Exhibit C

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Association of Plasma Human Immunodeficiency Virus Type 1 RNA Level with Risk of Clinical Progression in Patients with Advanced Infection

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Human immunodeficiency virus (HIV)-1 RNA level in plasma was evaluated as a surrogate marker for disease progression in a clinical trial of advanced HIV-1 infection. Baseline HIV-1 RNA level was an independent predictor of disease progression (relative hazard [RH] for each doubling of HIV-1 RNA level, 1.26; 95% confidence interval [CI], 1.03–1.54; $P = .02$), after adjusting for the week 4 change in HIV-1 RNA level, baseline CD4 cell count, syncytium-inducing phenotype, clinical status at study entry, and therapy randomization. A 50% reduction in HIV-1 RNA level was associated with a 27% decrease in the adjusted risk of disease progression during the study (RH, 0.73; 95% CI, 0.52–1.02; $P = .07$). The partial validation of HIV-1 RNA as a predictor for clinical end points has implications for the use of HIV-1 RNA in clinical trials and practice.

Laboratory markers of human immunodeficiency virus (HIV-1) replication are urgently needed as an alternative for clinical end points in the assessment of antiretroviral drug treatments. This is particularly important for studies of patients with earlier stages of infection, in whom clinical end points are infrequent. A marker of viral replication that predicted clinical progression could also further our understanding of the pathogenesis of HIV-1 infection, expedite the development of antiretroviral agents, and help optimize the use of antiretroviral therapy in clinical practice.

Plasma HIV-1 genomic RNA is a direct measure of viral replication [1, 2]. The level of plasma HIV-1 RNA is negatively correlated with the CD4 cell count [3–7], is positively correlated with disease stage and progression [8–13], and declines significantly with effective antiretroviral therapy [4–7, 14–18]. As such, the measurement of HIV-1 RNA holds promise as a useful marker for monitoring HIV-1 disease. Before HIV-1 RNA is considered as a suitable alternative to clinical end points, however, three strict validation criteria must be met. First, the baseline level of HIV-1 RNA must be associated with risk for clinical progression after controlling for virologic and immunologic factors that are independently associated with clinical progression. Second, an increase in HIV-1 RNA must correlate with an increased risk of disease progression, and a decrease in HIV-1 RNA must correlate with a decreased risk of disease progression. Third, all of the effects of treatment to either delay or accelerate the risk of clinical progression must be explained by the effects on the HIV-1 RNA level [19].

To address the validation of measurement of HIV-1 RNA, we undertook a retrospective virologic analysis of AIDS Clinical Trials Group (ACTG) protocol 116B/117 to determine if the clinical benefit of switching from zidovudine to didanosine seen in those zidovudine-experienced subjects was associated with a decrease in virus load by therapy, as assessed by plasma HIV-1 RNA [20]. Because about one-third of the 116B/117 subjects progressed to a clinical end point during the 2-year study period, and detailed virologic characterization of a subset of subjects' HIV-1 isolates was available [21, 22], we chose to assess HIV-1 RNA as a virologic marker for disease progres-

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sion by determining if HIV-1 burden measurements at study baseline and during therapy could explain, in part, the clinical and other virologic results of ACTG protocol 116B/117.

Methods

Patient selection. In total, 913 subjects who had received at least 16 weeks of previous zidovudine therapy were enrolled in ACTG protocol 116B/117 and followed a mean of 48 weeks for disease progression defined as a new AIDS event or death [20]. A subset of subjects for whom plasma samples were obtained for HIV-1 RNA quantitation were enrolled throughout the distribution of randomization dates for all subject participants enrolled. These subjects were followed in the study for a median of 304 days (range, 12–736). Plasma samples were available from 100 subjects at baseline; 71 of them had plasma samples at week 4, 72 at week 8, 66 at week 12, and 49 at week 24. Cryopreserved peripheral blood mononuclear cells (PBMC) or primary HIV-1 isolates were available from 98 of the 100 subjects for determining syncytium-inducing (SI) phenotype by the MT-2 cell assay. Genetic analysis of HIV-1 reverse transcriptase (RT) and plasma specimens suitable for HIV-1 RNA analysis were available for 74 of the 100 subjects whose baseline specimens yielded productive viral isolates by PBMC microculture [22]. In contrast, as previously reported, specimens for drug resistance and SI phenotype were obtained from the first 10 subjects enrolled at each participating clinical site [21, 22].

Sample preparation. Plasma and PBMC were collected after ficoll-hypaque density gradient centrifugation of heparinized whole blood according to standard methods of the ACTG Virology Group [23–25]. Plasma aliquots were stored at $-70°C$. PBMC were either cultured for HIV-1 or cryopreserved using standard methods [25].

