Sequence Clusters in Human Immunodeficiency Virus Type 1 Reverse Transcriptase Are Associated with Subsequent Virological Response to Antiretroviral Therapy

Andrew J. Leigh Brown,[1] Huldrych F. Günthard,[2,a] Joseph K. Wong,[2] Richard T. D'Aquila,[3] Victoria A. Johnson,[4] Daniel R. Kuritzkes,[5] and Douglas D. Richman[2]

[1]Centre for HIV Research, Institute of Cell, Animal and Population Biology, University of Edinburgh, Scotland; [2]Departments of Pathology and Medicine, University of California San Diego and San Diego Veterans Affairs Medical Center, La Jolla; [3]Massachusetts General Hospital and Harvard Medical School, Boston; [4]Department of Infectious Diseases, University of Alabama at Birmingham, and Birmingham Veterans Affairs Medical Center; [5]Division of Infectious Diseases, University of Colorado Health Sciences Center and Veterans Affairs Medical Center, Denver

Many amino acid (aa) sites in reverse transcriptase (RT) have been implicated in resistance to nucleoside (NRTI) and nonnucleoside antiretrovirals. Interactions between these in response to combination therapy remain poorly understood. In a trial (ACTG 241) of zidovudine/didanosine (ddI) versus zidovudine/ddI/nevirapine in nucleoside-experienced patients, baseline sequence data from the RT coding region was analyzed from 55 individuals. Sequences were clustered by use of a parsimony method and the virological responses (ratio of baseline viral load to viral load after of therapy) for each cluster were analyzed at week 8 and week 48. Both clusters and genotype at aa 215 were significantly associated with virological response at both time points, whereas viral load showed a stronger association with sequence clusters. Sequence clusters identified one group of patients who never developed high-level resistance to NRTIs despite prior nucleoside exposure and poor suppression of viral replication.

Antiretroviral drug therapy often selects for mutants in the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) with quantifiable phenotypic changes in the drug sensitivity. That single amino acid (aa) substitutions are sufficient to cause high-level resistance to the nucleoside analogue 3TC [1, 2] and to nonnucleoside inhibitors [3, 4] was first observed in vitro and subsequently detected in vivo [5, 6] by inspection of sequences obtained from patients failing monotherapy and verified by in vitro mutagenesis. In contrast, the acquisition of high-level resistance to zidovudine involves several changes in RT including T215Y/F [7, 8], whereas such resistance to the protease (PR) inhibitors indinavir and ritonavir requires at least 3–4 changes at up to 11 aa sites in PR [9–12]. Under these circumstances, where many sites may be involved and the contribution of individual sites is small, the identification of aa sites contributing to resistance becomes difficult. In some cases, it has been possible to identify individual sites by use of regression methods [11], but this approach assumes that individual sites act independently, which is not always the case [13].

Resistance to combinations of antiretrovirals is also characterized by changes at multiple aa sites, and as more complex regimens become widely adopted, the identification and analysis of changes contributing to these phenotypes is increasingly important. There are already examples of aa changes whose contribution to resistance is dependent on the presence of specific mutations at other sites, such as L210W in RT [14]. Other mutations in RT have been shown to interact to reduce the level of resistance (T215Y and M184V) [15]. In protease, M46I, which is often observed with the active site mutation V82T, contributes to resistance by counteracting the detrimental effects of the latter on catalytic efficiency [16]. These interactions have been detected on an individual basis; there is no general method in use for simultaneous analysis of multiple sites,

Received 8 February 1999; revised 27 May 1999; electronically published 8 September 1999.

Presented in part: 2d International Workshop on HIV Drug Resistance and Treatment Strategies, Lago Maggiore, Italy, 24–27 June 1998 (abstract 50).

Financial support: National Institutes of Health grants TW00767 (Fogarty Center), K 11 AI01361, AI 27670, AI 38858 AI 36214 (Center for AIDS Research), AI 29164, AI 32775, 96VC001, AI 40876, and AI 27767 (Center for AIDS Research); Research Center for AIDS and HIV Infection of the San Diego Veterans Affairs Medical Center and Core Laboratory Research Facilities of University of Alabama at Birmingham School of Medicine, University of Alabama at Birmingham Center for AIDS Research, and Birmingham Veterans Affairs Medical Center; and grant 84 AD-046176, Swiss National Science Foundation (H.G.).

