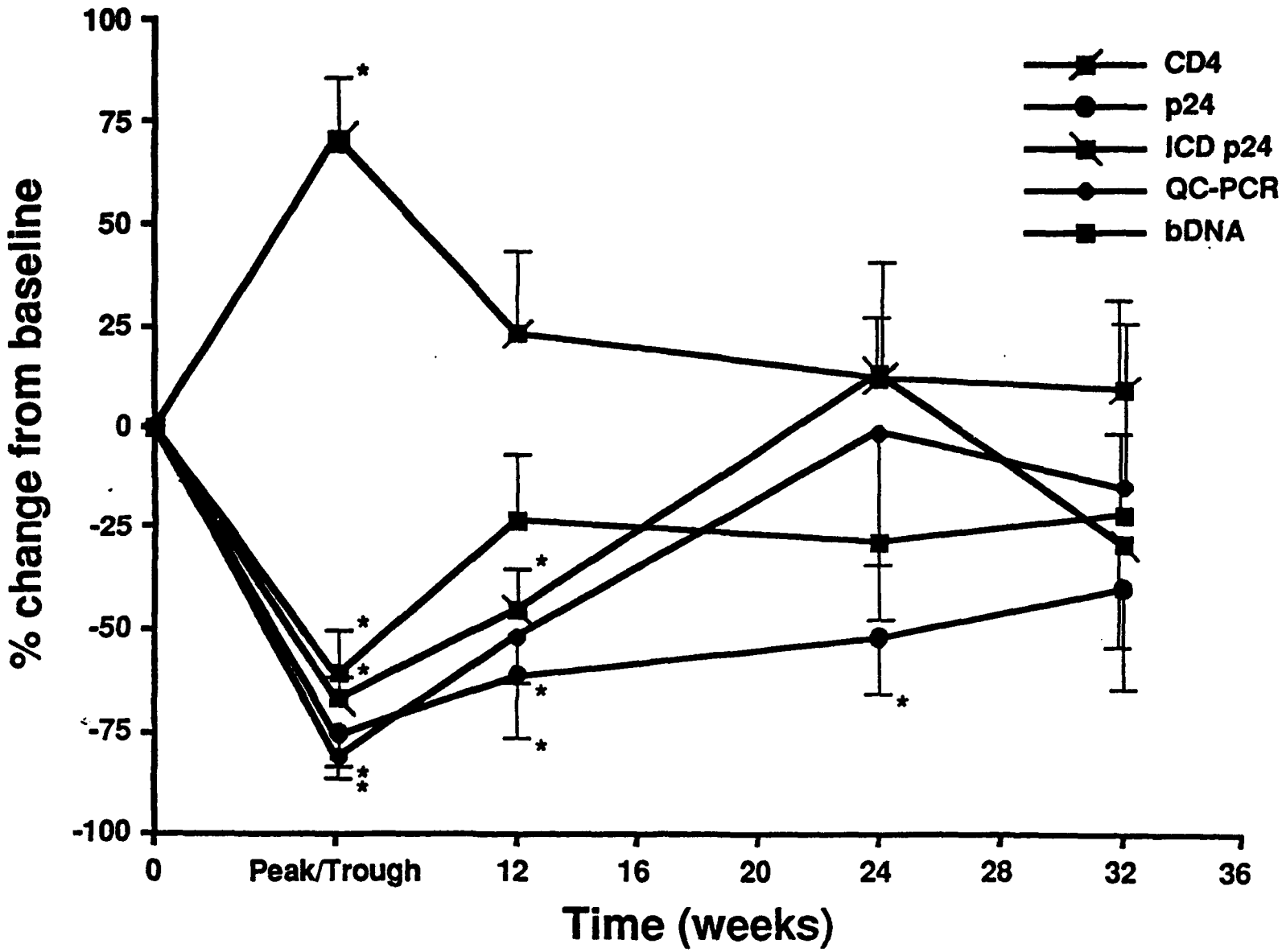

Figure 4: Virologic and immunologic response to combination Nevirapine therapy (all treatment arms combined - all 24 patients enrolled)