Assays of drug susceptibility, RT mutations at codons 215 and 41, and SI phenotype. Virus stocks were prepared from either primary isolates or cryopreserved PBMC, and infectivity was assessed by end-point dilution, followed by zidovudine susceptibility determination using the ACTG/Department of Defense drug susceptibility assay as described [21, 26]. The mutations at codons 215 and 41 of the HIV-1 RT gene that are associated with zidovudine resistance were determined by a selective polymerase chain reaction (PCR) analysis of $3–5 \times 10^6$ cells collected from HIV-1 stocks prepared in phytohemagglutinin-stimulated PBMC as described [22]. SI phenotype was assessed by cultivation of HIV-1 cell-free virus stocks with MT-2 cells in 96-well microtiter plates [27].

HIV-1 RNA analysis. Plasma HIV-1 RNA levels were determined from thawed heparinized plasma using two analytic methods: RT quantitative PCR amplification (Roche Molecular Systems, Alameda, CA) and branched DNA (bDNA) signal amplification (Chiron, Emeryville, CA) [17, 18, 28–31]. For the RT-PCR assay, HIV-1 RNA was purified from heparinized plasma by silica particle adsorption [32]; no pretreatment was required before bDNA testing. The RT-PCR assay was run at Roche Molecular Systems and the bDNA assay was run at the University of Washington. HIV-1 RNA copy numbers were assessed using the manufacturers' reference standards. HIV-1 assays differed in their lower level of sensitivity: 400 RNA copies/mL for the RT-PCR assay and 10,000 RNA Eq/mL for the bDNA assay [28, 30].

HIV-1 p24 antigen analysis. Frozen serum was thawed and analyzed by batch testing at National Institute of Allergy and Infectious Diseases Virology Quality Assurance Program–certified ACTG laboratories using consensus methods [25].

Statistical analysis. HIV-1 RNA data were treated as a continuous variable for comparing the RT-PCR and bDNA assays. Zidovudine susceptibility was measured as the IC_{50} and was considered as either a continuous or discrete variable (susceptibility, $IC_{50} < 0.2$ μM; moderate susceptibility, $IC_{50} \geqslant 0.2$ μM, < 1.0 μM; high-level resistance, $IC_{50} \geqslant 1.0$ μM). SI phenotype (SI vs. non-SI), diagnosis of AIDS at entry into the study (AIDS vs. AIDS-related complex and asymptomatic clinical infection), and drug treatment at randomization (didanosine vs. zidovudine) were evaluated as discrete data. Similarly, data on HIV-1 RT mutations at codons 215 and 41 were treated as categorical variables: Virus isolates were scored as having a mutation at either codon (mutant), having no detectable mutation (wild type), or being a mixture of viral types.

Associations between HIV-1 RNA levels and other virologic and immunologic markers of disease activity were evaluated using nonparametric methods. For association of HIV-1 RNA level with categorical variables, Wilcoxon or Kruskal-Wallis rank sum multiple comparison tests were used to evaluate differences in the rank of the distribution of factors known to be associated with disease progression. For assessing associations between continuous data such as HIV-1 RNA, zidovudine IC_{50}, and CD4 cell count, Spearman correlation coefficients were determined.

To evaluate unadjusted associations among the various virologic and immunologic factors and progression of disease, we used Cox proportional hazard models or contingency table analysis. For contingency table analysis, P values presented were for Mantel-Haenszel extension tests.

To evaluate a possible threshold effect of baseline HIV-1 RNA level (measured by RT-PCR) on risk of disease progression, we derived a dichotomous indicator variable at threshold levels of 200,000 ($10^{5.30}$) and 400,000 ($10^{5.60}$) RNA copies/mL. Bivariate Cox regressions were done that tested whether continuous measures of baseline HIV-1 RNA or threshold levels, or both, remained predictive of clinical progression. If a dichotomous indicator variable remained predictive of disease progression after adjustment for continuous measurement of baseline HIV-1 RNA level, we considered this level to possibly represent a level above which subjects were at higher risk for progression than those below this level.

The associations between HIV-1 RNA levels at baseline and changes in HIV-1 RNA level from baseline, with therapy and with clinical progression, were examined by Cox proportional hazard models or multiple Cox regression that provided adjustment for other virologic and immunologic factors, AIDS diagnosis at entry, and treatment assignment. Both HIV-1 RNA and CD4 cell counts were transformed to their natural logarithms to improve the fit of the regression models. Disease progression was defined from the clinical trial as the occurrence of a new, previously undiagnosed AIDS-defining event or death, whichever occurred first during the 2-year study period [20]. Results were expressed as the relative hazard (RH), which is the change in the risk for progression in subjects with a given factor relative to patients without that factor. For categorical data, subjects with increasing levels of a given

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Table 1. Demographics for all ACTG protocol 116B/117 subjects and those from the subsets with available plasma for HIV-1 RNA analysis.