[a] Present affiliation: Department of Internal Medicine, Division of Infectious Diseases, University Hospital Zurich, Zurich, Switzerland (H.G.).

Reprints or correspondence: Prof. Andrew J. Leigh Brown, Centre for HIV Research, University of Edinburgh, Waddington Building, West Mains Rd., Edinburgh EH9 3JN, Scotland (A.Leigh-Brown@ed.ac.uk).

The Journal of Infectious Diseases 1999; 180:1043–9

0022-1899/1999/18004-0015$02.00

though in other areas techniques have been introduced for the analysis of quantitative traits by use of molecular genetic data [17, 18].

We wished to determine the extent to which RT genotype at initiation of a particular course of antiretroviral therapy determined the response. To investigate this we analyzed sequence data obtained in the virology substudy of ACTG 241, a randomized, double-blind, placebo-controlled trial of zidovudine/ddI versus zidovudine/ddI/nevirapine, in 398 zidovudine-experienced patients with a $CD4^+$ cell count of $\leqslant$350 [19]. All patients had extensive prior zidovudine monotherapy and most also had additional experience with other nucleosides, as monotherapy or in combination with zidovudine. Triple therapy was shown to confer an 18% higher CD4 cell count and a 0.25 $\log_{10}$ lower plasma viral load than dual nucleoside therapy after 48 weeks in this trial. Patients enrolled at half of the trial sites (4 sites) underwent virological analysis, and sequences of RT were obtained from a selected subset of this group at 3 time points: baseline, week 8, and week 48 [20]. We have found that variation in response to therapy and in viral load is significantly related to baseline sequence and that joint analysis of complete sequence data is more informative than analysis of the key zidovudine-associated aa at position 215 taken on its own.

Materials and Methods

Sequence analysis. A 1.2-kb DNA fragment of HIV-1 *pol* was amplified by polymerase chain reaction (PCR) from culture lysates using conditions and primers described elsewhere [21, 22]. Sequences were obtained by use of high-density oligonucleotide arrays. Amplified *pol* gene sequences were labeled with fluorescein-rUTP (Boehringer Mannheim, Lewes, U.K.) with T3 RNA polymerase (Promega, Madison, WI). RNA fragments were hybridized to the "PRT 440" sense and antisense chips (Affymetrix, Santa Clara, CA), and the chip was scanned by a confocal laser microscope. A composite sequence of the *pol* gene (1023 bp) was generated by integrating the sense and the antisense chip data by use of the "rule algorithm" (GeneChip 2.0 software, Affymetrix). This includes the entire protease region (codons 1–99) and 726 bp of the RT region (codons 1–242). The baseline sequences from 55 patients were exported as text files from the GeneChip software and aligned by use of the Genetic Data Environment multiple sequence editor (GDE; Harvarad Genome Laboratory, Cambridge, MA). All subsequent analyses were performed on translated aa sequences of RT. Parsimony clustering was performed by use of 2 programs: PROTPARS, which identifies the most parsimonious topologies, and CONSENSE, which identifies clusters found in the majority of the 50 such topologies obtained (PHYLIP package version 3.5c; J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA) [23]. MACCLADE (Affymetrix) was used to display the individual aa distribution within the clusters. In this data set, most aa variants were acquired independently and in parallel in each patient because of the selection pressure exerted by antiretroviral drugs; therefore, PROTPARS was not used to reconstruct phylogenetic relationships, but to group the viral sequences on the basis of shared aa's at these sites and to choose the groups that minimized the overall number of steps. The Genbank accession numbers are AF 156033–AF 156087.

Viral quantitation. HIV plasma viral RNA was quantified with the Amplicor Monitor assay (Roche Molecular Systems, Branchburg, NJ). Virological response was defined as the ratio of the baseline viral load to the viral load at week 8 or week 48. Statistical analysis was performed on transformed ($\log_{10}$) values by use of SPSS (version 6.1.3 for Windows, SPSS, Inc., Chicago, IL).