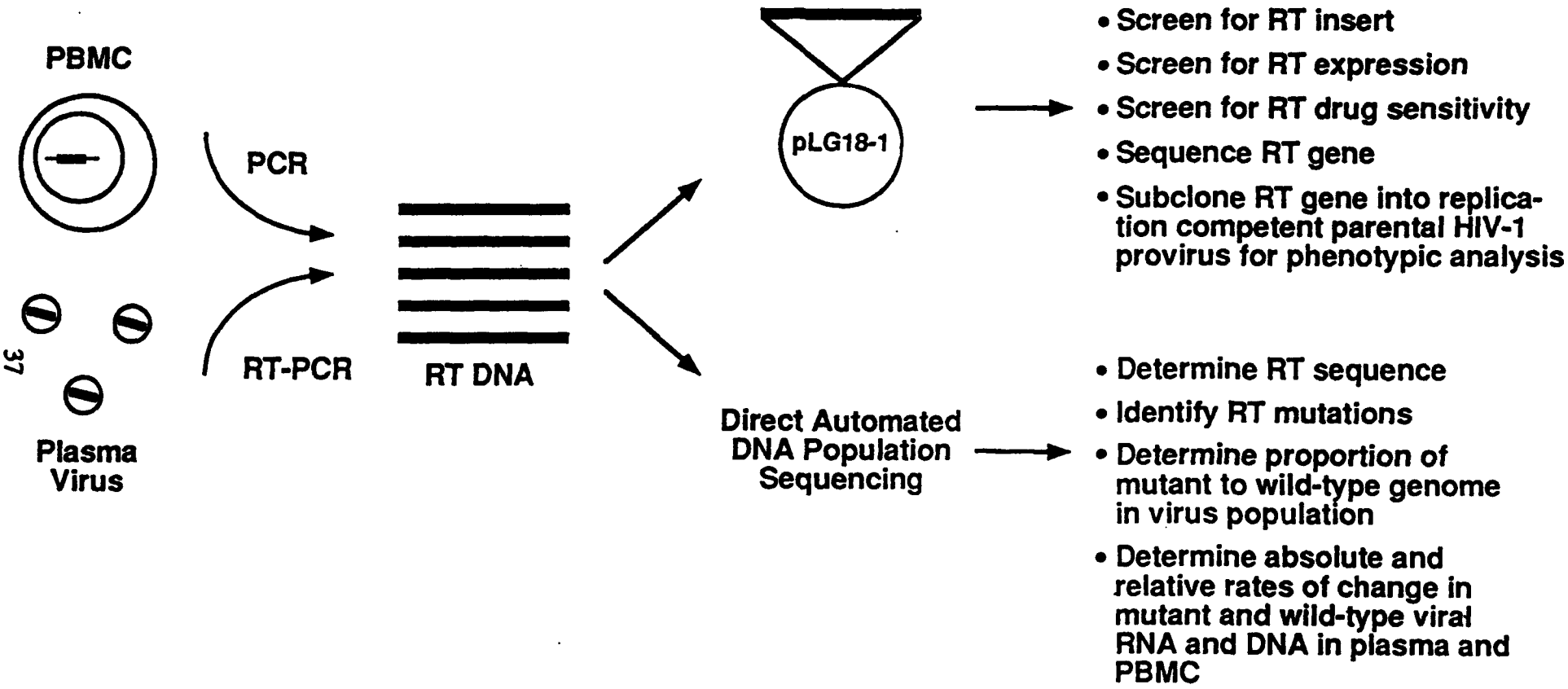

Figure 5: Molecular analysis of HIV-1 RT genes from *uncultured* PBMCs and plasma before and after Nevirapine therapy.

Sample ID	# Clones	Nonfunctional Clones	Functional Clones	Nevirapine Sensitive Clones	Nevirapine Resistant Clones
DBP-7	27	12	15	15	0
DBP-7**	119	98	21	21	0
DBP+140	121	81	40	25	15
DBP+140**	143	120	23	0	23
HVH-7	31	26	5	5	0
HVH+140	125	62	63	17	46
SHL-7	104	73	31	31	0
SHL+140	76	45	31	11	20
TED+43	87	67	20	14	6
TED+127	43	33	10	5	5

**Plasma-derived

Figure 6: *In situ* functional analysis (methodology described in Saag et al., *NEJM* 329:1065-1072, 1993) of HIV-1 RT clones derived from *uncultured* PBMCs and plasma before and after Nevirapine combination therapy.