Characteristic	All subjects* (n = 913)	Subset of subjects with available HIV-1 RNA values		
		Baseline (n = 100)	Week 4 (n = 71)	Week 24 (n = 49)
At study entry				
Age (median, years)	36	35	35	35
Homosexual (%)	79	86	84	88
Race/ethnicity (% white, non-Hispanic)	82	85	88	84
Sex (% male)	96	96	94	95
Diagnosis of AIDS at study entry (%)	30	37	32	43
CD4 cell count (median, cells/μL)	95	85	90	99
Serum HIV-1 p24 antigen (% with $\geq$10 pg/mL)	43	42	42	45
Prior zidovudine experience (median, months)	14	14	14	12
During clinical trial				
Progression to primary end point (%)[†]	37	45	44	43
Survival at 1 year of follow-up (%)	61	55	64	76[‡]

NOTE. ACTG, AIDS Clinical Trials Group.
* Data from [20].
[†] Progression was defined as new AIDS-defining event or death during study period.
[‡] Comparison with subset of subjects at baseline, $P = .01$.

factor were compared with those with the lowest level (referent group) with respect to clinical outcome. For continuous data (baseline HIV-1 RNA level, change in RNA during therapy, CD4 cell count), RHs were expressed for doubling of the marker level. A factor was considered an independent risk factor for progression if the factor remained a significant predictor of disease progression after adjustment for other predictors of progression. Estimates of RH were given with 95% confidence intervals (CI) and, where appropriate, the corresponding P value of significance.

Results

Subject characteristics. With the exception of survival at 1 year of follow-up, there were no statistically significant differences in baseline characteristics between the study subsets and the entire study population (table 1). The distributions of study randomization dates between the study subsets and the entire study population were also compared and found not to be different across study centers (data not shown).

Baseline HIV-1 RNA measurement by RT-PCR and bDNA assays. The two assay methods used for quantifying plasma HIV-1 RNA were compared for the 97 subjects for whom baseline plasma specimens were tested by both assays. The two assays were significantly correlated (Spearman rank correlation coefficient $r = .79$, data not shown). Thirty subjects (30%) had <10,000 RNA Eq/mL by the bDNA assay and were considered left-censored for the purposes of analysis that used the bDNA-derived values. To evaluate the effect of censoring HIV-1 RNA on estimating the association of HIV-1 RNA copy number with disease progression, we conducted an analysis whereby the 30 censored specimens at baseline were assigned values of either

9999 HIV-1 RNA Eq/mL or 1 HIV-1 RNA Eq/mL of plasma. There was no significant difference between the magnitude of the RH of disease progression with HIV-1 RNA level when comparing RH estimates using either assigned value (data not shown).

HIV-1 RNA level at study baseline. The baseline plasma specimens tested for HIV-1 RNA by both the bDNA and RT-PCR assays were stratified into eight ranges according to the bDNA-derived RNA level, and the frequency of disease progression was plotted for each partition (figure 1). By contingency table analysis, there was a positive association between RNA copy number and the frequency of disease progression regardless of assay used ($P < .003$ for RT-PCR, $P < .001$ for bDNA).

Disease progression occurred in 73% of subjects with >218,000 ($10^{5.33}$) HIV-1 RNA copies/mL compared with 31% with $\leq$218,000 HIV-1 RNA copies/mL ($P < .001$, pairwise comparison using RT-PCR-derived RNA values; figure 1). Because this suggested that there might be a change in the hazard relationship at higher HIV-1 RNA levels, we explored the possible relationship of threshold using Cox regression models that included indicator variables of RNA levels >200,000 ($10^{5.30}$) and >400,000 ($10^{5.60}$) copies/mL. There was a statistically significant increased RH for disease progression at these two threshold levels: RH for $10^{5.30}$ RNA copies/mL, 3.40 (95% CI, 1.20–9.62); RH for $10^{5.60}$ RNA copies/mL, 2.82 (95% CI, 1.01–7.87).

Associations of the baseline HIV-1 RNA copy number and other predictors of clinical progression were evaluated using nonparametric analysis methods. The baseline HIV-1 RNA

copy number and CD4 cell count were significantly correlated (Spearman $r = -.31$; $P = .001$). HIV-1 RNA copy number was associated with an entry diagnosis of AIDS but not with SI phenotype (table 2).

The detection of codon 215 mutation either alone or with codon 41 was significantly associated with increased virus load compared with the wild type codons ($P = .05$; table 2). There was no correlation, however, between the baseline HIV-1 RNA copy number and zidovudine susceptibility when analyzed either as a continuous variable using IC_{50} (Spearman $r = -.06$; $P = .52$) or as a discontinuous variable obtained by dividing the IC_{50} data into three categories: susceptible, moderately susceptible, or resistant (table 2).