Results

Baseline sequence variation: aa at residue 215. Sequences of the RT coding region between aa 1 and aa 241 were obtained from isolates established from baseline samples provided by 55 patients. Plasma viral RNA data were available for all 55 patients at week 0, 54 at week 8, and 50 at week 48. The virological response was measured as the $\log_{10}$ of the ratio of plasma viral RNA at baseline to that at week 8 (or 48) (table 1). Substantial diversity was detected in the aa sequences obtained from baseline samples. At more than 35 positions a variant aa was observed in at least 5 patients. The presence of aa's known to confer resistance to antiretroviral drugs was recorded. Thus, of these 55, 31 had R at position 70; at residue 215, 32 had Y and 9 had F; at residue 41, 24 had L. In view of the key role of T215Y/F in conferring resistance to zidovudine, which all patients received, we analyzed viral load data in terms of aa at this position in the baseline sequences. There were significant differences, with individuals carrying 215T (wild type) having a lower plasma HIV RNA at week 8 and week 48, but not at baseline (table 2). Analyzing the virological response to treatment revealed highly significant differences in the median value for week 8, with the value for the 215T being 5-fold higher than those for 215F or 215Y. The difference was much smaller at week 48, but was still significant.

Baseline sequence variation: sequence clusters. In view of the extensive variation in baseline sequences at sites other than 215 which are known to be important for antiretroviral resistance, a technique was adopted that would include information on all variable sites, because we felt that incorporation of multiple sites to the classification could enhance the prediction of response. Accordingly, patients were clustered on the basis of shared acquired aa changes at variable sites in RT, adopting a maximum parsimony approach to minimize the overall diversity among groups. The procedure yielded 3 primary groups (figure 1). These groups overlap with, but are not identical to, those obtained from aa at 215, since most patients in group 1 are 215T, patients in group 2 are 215F (8/13) or 215Y (5/13), and almost all in group 3 are 215Y (27/28). However, in addition, most patients in groups 1 and 3 were 219K, whereas group 2 was 219Q, and groups 1 and 2 were 41M and 70R, with group 3 being predominantly 41L and 70K. Because group

Table 1. Virological data for 55 patients in ACTG 241.

Patient no.	Group	Log_{10} RNA at week no.				
		0	8	48	0/8	0/4
1	1	5.27	3.99	4.45	1.2752	.8172
2	1	4.02	2.53	2.45	1.4856	1.5699
3	1	4.05	.00	2.82	4.0515	1.2320
4	1	4.86	5.06	4.96	−.2009	−.0999
5	1	4.21	3.24	NA	.9777	NA
6	1	4.12	.00	3.30	4.1153	.8142
7	1	3.72	2.64	2.94	1.0759	.7748
8	1	4.99	4.35	4.65	.6396	.3390
9	1	3.67	2.92	3.68	.7422	−.0147
10	1	3.15	3.26	4.07	−.1091	−.9287
11	1	5.13	4.79	5.02	.3435	.1196
12	1	4.63	.00	4.68	4.6314	−.0525
13	1	3.51	2.83	3.20	.6780	.3064
14	1	4.58	.00	3.84	4.5784	.7408
15	2	5.53	5.22	NA	.3086	NA
16	2	4.08	3.49	3.84	.5843	.2434
17	2	5.48	5.14	NA	.3410	NA
18	2	6.39	5.85	6.19	.5356	.1983
19	2	5.51	5.20	5.76	.3077	−.2579
20	2	4.85	4.79	4.98	.0530	−.1292
21	2	5.57	5.52	5.57	.0513	.0028
22	2	3.52	4.11	4.58	−.5961	−1.0602
23	2	4.91	4.69	4.74	.2155	.1634
24	2	4.20	4.53	4.71	−.3291	−.5174
25	2	4.38	3.77	4.72	.6039	−.3458
26	2	5.24	2.85	5.57	2.3968	−.3241
27	2	5.66	4.80	4.87	.8601	.7907
28	3	5.36	5.05	NA	.3045	NA
29	3	6.05	NA	6.04	NA	.0096
30	3	4.62	4.24	4.52	.3800	.1010
31	3	6.44	4.70	6.22	1.7449	.2279
32	3	4.48	3.73	5.34	.7513	−.8608
33	3	5.16	5.49	5.66	−.3254	−.4953
34	3	4.90	5.48	4.60	−.5841	.2949
35	3	4.98	5.14	4.73	−.1661	.2519
36	3	5.36	5.33	5.23	.0292	.1231
37	3	3.67	5.24	5.48	−1.5705	−1.8060
38	3	5.29	5.02	5.19	.2709	.1009
39	3	4.28	5.06	4.79	−.7838	−.5098
40	3	5.26	5.43	NA	−.1737	NA
41	3	4.84	4.88	5.22	−.0404	−.3880
42	3	4.98	4.78	5.19	.2015	−.2080
43	3	4.74	4.06	3.78	.6792	.9616
44	3	4.83	4.87	5.19	−.0454	−.3612
45	3	5.97	5.47	5.72	.5041	.2511
46	3	5.04	4.75	4.19	.2937	.8505
47	3	3.75	2.51	4.07	1.2446	−.3177
48	3	4.84	5.48	5.62	−.6396	−.7793
49	3	4.73	5.50	5.84	−.7636	−1.1037
50	3	5.43	5.36	4.01	.0757	1.4216
51	3	3.21	2.60	3.21	.6128	.0000
52	3	4.90	5.06	4.71	−.1660	.1880
53	3	4.74	4.13	4.07	.6121	.6743
54	3	4.37	3.86	5.18	.5148	−.8041
55	3	4.36	4.65	5.52	−.2966	−1.1687