Virus	Genotype																Therapy
	35	39	41	67	69	70	74	101	135	181	188	190	210	215	219	228	
HXB (wt)	V	T	M	D	T	K	L	K	I	Y	Y	G	L	T	K	L	None
SHL (d-7)	•	E	L	•	•	•	•	•	•	•	F	•	W	N	•	•	ddI
SHL (d+140)	•	E	L	•	•	•	•	•	•	•	L*	•	W	Y	•	H	ddI + Nev.
HVH (d-7)	•	•	•	•	•	•	•	•	•	•	•	•	W	Y	•	•	ddI + AZT
HVH (d+140)	•	•	L	•	•	•	•	•	•	C*	•	A*	W	Y	•	H	ddI + AZT + Nev.
TED (d-14)	•	•	•	•	•	•	•	•	T	•	•	•	•	Y	•	•	ddI
TED (d+43)	•	•	L	•	•	•	•	•	T	C*	•	•	•	Y	•	•	ddI + AZT + Nev. (7d)
TED (d+127)	•	•	L	•	•	•	•	•	T	C*	•	•	•	Y	•	R	ddI + AZT + Nev. (91d)
DBP (d-7)	•	•	L	•	•	•	•	•	T	•	•	•	•	F	•	•	ddI + AZT
DBP (d+140)	I	•	L	N	P	R	•	•	T	•	•	A*	W	Y	E	•	ddI + AZT + Nev.

Figure 7: Reverse transcriptase mutations in *cultured* HIV-1 strains from patients treated with combination antiviral therapy.