Although the above analysis suggested that there was a nonlinear relationship between baseline virus load and hazard of disease progression, it was convenient to analyze the baseline RNA level as a linear parameter in modeling the hazard associated with the therapy-induced change in HIV-1 RNA. The reason for this was that we wished to control for the baseline HIV-1 RNA level as a continuous measure for investigating the relationship between hazard and change in HIV-1 RNA. Analyses were done using the baseline RNA level as a continuous variable with or without the two threshold levels and with just the thresholds; this did not affect the subsequent findings for the analysis that used HIV-1 RNA as a continuous variable (excluding the threshold). There was an unadjusted 23% in-

Table 2. Relationship of baseline HIV-1 RNA copy number measured by RT-PCR assay with other independent predictors of clinical progression.

Variable	n	HIV-1 RNA copy number/mL of plasma*			P
		Median	25th–75th percentile		
Zidovudine susceptibility					.87[†]
Susceptible ($<0.2\ \mu M$)	57	95,800	37,400	330,000	
Moderate ($0.2-<1.0\ \mu M$)	24	69,600	39,800	183,000	
Resistant ($\geq 1.0\ \mu M$)	16	125,000	46,000	532,000	
pol codon 215 and 41 genotype[‡]					.050[§]
215WT/41WT	13	74,600	47,800	139,000	
215 MT/41WT	24	365,000	105,000	672,000	
215MT/41MT	25	191,000	103,000	724,000	
MT-2 cell tropism					.65[¶]
Syncytium-inducing	62	71,600	44,800	320,000	
Non–syncytium-inducing	33	120,000	37,400	276,000	
Clinical diagnosis					.036[**]
Asymptomatic	6	35,400	12,600	133,000	
Symptomatic	55	69,400	29,600	226,000	
AIDS	36	139,000	59,400	436,000	

NOTE.   RT-PCR, reverse transcriptase–polymerase chain reaction amplification; WT, wild type codon; MT, mutant codon.
* HIV-1 RNA levels were derived using manufacturer's standard.
[†] Wilcoxon test comparing resistant to susceptible and moderately susceptible.
[‡] There was only 1 specimen with codon mutations 215WT/41MT; therefore, this category was not analyzed.
[§] Kruskal-Wallis test comparing 215MT to 215WT.
[¶] Wilcoxon test comparing syncytium-inducing to non–syncytium-inducing phenotype.
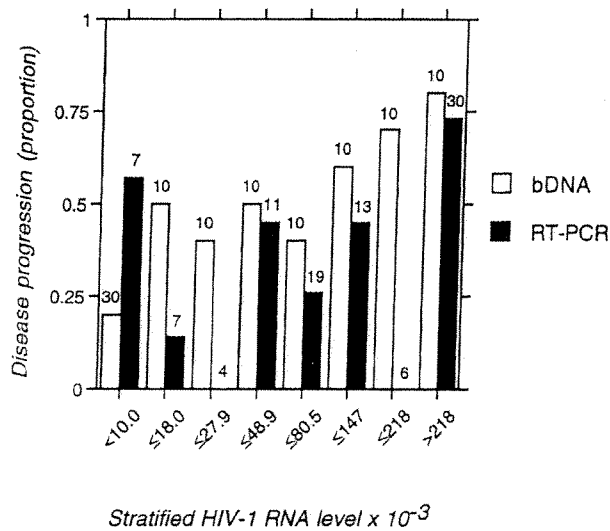[**] Wilcoxon test comparing AIDS to asymptomatic and symptomatic.

Figure 1. Frequency of disease progression or death by baseline HIV-1 RNA range for 100 subjects with branched DNA (bDNA) values (RNA Eq/mL of plasma) and 97 subjects with RT-PCR (reverse transcriptase–polymerase chain reaction amplification) values (RNA copies/mL of plasma). bDNA values $\geq 10,000$ RNA Eq/mL were stratified into ranges that contained 10 subjects, and RT-PCR–derived RNA values were assigned according to ranges set by bDNA data. No. of subjects constituting each stratum are shown above each bar. Cochran-Mantel-Haenszel nonzero correlation: $P < .001$ for bDNA and $P = .003$ for RT-PCR.

crease in RH of disease progression for every 2-fold increase in HIV-1 RNA copy number for persons entering the study ($P = .001$; table 3). In contrast, each 2-fold increase in CD4 cell count at baseline was associated with a 27% reduction in unadjusted RH of disease progression. Both the SI phenotype and diagnosis of AIDS at study entry were associated with a >2-fold increased unadjusted RH of disease progression.

Because unadjusted estimates of association may not provide important information about the relative contributions of other potential predictors to disease progression, multiple regression analysis was used to adjust the association of HIV-1 RNA copy number with disease progression or death for other virologic and immunologic host factors for 97 subjects with complete baseline data available (table 3) [21, 22]. After adjusting for these other markers of disease progression, analysis suggested that a ≥ 2-fold level of HIV-1 RNA copy number at study entry (baseline) was associated with a 13% increase in the adjusted RH for disease progression ($P = .12$). The association of CD4 cell count and diagnosis of AIDS at study entry remained statistically significant after adjusting for the other variables, and the magnitude of association for SI phenotype with

Table 3. Relative hazard of clinical progression or death for 97 subjects with baseline HIV-1 RNA and other risk factors.