NOTE. Patient numbers and sequence clusters are as shown in figure 1. Plasma viral RNA (log_{10}) and ratios are given for week 0, week 8, and week 48 time points for all patients where these were available. NA, not available.

2 was 41M and 70R, whereas group 3 was not, five 215Y sequences were allocated to group 2. Thus, the inclusion of data from all variable sites leads to a new configuration of patient groups for analysis.

Associations between sites. To explore the interdependence of variation at multiple sites further, a test for independence between all variable sites was performed by using the mutual information statistic described by Korber et al. [24]. Three pairs of aa sites were found to be significantly associated ($P < .05$): 41 and 215; 41 and 70; and 210 and 211. In addition, associations between 70 and 215, 70 and 219, and 210 and 215 approached significance ($.05 < P < .1$; table 3). The identities of the associated aa's at positions 41, 70, 215, and 219 have been described elsewhere. It is interesting to note that in groups 1 and 2, only leucine was observed at position 210—all 13 patients with tryptophan and 2 with glycine were members of group 3. Thus, the statistical analysis of covariation confirms the validity of the sequence groups defined above.

Response to therapy. Sequence groups were analyzed for variation in plasma HIV RNA and in virological response to therapy, as described above. Group 1 patients had significantly lower viral load at all time points, but particularly at week 8 and week 48 (table 4). In addition, the ratios of the week 0 to week 8 viral loads revealed substantial, highly significant, differences among groups (table 4; $F[df = 2] = 10.9$, $P < .0001$). Thus, at week 8, group 1 responded significantly better to therapy than the other 2 groups with a median of 10.7 compared with 2.0 and 1.2 for groups 2 and 3, respectively. Again, the difference in the ratios at week 48 was smaller, but still significant ($F[df = 2] = 3.45$, $P < .05$).

The analysis of variance revealed that sequence groups explained more of the total variance in plasma HIV RNA among patients than do the groupings based solely on aa at 215. At week 0, the clusters explained 15% of the variance, whereas position 215 only explained 8%. At week 8, the corresponding figures were 26% and 17%, whereas at week 48 they were 32% and 22%. However, the difference between aa 215 groups and baseline sequence clusters was less marked for week 0/week 8 viral load ratios: the proportions of variance explained were 30% and 27%, respectively, and there was no difference at all in the week 0/week 48 ratio, both explaining 14%.

Relation to changes at resistance-associated sites. To de-

Table 2. Association of codon 215 amino acid at baseline with viral load and virological response in ACTG 241.

Week	*n*	215 T	215 F	215 Y	*P*[a]
Viral load					
0	54[b]	3.8×10^4	1.9×10^5	7.0×10^4	NS
8	53	6.8×10^2	1.5×10^5	6.2×10^4	.01
48	50	1.2×10^4	3.2×10^5	9.5×10^4	.01
Virological response					
0/8	53	11.9	2.0	1.7	.01
0/48	50	2.2	1.0	1.0	.05

NOTE. Median values are presented, together with sample sizes. NS, not significant.

[a] Significance levels in a one-way analysis of variance performed on transformed data.

[b] One patient with valine at 215 omitted.