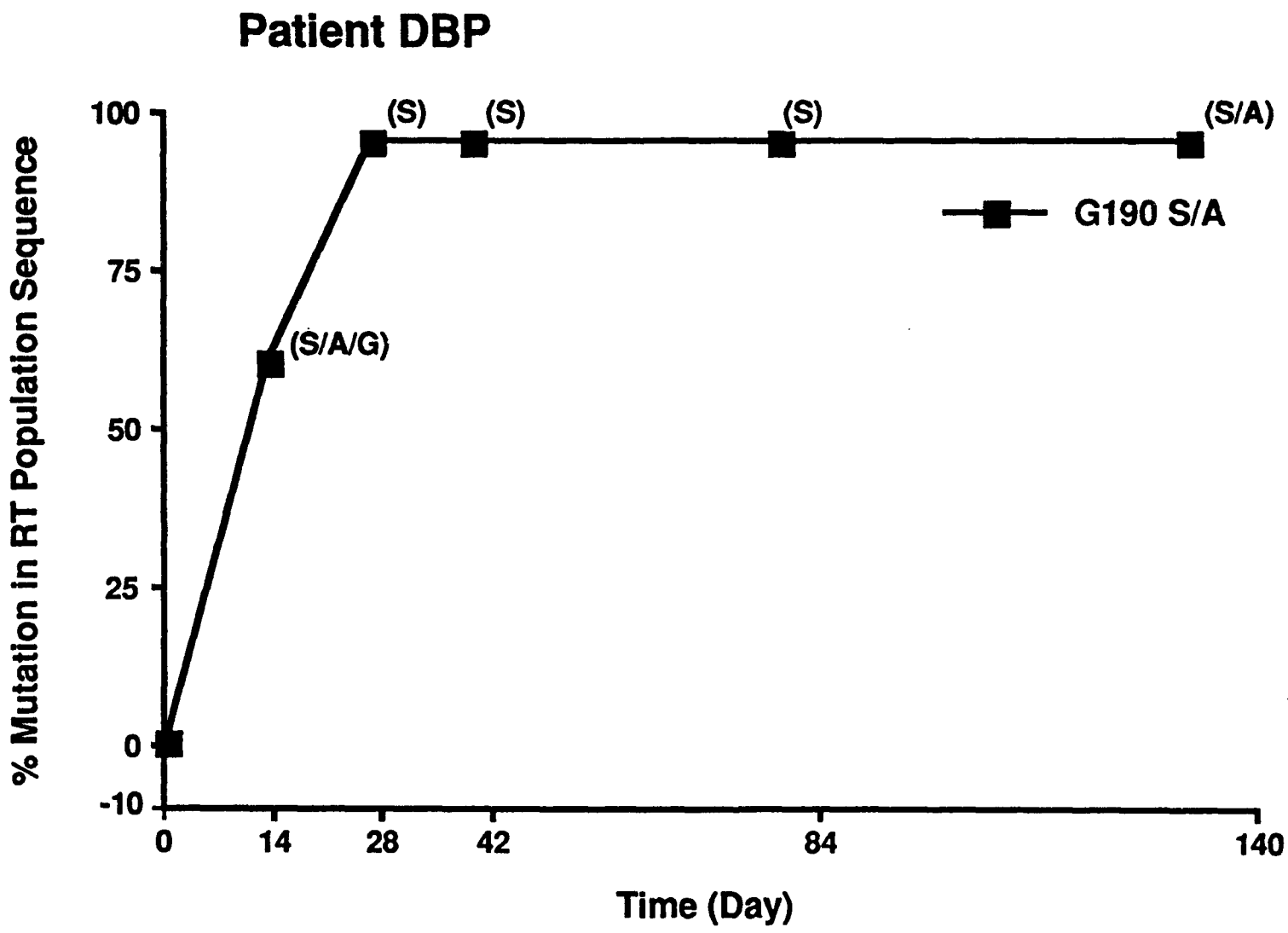

Figure 8: Proportional change in mutants in HIV-1 RT population sequence derived from uncultured plasma (viral RNA) following the additional of Nevirapine to combination AZT + ddI therapy.