Variable	Relative hazard (95% CI) for clinical progression to primary end point*		$P^{\dagger}$
	Unadjusted	Adjusted	
Baseline RNA copy numbers[‡§¶]	1.23 (1.07–1.42)	1.13 (0.97–1.32)	.12
Baseline CD4 cell counts[§§]	0.73 (0.64–0.84)	0.77 (0.64–0.92)	.004
Syncytium-inducing phenotype	2.10 (1.06–4.15)	2.12 (0.97–4.63)	.06
Diagnosis of AIDS at study entry	2.89 (1.60–5.23)	2.50 (1.33–4.69)	.004
Didanosine therapy	0.74 (0.41–1.34)	0.67 (0.36–1.25)	.21

NOTE. CI, confidence interval.

* Unadjusted and adjusted relative hazard of progression or death were determined by Cox proportional hazards model. Unadjusted relative hazard is the risk of progression to primary end point or death conferred by that variable alone; adjusted relative hazard is risk conferred by that variable after controlling for all other variables in table. Relative hazard for each variable is based on analysis of entire population for whom measures of each variable were available.

[†] For adjusted model.

[‡] HIV-1 RNA was measured by both reverse transcriptase–polymerase chain reaction (RT-PCR) assay (Roche) and branched DNA (bDNA) assay (Chiron); however, since 30 subjects were censored at baseline because of lower sensitivity for bDNA assay (<10,000 RNA equivalents/mL), only RT-PCR–derived RNA values are shown. Relative hazard values derived from both assays were not significantly different (data not shown).

[§] Relative hazard associated with each doubling (0.3 $\log_{10}$ change) of RNA copy number or CD4 cell count at baseline.

[¶] Adjusted relative hazard for clinical progression of 1.13 indicates 13% increased risk for each doubling in baseline HIV-1 RNA level; similarly, adjusted relative hazard of 0.77 indicates 23% reduction in risk for each doubling in the baseline CD4 cell count.

disease progression remained essentially unchanged, although it did not achieve statistical significance. Treatment assignment, either by itself or after adjustment, was not significantly associated with disease progression; however, the magnitude of association with disease progression was in agreement with other findings for the ACTG protocol 116B/117 study [20–22].

Serum HIV-1 p24 antigen levels were correlated with HIV-1 RNA copy number (Spearman $r = .78$), but p24 antigen level at baseline or the change in p24 antigen level from baseline after therapy was not significantly associated with disease progression either by themselves or after adjustment. Therefore, p24 antigen was not modeled further.

Change in plasma HIV-1 RNA level from baseline with therapy. The plasma HIV-1 RNA level declined by a median of 0.2 $\log_{10}$ during therapy for subjects who were switched to didanosine (figure 2A) but not for those who continued zidovudine (figure 2B). The relative changes in plasma HIV-1 RNA level over the 24 weeks of this study were similar for both the RT-PCR and bDNA assays. For example, the relative change in HIV-1 RNA from baseline remained significantly different

at week 12 by both RT-PCR ($P = .018$) and bDNA ($P = .005$) but not at week 24 ($P > .06$ by both HIV-1 RNA determinations). After 2 weeks of didanosine therapy, there was a small increase in the CD4 cell count associated with the decline in HIV-1 RNA; however, the increase in CD4 cell number from baseline was not sustained at 8 weeks (figure 2A). There was no increase in CD4 cell number for subjects who continued to receive zidovudine (figure 2B).

The adjusted associations of the baseline HIV-1 RNA level, change in HIV-1 RNA level from baseline after 4 weeks of therapy, and other potential markers of disease progression with new opportunistic infection or death are presented in table
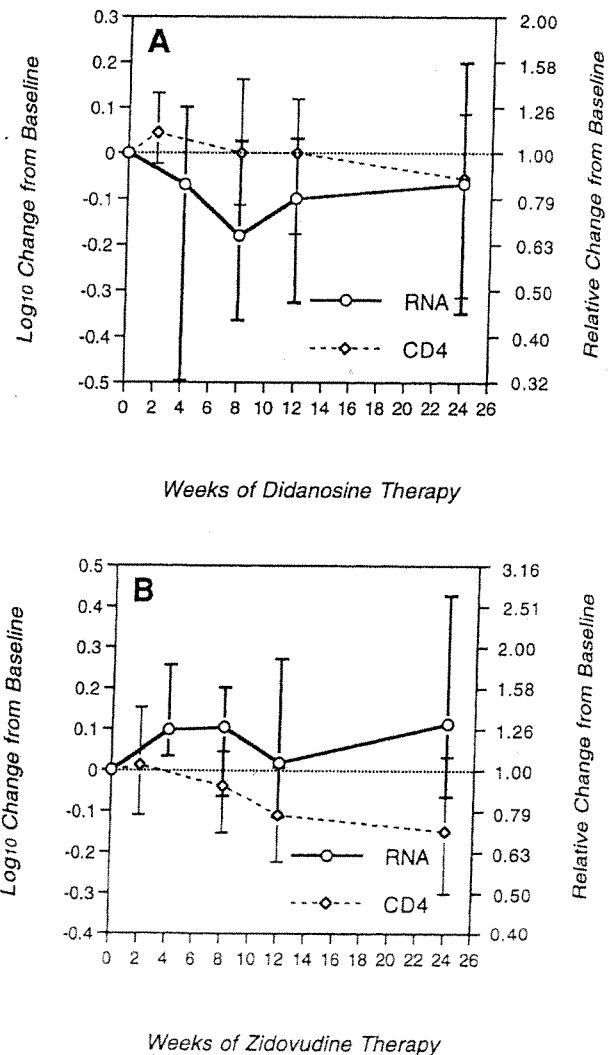