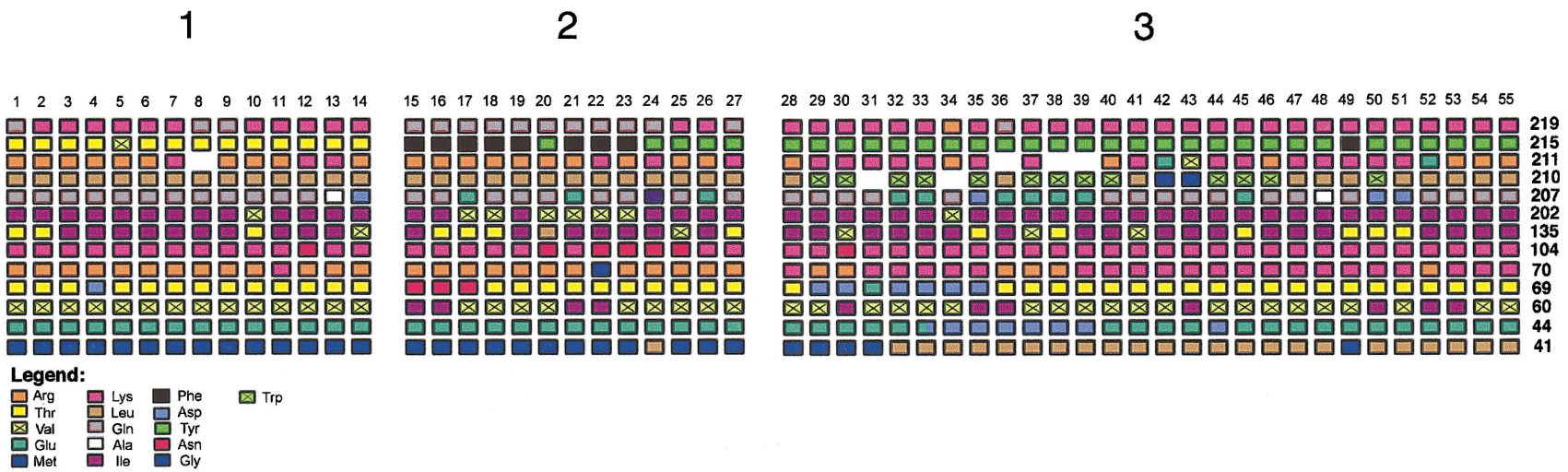

Figure 1. Reverse transcriptase (RT) sequence clusters at baseline. Figure shows amino acids at 13 variable sites (rows) in the RT domain sequences of human immunodeficiency virus type 1 isolates established at baseline from 55 patients (columns) entered into ACTG 241. Patient no. (table 1) is given above each column and 3 clusters identified on basis of shared amino acids are indicated. Color code used for amino acids is indicated in legend.

Table 3. Associations between amino acid sites detected by mutual information [24].

Amino acid site *a*	Amino acid site *b*	Mutual information	P[a]
41	215	.516	<.01
41	70	.350	<.02
210	211	.345	<.05
70	215	.303	<.07
70	219	.300	<.08
210	215	.293	<.1

[a] Probability of obtaining this value by chance, estimated from bootstrap resampling.

termine whether the differences among sequence groups were related to the appearance of genotypic resistance, we examined the distribution of resistance-associated mutations observed at week 48 among the groups established by the baseline sequences. There was little change in the distribution of nucleoside resistance-associated aa's, with the exception of position 215. Of 10 group 1 patients for whom sequences were available at both time points, 3 had acquired the T215Y and 2 the T215F mutations. In group 2, there were, surprisingly, 3 changes from 215Y to 215F of 5 patients, and one 215F to 215T of 8. At position 41, 2 patients of 9 in group 1, sequenced on both occasions, acquired 41L, as did 2 of 12 in group 2.

With regard to the distribution of known nonnucleoside antiretroviral (NNRTI) resistance-associated mutations at week 48, aa positions 101, 103, 106, 181, and 190 were tested for association with baseline groups. When treatment assignment was taken into account, there was only 1 significant difference in the distribution of any of these mutations among sequence. The NNRTI resistance-associated mutation 103N was more commonly found in group 1 patients, and this remained significant when treatment effects were removed (see below).