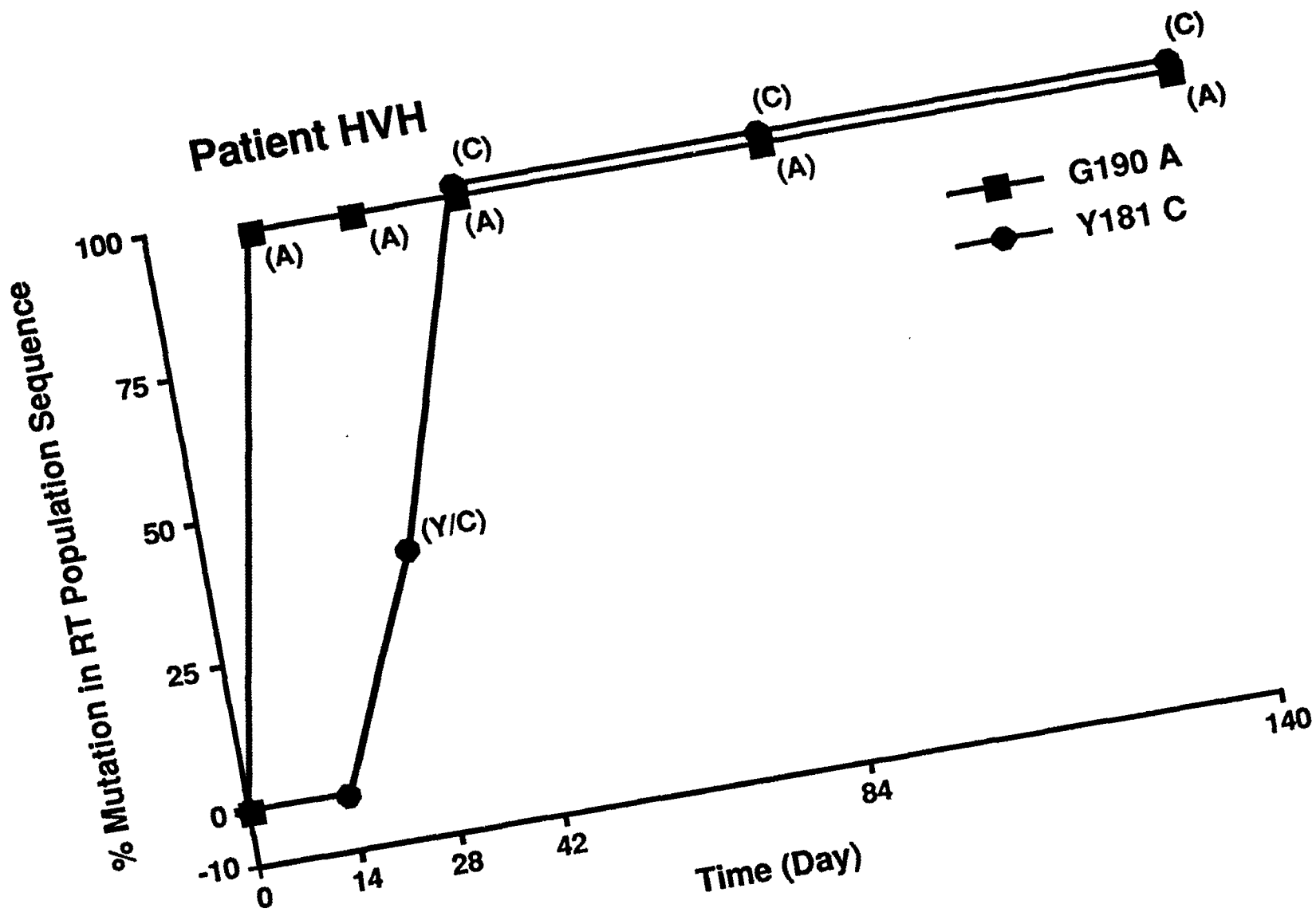

Figure 9: Proportional change in mutants in HIV-1 RT population sequence derived from uncultured plasma (viral RNA) following the addition of Nevirapine to combination AZT + ddI therapy.