Weeks of Didanosine Therapy

Weeks of Zidovudine Therapy

Figure 2. Relationship between HIV-1 RNA (measured by reverse transcriptase–polymerase chain reaction amplification) and CD4 cell count during 24 weeks of monotherapy with (**A**) didanosine or (**B**) zidovudine. Data are median changes and interquartile ranges (left ordinate) and relative changes from baseline (right ordinate) for $\log_{10}$-transformed HIV-1 RNA and CD4 cell values.

Table 4. Adjusted relative hazard of clinical progression or death for 65 subjects with a change in HIV-1 RNA copy number at week 4 from baseline and other risk factors.

Variable	Adjusted relative hazard* (95% CI)	P
Baseline RNA copy number[†][‡]	1.26 (1.03–1.54)	.02
RNA copy number change at week 4[§]	0.73 (0.52–1.02)	.07
Baseline CD4 cell count[‡]	0.83 (0.66–1.03)	.08
Syncytium-inducing phenotype	2.73 (0.90–8.34)	.08
Diagnosis of AIDS at study entry	1.58 (0.73–3.42)	.25
Didanosine therapy	0.99 (0.41–2.39)	.97

NOTE. CI, confidence interval.

* Adjusted relative hazard of progression or death was determined by Cox proportional hazards model. Adjusted relative hazard is risk conferred by that variable after controlling for all other variables in table. Relative hazard for each variable is based on analysis of entire population for whom measures of each variable were available.

[†] HIV-1 RNA was measured by both reverse transcriptase–polymerase chain reaction (RT-PCR) assay (Roche) and branched DNA (bDNA) assay (Chiron); however, since 30 subjects were censored at baseline because of lower sensitivity for bDNA assay (<10,000 RNA equivalents/mL), only RT-PCR–derived RNA values are shown. Relative hazard values derived from both assays were not significantly different (data not shown).

[‡] Relative hazard associated with each doubling (0.3 $\log_{10}$ decrease) of RNA copy number or CD4 cell count at baseline.

[§] Adjusted relative hazard for clinical progression of 1.26 indicates 26% increased risk for each doubling (0.3 $\log_{10}$ increase) in baseline HIV-1 RNA level; similarly, adjusted relative hazard of 0.73 indicates 27% reduction in risk for each halving (0.3 $\log_{10}$ decrease) in HIV-1 RNA copy number following 4 weeks of therapy.

4. The addition of the change in plasma HIV-1 RNA level at week 4 from baseline into the Cox model strengthened the association between the baseline HIV-1 RNA level as an independent predictor of disease progression, whereby each 50% reduction in HIV-1 RNA copy number from baseline was associated with a 25% decrease in the unadjusted RH of disease progression ($n = 97$; unadjusted RH, 0.75; 95% CI, 0.59–0.96; $P = .02$), which approached statistical significance after adjustment for the other variables associated with disease progression ($n = 65$; RH, 0.73; 95% CI, 0.52–1.02; $P = .07$). The magnitude and direction of the RHs for the other predictors of disease progression (baseline HIV-1 RNA level, CD4 cell count, SI phenotype, diagnosis of AIDS at study entry, and therapy assignment to didanosine) remained unchanged from the previous regression model shown in table 3. The change in CD4 cell count at all time points assessed was not significantly associated with clinical outcome when either unadjusted or adjusted for baseline CD4 cell count and, therefore, was not included in the regression model (data not shown).

Discussion

The results from this study support the hypothesis that plasma HIV-1 RNA may be a surrogate marker for clinical outcome based on the following criteria: The baseline HIV-1 RNA level was an independent predictor of clinical progression, and a decrease in HIV-1 RNA by therapy was associated with clinical benefit. A decrease in plasma HIV-1 RNA was seen with didanosine but not with zidovudine therapy, and switching to didanosine was clinically beneficial to these subjects [20]. A third and essential criterion for strict surrogacy, that a change in HIV-1 RNA level should explain almost all of the treatment effect on disease progression, was not assessable with the current study analysis.