Analysis by treatment arm. Allocation to treatment arms differed significantly among sequence groups. Group 1, which maintained the greatest suppression of viral replication, had the highest proportion of patients (50%) on the dual-nucleoside arm. The number assigned to arm A was the same in groups 2 and 3 (table 5). The distribution of prior nucleoside therapy across groups is even more striking, with 12 of 14 patients in group 1 having received only zidovudine (and no other nucleoside) before entering the clinical trial. The proportions in groups 2 and 3 with only prior zidovudine were 6/13 and 6/28, respectively. Because group 1 individuals had a lower plasma viral load (table 2), they were less likely to have had to switch from zidovudine monotherapy to other nucleosides. This good response is maintained despite half the patients in the group receiving the less effective therapy during the trial.

Synonymous nucleotide changes appear at different rates in the sequence groups. We have analyzed [22] the relationship between virological response in ACTG 241 and nucleotide distance in RT from baseline to week 8. We have performed the same analysis broken down by the 3 sequence groups defined here and find that there are significant differences between them in the within-patient synonymous, but not nonsynonymous, evolutionary distance from baseline sequence to week 8 on therapy (table 6; $P < .05$). Group 1 shows a significantly higher evolutionary distance in synonymous sites between baseline and week 8 than do the other 2 groups; that is, patients with the strongest response to therapy on the basis of decrease in plasma RNA at week 8 show greater evolutionary change from baseline to week 8 in synonymous sites than do patients who respond less well.

Discussion

Although individual mutations in the RT coding sequence of HIV responsible for a reduction in susceptibility to RT inhibitors are well known, a detailed understanding of viral factors affecting response to combination therapy is a more demanding objective. Statements can be made concerning the probable outcome of zidovudine monotherapy on the basis of the presence or absence of mutations at positions 215 and 41 [7]. In an analysis of isolates obtained during ACTG 116B/117 trials that compared continuing zidovudine with ddI in zidovudine-experienced individuals, Japour et al. [25] showed that the presence of mutations at positions 215 and 41 was predictive of clinical progression, conferring an adjusted relative hazard of 1.6, though the benefit of a switch to didanosine was independent of mutations at these sites. Thus, the presence of zidovudine-resistant mutations at baseline appeared to constitute a risk factor for progression for patients receiving ddI on trial as well as for those receiving zidovudine.

In combination therapy it is not yet clear what information is required to predict a response. We analyzed whether baseline sequences can be informative in predicting outcome to therapy by using 2 approaches. First, we tested the predictive capacity of genotype at individual aa sites, including 215. Second, we applied a novel method based on parsimony clusters of aa sequences. We found that both were predictive in this case. It is possible to compare the performance of the methods on the basis of the proportion of variance in virological response (ratio of baseline viral load to viral load at week 8 or week 48 of therapy) that is explained. For aa 215 this was about 30% for response at week 8, but not more than 14% in case of week 48 response. The parsimony clusters explained a similar propor-

Table 4. Association of baseline sequence cluster with viral load and virological response in ACTG 241.

Week	*n*	Group 1	Group 2	Group 3	P[a]
Viral load					
0	55[b]	1.5×10^4	1.7×10^5	7.4×10^4	.05
8	54	7.6×10^2	6.2×10^4	1.1×10^5	.001
48	50	6.9×10^3	7.4×10^4	1.5×10^5	.001
Virological response					
0/8	54	10.7	2.0	1.2	.001
0/48	50	2.2	.7	1.0	.05

[a] Analysis of variance significance levels.
[b] One patient with valine at 215 omitted.

Table 5. Treatment arm and prior exposure by sequence group.

	Arm		Prior therapy	
Sequence group	A	B	ZDV	ZDV+
Cluster 1	7		6	1
		7	6	1
Cluster 2	4		3	1
		9	3	6
Cluster 3	4		3	1
		24	3	20[a]

NOTE. Treatment: arm A, ZDV + ddI; arm B, ZDV + ddI + nevirapine. Prior exposure: ZDV, zidovudine alone; ZDV+, zidovudine + one other nucleoside.

[a] Includes +1 patient with no prior treatment.

tion of the variation. When viral load itself was examined, baseline sequence clusters explained a higher proportion of the variation in viral load during therapy than baseline genotype at 215 alone. We conclude that the response to the therapy received in this trial at week 8 could be adequately predicted on the basis of 215 genotype alone, but that overall virological status was better explained when sequence data from multiple sites were included.

