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Reduced Susceptibility of Human Immunodeficiency Virus Type 1 (HIV-1) from Patients with Primary HIV Infection to Nonnucleoside Reverse Transcriptase Inhibitors Is Associated with Variation at Novel Amino Acid Sites

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Recently, significant numbers of individuals with primary human immunodeficiency virus (HIV) infection have been found to harbor viral strains with reduced susceptibility to antiretroviral drugs. In one study, HIV from 16% of such antiretroviral-naive individuals was shown to have a susceptibility to nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) between 2.5- and 10-fold lower than that of a wild-type control. Mutations in the RT domain that had previously been associated with antiretroviral resistance were not shared by these strains. We have analyzed by logistic regression 46 variable amino acid sites in RT for their effect on susceptibility and have identified two novel sites influencing susceptibility to NNRTIs: amino acids 135 and 283 in RT. Eight different combinations of amino acids at these sites were observed among these patients. These combinations showed a 14-fold range in mean susceptibility to both nevirapine and delavirdine. In vitro mutagenesis of the control strain combined with a phenotypic assay confirmed the significance of amino acid variation at these sites for susceptibility to NNRTIs.

Since the first reports of sexual transmission of antiretroviral-resistant strains of human immunodeficiency virus (HIV) (1, 3, 5), there has been concern over the extent to which the possibility of infection with drug-resistant strains might prejudice the successful treatment of HIV-infected individuals. Studies of transmission occurring when zidovudine (ZDV) monotherapy was the predominant treatment indicated that mutations at amino acid sites associated with ZDV resistance were present in up to 10% of untreated individuals (16, 19). Antiretroviral-naive HIV-infected individuals with mutations associated with reduced susceptibility to nonnucleoside reverse transcriptase (RT) inhibitors (NNRTI) and protease (PR) inhibitors have also been reported (8, 10).

The introduction of potent antiretroviral therapy has had a major impact on the morbidity and mortality associated with HIV disease (15), but the efficacy of combination therapy is impaired if strains resistant to the component drugs are present (14). Recent studies of the prevalence of reduced susceptibility to antiretrovirals among patients with primary HIV infection have addressed the question in patients infected since combination therapy became the standard of care (21, 26). The studies of Boden et al. (2) and Little et al. (13) analyzed 80 and 141 patients, respectively, all infected in major cities in the United States. These studies revealed that while strains with large reductions in susceptibility were infrequent in primary infection (~3%), strains of intermediate susceptibility were more common. In particular, Little et al. (13) found, using a highly reproducible phenotypic assay, that 16% of subjects were infected with strains showing susceptibility to NNRTI between 2.5- and 10-fold lower than that of the wild-type control (NL4-3). Surprisingly, only in one case was this phenotypic difference associated with a mutation at a site previously associated with drug resistance.

At least two possible explanations for the appearance of moderate reductions of susceptibility in significant numbers of individuals in this study can be proposed. According to the first, suggested by the association of many of these individuals with a subset of the clinics participating in this study, a proportion of these individuals are infected with phylogenetically related viral strains through previously unknown transmission networks, and these strains share reduced susceptibility as a result of their common ancestry. Under the second hypothesis, the high prevalence of reduced susceptibility is due to polymorphic variants at amino acid sites which have not previously been associated with antiretroviral resistance, acting either alone or in combination. We have assessed the merits of the first of these two alternatives by performing a phylogenetic analysis of the nucleotide sequences of the viral RT and PR

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coding regions of the *pol* gene, and we have investigated the second by a logistic regression analysis of the association between variation at polymorphic amino acid sites and phenotype for each drug. The former analysis did not support the possibility that strains with reduced susceptibility to NNRTIs shared a common infection source. The latter indicated that novel sites, not previously linked with resistance to these antiretrovirals, were significantly associated with reduced drug susceptibility. The mutations identified were then tested by in vitro mutagenesis studies, which confirmed both their identity and the scale of effect.

MATERIALS AND METHODS

Patient characteristics. Sequence data were determined for the RT and PR coding regions from plasma samples collected from a subset of 110 patients from the original cohort of 141 subjects (13). All subjects signed an informed consent for study participation which had been approved by the local Institutional Human Subjects Committee. Patients with clinical or laboratory evidence of primary HIV infection were referred to participating study centers. Documentation of HIV seroconversion was available for 71% of the original 141-patient cohort. Primary HIV infection was presumed in the remaining subjects based on documentation of a positive HIV serology following an acute retroviral illness and a negative HIV antibody test (undocumented) during the previous 12 months. Study participants were predominantly men who reported a history of sex with men; they were enrolled between 1989 and 1998, though only 19 (17%) were identified prior to 1996. A baseline plasma sample was collected from each of the 110 subjects an average of 62 days (range, 0 to 279 days) after the estimated date of HIV infection and stored at −70°C. None of the subjects had received more than 7 days of antiretroviral therapy prior to study entry and analysis of antiretroviral susceptibility.