Disease progression was seen in all HIV-1 RNA stratification ranges for at least one of the two HIV-1 RNA detection assays, including 20% of subjects by bDNA and 60% of subjects by RT-PCR who were classified in the lowest category of ≤10,000 HIV-1 RNA Eq/mL. The unadjusted effect of having a 2-fold higher level of RNA at baseline was to increase the RH of disease progression by 23% (RH, 1.23; 95% CI, 1.07–1.42); this increased RH remained essentially unchanged after adjustment for several other virus and host parameters, including the HIV-1 RNA response to therapy (RH, 1.26; 95% CI, 1.03–1.54; $P = .02$). The observation that the baseline level of HIV-1 RNA was independently predictive of disease progression in our population with advanced HIV-1 infection complements and extends several studies that have shown long-term clinical survivors or slow progressors to have median plasma HIV-1 RNA levels of fewer than ~70,000 ($10^{4.85}$) RNA copies (or ~35,000 [$10^{4.54}$] RNA Eq)/mL [10, 13, 33–35]. The different estimates of HIV-1 RNA copy number associated with disease progression arise from the noncomparability of the RT-PCR and bDNA methods because of variability in specimen collection and storage and lack of a common HIV-1 RNA reference standard.

The 50% reduction in HIV-1 RNA copy number that followed 4 weeks of therapy was associated with a statistically significant 25% reduction in the risk of disease progression (unadjusted RH, 0.75; 95% CI, 0.59–0.96). Because of the limited number of subjects contributing complete data, this risk remained unchanged in magnitude but became less significant ($P = .07$) when adjusted for other virologic and immunologic factors that were independent variables for disease progression. Substantiating this association will require a larger sample size, clinical trials with more potent antiviral agents effecting a greater treatment response, or a meta-analysis of other clinical trials [36].

In an earlier study, the mutation at codon 215 alone did not predict an increased risk of disease progression, although the presence of mutations in both codons 41 and 215, as well as the phenotype of high-level zidovudine resistance, did increase the risk of disease progression [22]. The association between baseline HIV-1 RNA level and the mutation at codon 215 may indicate that the detection of this mutation is a reflection of viral replication [16, 37]. The detection of codon 215 mutation either alone or with codon 41 was significantly associated with increased virus load compared with the wild type codons (table 2). This finding is consistent with other data showing that higher virus load is associated with the codon 215 mutant virus in plasma [38].

There was no apparent association observed between the zidovudine susceptibility phenotype and HIV-1 RNA level. This lack of association between HIV-1 RNA level and the susceptibility phenotype may have been due to the use of a cutoff for susceptible isolates of $<0.2 \ \mu M$, which may have misclassified some isolates as susceptible when these isolates had the mutation at codon 215 [22]. Given the more extensive phenotypic and genotypic analysis in ACTG protocol 116B/117 reported elsewhere [21, 22], it would appear that the contributing factors necessary to define the different clinical response between the zidovudine and didanosine treatment arms comprised a combination of factors including the baseline HIV-1 RNA level and CD4 cell count, zidovudine susceptibility as assessed by either viral phenotype or genotype, the differential response in virus load between zidovudine and didanosine, and the effect of SI viral phenotype.

Our study complements the results of the Department of Veterans Affairs Cooperative Study 298A (VACS 298A), which showed that the baseline CD4 cell count and plasma HIV-1 RNA level were both predictive of disease progression and that a decrease in plasma HIV-1 RNA level, after 1 month of zidovudine therapy, was strongly correlated with improved clinical outcome [8]. The VACS 298A subjects were zidovudine-naive and asymptomatic and had higher median CD4 cell counts than did the subjects in ACTG protocol 116B/117. In contrast to the VACS 298A study, ours controlled for SI viral phenotype, which conferred a significant independent increased risk of death in the protocol 116B/117 study population [21, 22].

Other virologic and host factors (e.g., CD4 cell count, SI viral phenotype, and a diagnosis of AIDS at study entry) were also linked to clinical outcome and should be considered when evaluating the potential significance of a change or lack of change in plasma HIV-1 RNA following a modification in antiretroviral therapy. It is very unlikely that a single virologic (or immunologic) parameter will suffice for fully characterizing the response to antiretroviral therapy for either clinical trials or patient management in the clinical setting. When we modeled baseline plasma HIV-1 RNA copy number and change in plasma HIV-1 RNA copy number by therapy with other independent predictors of risk for disease progression, the diagnosis of AIDS at study entry was no longer a significant predictor of risk for disease progression, and the magnitude of the RH for treatment assignment approached unity (table 4). This suggested that a change in virus load in response to therapy explained, in part, the effect of treatment due to the switch from zidovudine to didanosine, even though we could not quantify the magnitude of this contribution.