The observations on viral load are in line with results obtained in an earlier study of 198 patients, which identified high plasma load at baseline as a risk factor for disease progression [20]. We also found that patients performing better virologically included a relatively high proportion of individuals on the less effective therapy. The differences in response of these patients is therefore independent of treatment. However, most of these individuals had only been treated with one, rather than 2 (or more) nucleosides prior to entry into the trial. Taken together, this suggests that these individuals represent a subset of individuals whose viral load was maintained at a low level both before and during this trial, independent of treatment. One possible reason might be heterozygosity for the 32-bp deletion in the CCR5 chemokine receptor gene, which has been shown to reduce the rate of progression in HIV-infected patients [26, 27]. There was no evidence for association of CCR5 heterozygotes with any group. Of the 5 detected among the patients analyzed here (49 were among those scored previously for CCR5 [28]), 2 were found in group 1, and 3 in group 3. In addition, these individuals did not appear to be slow progressors, because all of them possessed virus of an syncytium-inducing phenotype.

Table 6. Evolutionary distance from baseline sequence to week 8 and week 48 sequences [22].

	Synonymous (%)		Nonsynonymous (%)		Overall (%)	
Sequence group	Week 8	Week 48	Week 8	Week 48	Week 8	Week 48
Cluster 1	4.7	3.8	0.88	0.9	2.4	2.3
Cluster 2	2.6	3.0	0.54	0.71	1.6	1.8
Cluster 3	2.7	2.0	0.54	1.1	1.7	1.6

NOTE. Overall median genetic distances are shown for each baseline sequence group, together with those for synonymous and nonsynonymous sites.

It is noteworthy that few of the patients in group 1 subsequently acquired M41L during study treatment, despite continuing viral replication (table 1). One hypothesis that could account for these observations and the greater genetic difference observed between week 0 and week 8 virus in group 1 (table 5) invokes compartmentalized replication of HIV during study treatment. The apparently rapid genetic divergence at synonymous sites could reflect the prior differentiation of a viral subpopulation months or years earlier, which evolved in the absence of drug selection. It has been shown that viral populations in the brain, a potential sanctuary from drug exposure, can be genetically distinct [29, 30] and would also be more likely to remain drug-susceptible. Other anatomic privileged sites have been hypothesized, including the testis; recently, it has been suggested that such compartments could be significant in the evolution of resistance [31]. Compartmentalization within the same organ is also possible, given the observations that replication-competent provirus in resting memory $CD4^+$ T lymphocytes remains drug-susceptible. If there is some level of isolation between the viral populations in such compartments even between individual white pulps within the same spleen, as has been described elsewhere [32], continuous gradual genetic divergence would be expected at synonymous sites. Thus, the virus that was detected at week 8 in group 1 patients might represent spillover from such compartments into the periphery, giving rise to the drug-susceptible viruses isolated therein. Closer characterization of the release of this persistently sensitive, genetically distinct virus will be important for predictions relating to HIV eradication through potent antiretroviral therapy.

Acknowledgments

We thank Jacques Corbeil for his help in the production of figure 1 and George Hanna for careful reading of the manuscript.

References

1. Boucher CA, Cammack N, Schipper P, et al. High-level resistance to (-) enantiomeric 2'-deoxy-3'-thiacytidine is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother **1993**;37:2231–4.
2. Tisdale M, Kemp SD, Parry NR, Larder BA. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc Natl Acad Sci USA **1993**;90:5653–6.
3. Nunberg JH, Schleif WA, Boots EJ, et al. Viral resistance to human immunodeficiency virus type 1–specific pyridinone reverse transcriptase inhibitors. J Virol **1991**;65:4887–92.
4. Richman D, Shih CK, Lowy I, et al. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc Natl Acad Sci USA **1991**;88:11241–5.
5. Schuurman R, Nijhuis M, van Leeuwen R, et al. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J Infect Dis **1995**;171:1411–9.
6. Richman DD, Havlir D, Corbeil J, et al. Nevirapine resistance mutations of

human immunodeficiency virus type 1 selected during therapy. J Virol **1994**; 68:1660–6.