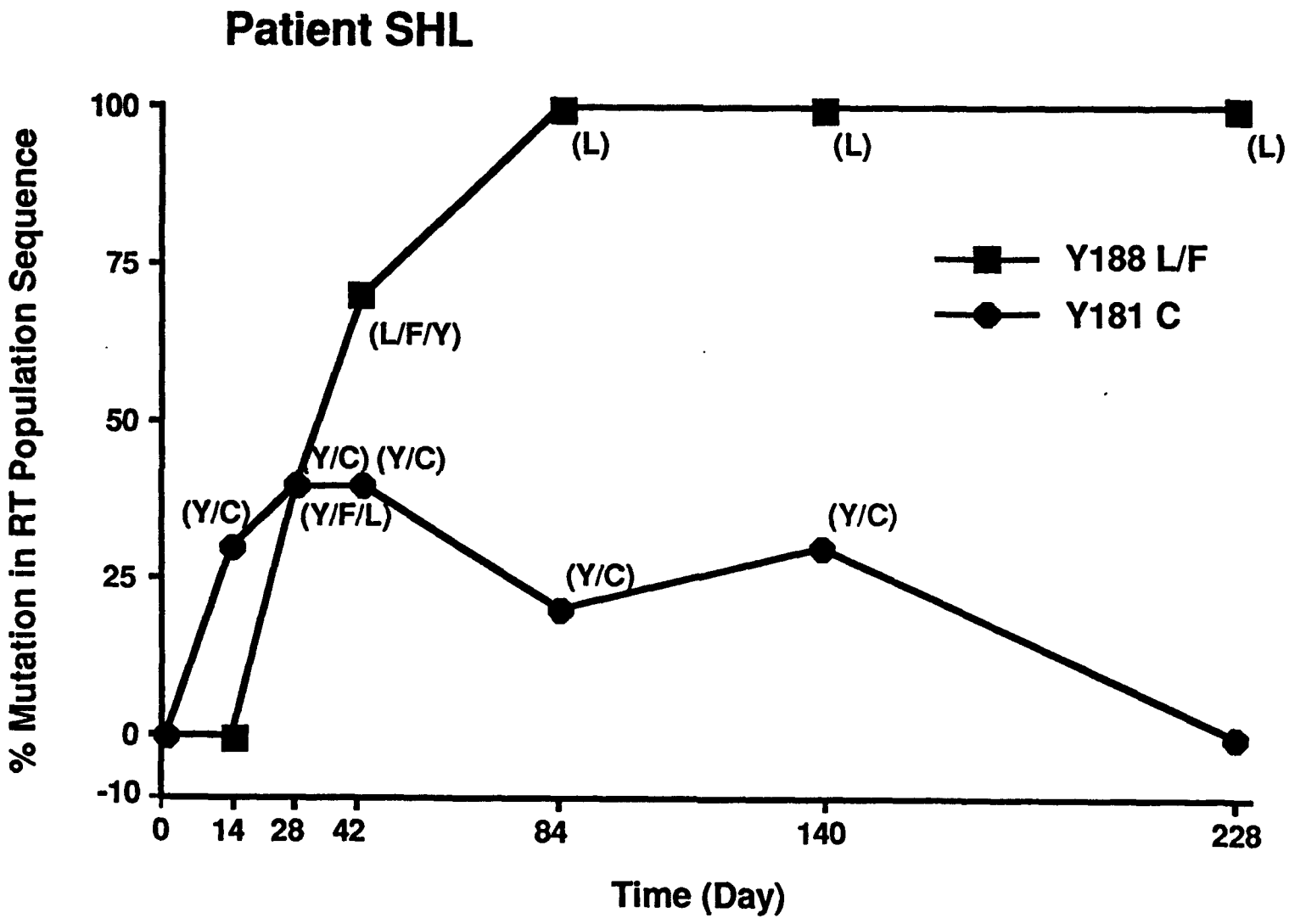

Figure 10: Proportional change in mutants in HIV-1 RT population sequence derived from uncultured plasma (viral RNA) following the addition of Nevirapine to ddI therapy.

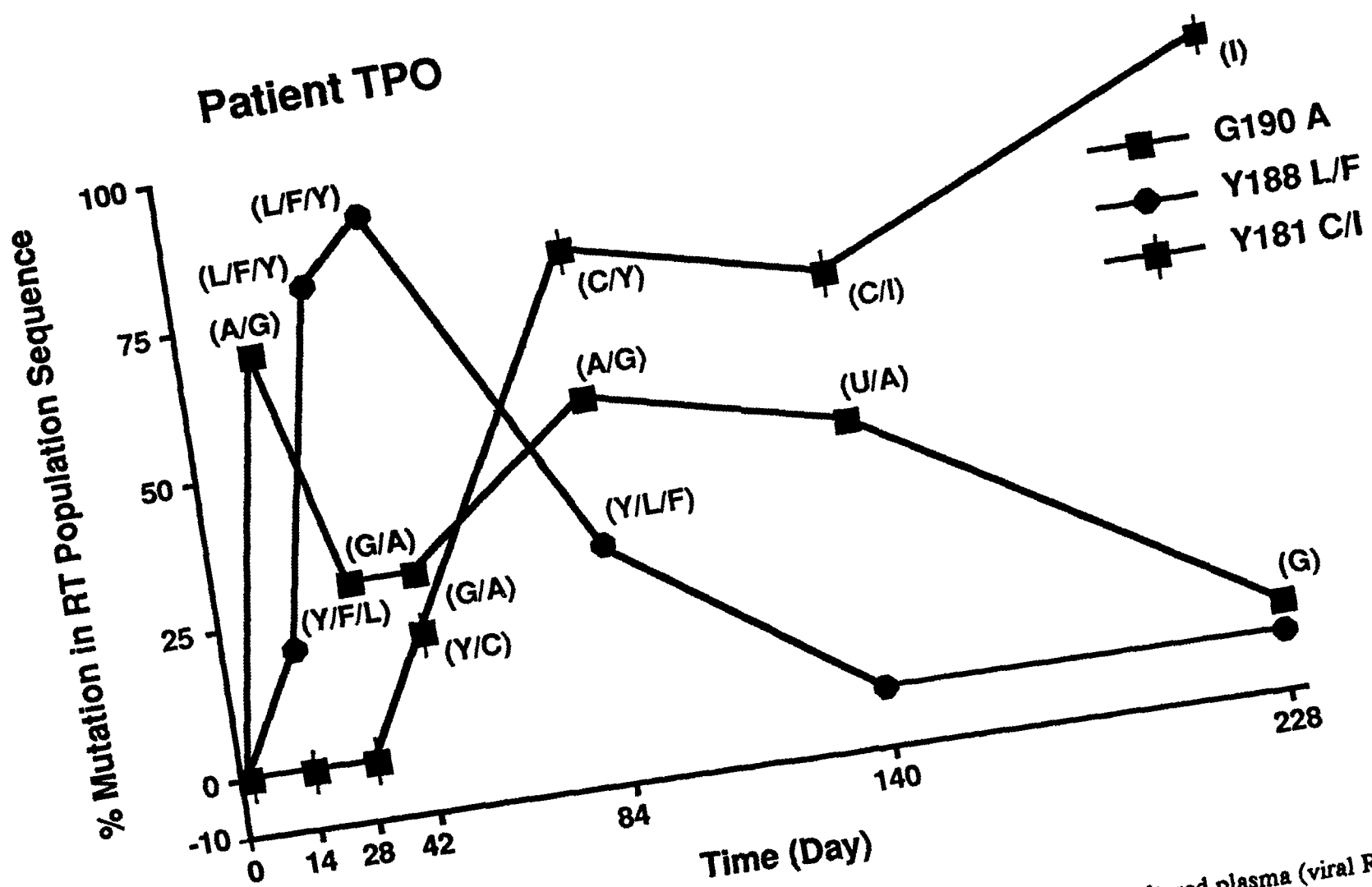

Figure 11: Proportional change in mutants in HIV-1 RT population sequence derived from uncultured plasma (viral RNA) following the addition of Nevirapine to combination AZT + ddI therapy.