Our study provides further support for the use of quantitative plasma HIV-1 RNA measurement in HIV-1 infection even though the study results must be viewed cautiously before making specific recommendations for the use of HIV-1 RNA measurement in routine clinical practice. Moreover, our analysis, together with the analysis of ACTG protocol 116A [39], VACS 298 [8], and a recent European study [40], provide

consistent results over a broad range of CD4 cell counts, arguing for the generalizability of our results to patients with <500 CD4 cells/μL.

There is now a pattern emerging to suggest that patients with high virus load are at increased risk for disease progression [9, 11–13, 35, 41]. Viral RNA levels $>50,000$ RNA copies/mL of plasma by RT-PCR ($\sim 25,000$ RNA Eq/mL by bDNA) are associated with both HIV-1 p24 antigen positivity and recovery of cell-free infectious virus from plasma (Coombs RW, unpublished data), both of which are associated with an increased risk for disease progression [19, 42–47].

The analysis comparing the unadjusted RH of disease progression for incremental increases in the HIV-1 RNA level suggested that persons with high viral RNA levels $>200,000$ ($10^{5.30}$) HIV-1 RNA copies/mL were at a 3-fold higher risk for disease progression than were those below this level. This HIV-1 RNA threshold for increased risk of disease progression is within the copy number range reported to distinguish rapid progressors from slower disease progressors [13] and suggests, moreover, that there was a nonlinear relationship between HIV-1 RNA level and RH of disease progression for our study subjects. Further attention should be given to defining specific virologic thresholds for increased risk of disease progression and, possibly, a response to therapy for different patient populations, particularly since the presence of such levels of virus may have clinical utility in the management of patients, much like CD4 cell thresholds exist for defining the increased risk of specific opportunistic infection. An accurate definition of these virus levels may be particularly helpful, since the HIV-1 RNA level that is established within 1 year after seroconversion may define the future risk of disease progression [12, 13, 35] and benefit from antiretroviral therapy given that current antiretroviral drugs have only limited activity [48].

There were several limitations to our study. First, the patients in our substudy were less advanced clinically than those reported in two other ACTG protocol 116B/117 virology substudies [21, 22]. Plasma was requested after the study had begun to accrue subjects; consequently, subjects who were enrolled first had isolates available for viral phenotyping while subjects enrolled later had only plasma available for HIV-1 RNA measurement. Thus, the subgroup in whom susceptibility phenotype and genotype was studied appeared more advanced clinically, as judged by median CD4 cell counts of 48/μL and 61/μL, respectively [21, 22].

Second, the plasma specimens were not collected for the optimal detection of HIV-1 RNA [49]. Heparin was used as an anticoagulant in accordance with specimen collection procedures in effect at the start of the 116B/117 protocol, and strict adherence to the rapid processing of plasma to stabilize the HIV-1 RNA was not done. Despite these limitations and reported differences in accuracy and reproducibility between the two assays [31], both the RT-PCR and bDNA measurements produced RH estimates that were indistinguishable. A formal comparison of the RT-PCR and bDNA data is in progress.

Third, our retrospective study did not have sufficient statistical power to thoroughly address all aspects required for the complete clinical validation of plasma HIV-1 RNA as a surrogate marker for clinical end point. Evaluating the proportion of the treatment effect attributable to the change in HIV-1 RNA level by therapy was problematic given the low power of our study and the corresponding instability in the estimate of the treatment effect. To assess further the marker surrogacy of HIV-1 RNA, studies with a stronger treatment effect than was seen in our study are required; with relatively small treatment effects it may be necessary to perform analysis across several studies to improve the sample size and standard error estimate of the treatment effect [36, 50]. Nevertheless, we have provided insight into the role of plasma HIV-1 RNA measurements in this specific population of subjects with advanced HIV-1 disease. The statistical analysis and results were consistent with a model of HIV-1 infection that is driven by viral replication, phenotype, and immunologic changes that eventually lead to immunosuppression and an increased risk of disease progression [1, 2, 10, 11, 51].

In summary, we have shown that the baseline level of HIV-1 replication, as measured by the plasma-associated HIV-1 RNA level, was independently associated with the risk of disease progression. A decrease in HIV-1 RNA level was seen only in the didanosine treatment group, which also had a better clinical response than did the zidovudine treatment group. The change in plasma HIV-1 RNA by antiretroviral therapy approached statistical significance as an independent predictor for the risk of disease progression. When these two parameters were modeled with other independent predictors of disease progression, the treatment effect due to switching from zidovudine to didanosine was more fully but not completely explained. However, other factors in addition to HIV-1 RNA level must be sought to explain the full clinical benefit observed after a switch from zidovudine to didanosine in this population of patients with advanced HIV-1 infection.

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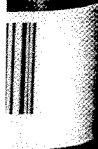
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