7. Kellam P, Boucher CA, Tijnagel JM, Larder BA. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. J Gen Virol **1994**; 75:341–51.
8. Richman DD, Grimes JM, Lagakos SW. Effect of stage of disease and drug dose on zidovudine susceptibilities of isolates of human immunodeficiency virus. J Acquir Immune Defic Syndr **1990**; 3:743–6.
9. Condra JH, Schleif WA, Blahy OM, et al. Dynamics of acquired HIV-1 clinical resistance to the protease inhibitor MK-639. J Acquir Immune Defic Syndr **1995**; 10:72.
10. Condra JH, Schleif WA, Blahy OM, et al. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **1995**; 374:569–71.
11. Condra JH, Holder DJ, Schleif WA, et al. Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. J Virol **1996**; 70:8270–6.
12. Molla A, Korneyeva M, Gao Q, et al. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat Med **1996**; 2:760–6.
13. Leigh Brown AJ, Korber BT, Condra JH. Associations between amino acids in the evolution of HIV-1 protease sequences under indinavir therapy. AIDS Res Hum Retroviruses **1998**; 15:247–53.
14. Harrigan PR, Kinghorn I, Bloor S, et al. Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. J Virol **1996**; 70:5930–4.
15. Larder BA, Kemp SD, Harrigan PR. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **1995**; 269:696–9.
16. Schock HB, Garsky VM, Kuo LC. Mutational anatomy of an HIV-1 protease variant conferring cross-resistance to protease inhibitors in clinical trials. Compensatory modulations of binding and activity. J Biol Chem **1996**; 271:31957–63.
17. Templeton AR, Boerwinkle E, Sing CF. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in Drosophila. Genetics **1987**; 117:343–51.
18. Templeton AR. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping or DNA sequencing. V. Analysis of case/control sampling designs: Alzheimer's disease and the Apoprotein E locus. Genetics **1995**; 140:403–9.
19. D'Aquila RT, Hughes MD, Johnson VA, et al. Nevirapine, zidovudine and didanosine compared with zidovudine and didanosine in patients with HIV-1 infection: a randomized, double-blind, placebo-controlled trial. Ann Intern Med **1996**; 124:1019–30.
20. Hughes MD, Johnson VA, Hirsch MS, et al. Monitoring plasma HIV-1 RNA levels in addition to $CD4^+$ lymphocyte count improves assessment of antiretroviral therapeutic response. Ann Intern Med **1997**; 126:929–38.
21. Günthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD. Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. AIDS Res Hum Retroviruses **1998**; 14:869–76.
22. Günthard HF, Wong JK, Leigh Brown AJ, et al. Higher selection pressure from antiretroviral drugs in vivo results in increased evolutionary distance. Virology (in press).
23. Felsenstein J. PHYLIP-phylogeny inference package (version 3.2). Cladistics **1989**; 5:164–6.
24. Korber BT, Farber RM, Wolpert DH, Lapedes AS. Covariation of mutations in the V3 loop of human immunodeficiency virus type 1 envelope protein: an information theoretic analysis. Proc Natl Acad Sci USA **1993**; 90: 7176–80.
25. Japour AJ, Welles S, D'Aquila RT, et al. Prevalence and clinical significance of zidovudine resistance mutations in human immunodeficiency virus isolated from patients after long-term zidovudine treatment. J Infect Dis **1995**; 171:1172–9.
26. Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Science **1996**; 273:1856–62.
27. Smith MW, Dean M, Carrington M, et al. Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Science **1997**; 277:959–64.
28. D'Aquila RT, Sutton L, Savara A, Hughes MD, Johnson VA, ACTG 241 Virology Team. CCR5/delta(ccr5) heterozygosity: a selective pressure for the syncytium-inducing human immunodeficiency virus type 1 phenotype. J Infect Dis **1998**; 177:1549–53.
29. Hughes ES, Bell JE, Simmonds P. Investigation of the dynamics of the spread of human immunodeficiency virus to brain and other tissues by evolutionary analysis of sequences from the p17gag and env genes. J Virol **1997**; 71:1272–80.
30. Wong JK, Ignacio CC, Torriani F, Havlir D, Fitch NJ, Richman DD. In vivo compartmentalization of human immunodeficiency virus: evidence from the examination of pol sequences from autopsy tissues. J Virol **1997**; 71:2059–71.
31. Kepler TB, Perelson AS. Drug concentration heterogeneity facilitates the evolution of drug resistance. Proc Natl Acad Sci USA **1998**; 95:11514–9.
32. Cheynier R, Henrichwark S, Hadida F, et al. HIV and T cell expansion in splenic white pulps is accompanied by infiltration of HIV-specific cytotoxic T lymphocytes. Cell **1994**; 78:373–87.