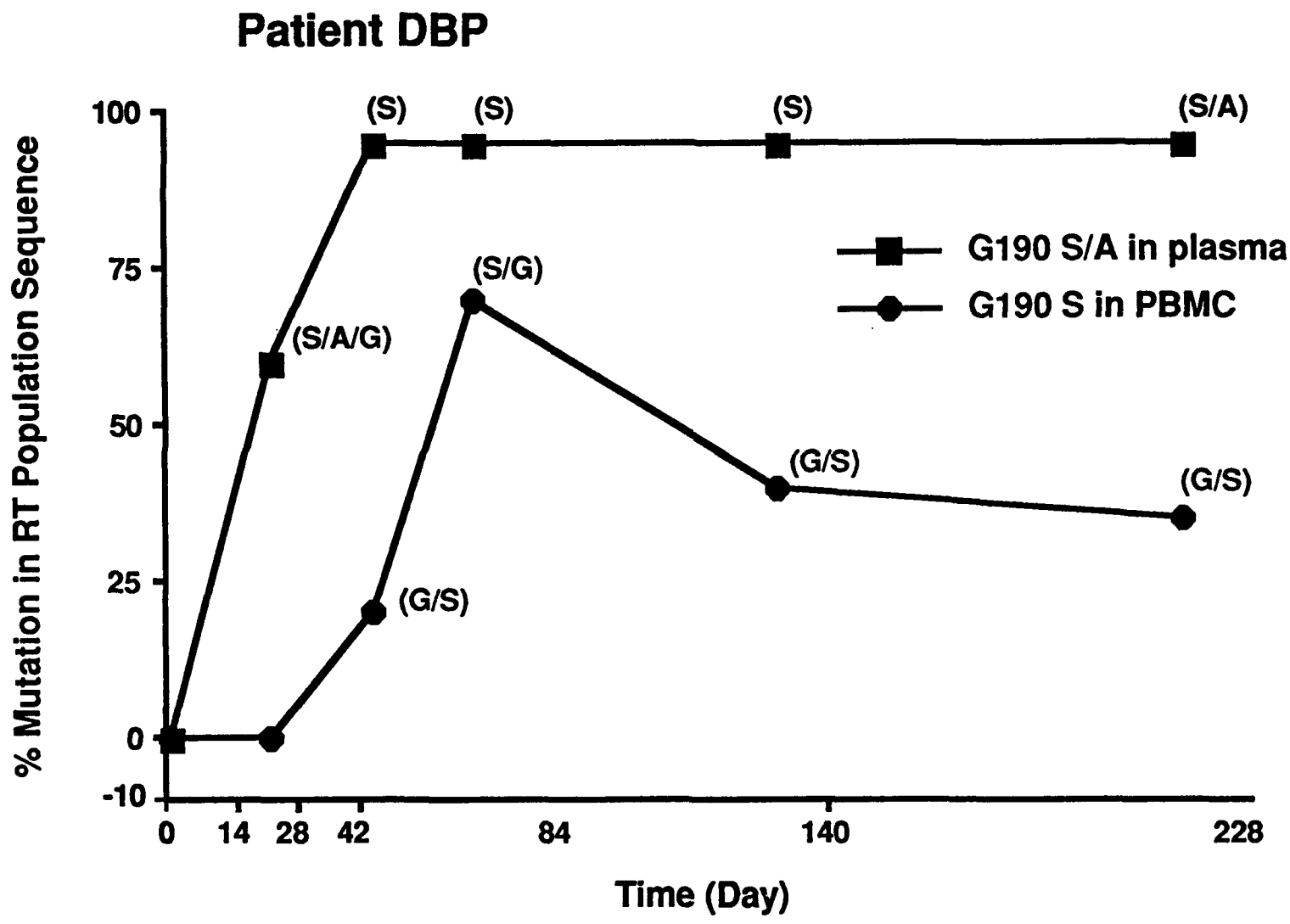

Figure 12: Comparison of rates of change in mutation frequencies between HIV-1 RNA and DNA from plasma and PBMC following addition of Nevirapine to combination AZT + ddI therapy.

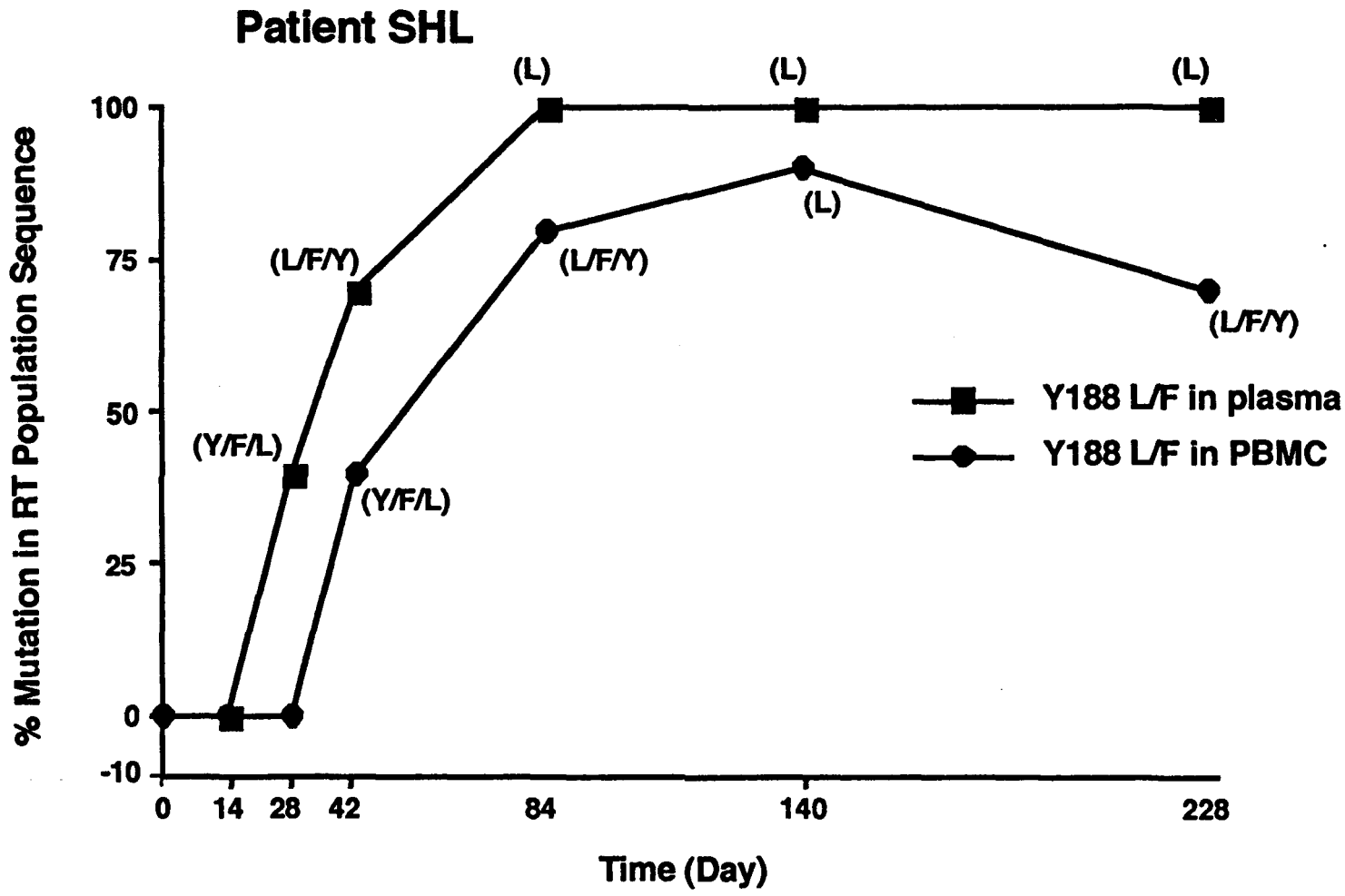

Figure 13: Comparison of rates of change in mutation frequencies between HIV-1 RNA and DNA from plasma and PBMC following addition of Nevirapine to ddI therapy.