Phenotypic determination of antiretroviral susceptibility. Patient virus drug susceptibility was measured by the PhenoSense HIV assay (17). Briefly, PR and RT coding sequences were amplified by RT-PCR and cloned into a recombinant HIV vector containing a luciferase reporter gene using restriction enzymes *Apa*I and *Pin*AI. The resistance test vectors (RTVs) were transfected into 293 cells; virus was harvested and used to infect fresh 293 cells in the presence and absence of drug. The concentration of drug required to inhibit viral replication by 50% in a single cycle assay was determined with reference to that of a drug-sensitive reference strain (CNDO) containing PR and RT coding sequences from the laboratory HIV strain NL4-3. Overall, the assay is reproducible within a 2.5-fold range (17). With respect to NNRTIs specifically, the coefficient of variation (standard deviation/mean) for 27 replicates of multiple patient and three reference samples lay between 7 and 12% (N. Hellmann, et al., Abstr. 3rd Int. Workshop HIV Drug Resist. Treat. Strategies, abstr. 51, 1999). Susceptibility to a panel of 15 antiretrovirals was performed for patient virus samples. Susceptibility to delavirdine, efavirenz, and nevirapine was determined for the site-directed mutants in RT.

Genotype determination was performed by consensus ABI sequencing of, in most cases, the PCR amplicon used in the PhenoSense assay, using *Taq* polymerase and Big Dye terminators (Perkin-Elmer, Foster City, Calif.) with sequences edited using Sequencher software (Gene Codes Corp., Ann Arbor, Mich.). Additional genotyping was obtained for some susceptible patients by direct consensus sequencing of the PCR-amplified product from plasma viral RNA. Nucleotide sequence data were aligned using the BioEdit version 4.5.8 (T. Hall, North Carolina State University) and GDE (24) screen-based multiple sequence editors, translated, and exported as ASCII files.

Site-directed mutagenesis. Mutations were introduced into the RT coding region of the reference vector (CNDO) using the megaprimer method (22). Briefly, a sense primer spanning the mutation was used in an amplification reaction with an antisense primer that anneals to sequences 3′ of the mutated region and spans the *Pin*AI site present in the NL4-3 RT coding region. The product of the first amplification reaction is used as a megaprimer in a second PCR in combination with a sense primer that anneals to sequences 5′ of the mutated region and spans the *Apa*I site in the *gag* coding region of NL4-3. The product generated in the second PCR containing the mutated sequence is cloned into the reference vector using the *Apa*I and *Pin*AI sites. The sequence of the entire *Apa*I-to-*Pin*AI segment of each clone was confirmed by DNA sequencing.

Phylogenetic analysis. Phylogenetic analysis was performed using maximum-likelihood (6) and neighbor-joining (20) methods as implemented in the PHYLIP (7) and TREECON (25) software packages, respectively. One thousand bootstrap resamples of the sequence data set were generated for the neighbor-joining analysis.

Statistical analysis. Aligned amino acid sequence files were imported into SPSS version 9.0 (SPSS Inc., Chicago, Ill.) and edited to remove invariant sites. Statistical analysis was performed on all amino acid sites where a nonconsensus amino acid was observed in 5 or more individuals out of 110 studied. Effects of individual amino acids were assessed by logistic regression and one-tailed exact tests. Combinations of sites contributing to the variation in susceptibility were identified by logistic regression with stepwise selection and by linear regression on log-transformed fold change values as described elsewhere (18).

Nucleotide sequence accession numbers. Nucleotide sequences have been submitted to GenBank under accession no. AF301265 to AF301374.

RESULTS

Phenotypic analysis determined that 13 out of 110 patients studied had moderate reductions in susceptibility to nevirapine, between 2.5- and 10-fold lower than that of the control strain, NL4-3, and 19 patients had reduced susceptibility to delavirdine. Two individuals were identified as having reduced susceptibility to efavirenz. Moderate reductions in susceptibility to NNRTIs were not associated with primary resistance-associated mutations at any of amino acid positions 98, 101, 103, 106, 108, 181, 188, and 190 (13).

Amino acid variation in RT in primary HIV infection. Among the 302 amino acid sites analyzed in RT, 102 showed a variant in one or more subjects, and at 63 sites the variant was present in two or more subjects. Sites where the mutant amino acid was present in five or more subjects were included in the regression analysis; 46 amino acid sites met this criterion, of which 24 had more than one non-wild-type amino acid within this data set.

Phylogenetic clustering. The phylogenetic analysis was performed on the same 906-bp region of RT used in the analysis of susceptibility. The tree presented (Fig. 1) was obtained from analysis of all sites, but including only third base positions did not change its structure (data not shown). Two sequences were clearly distinct from all others in the tree. These were identified as subgroup C viruses by comparison to reference strains (data not shown). Although the individuals from whom these strains were obtained were diagnosed with primary HIV infection in the United States, both had identified HIV exposure in Africa. Neither virus showed reduced susceptibility to any antiretroviral tested.

No general clustering of patients with reduced susceptibility was apparent from the tree, but there are a small number of clusters with strong bootstrap support. Two of these groupings, both from Los Angeles, each comprised pairs of patients both of whom showed reduced susceptibility. Although supported in 100% of bootstraps, these sequences were distinct, differing at 1.39 and 1.08% of nucleotide positions, respectively, out of 906. Two other small clusters of drug-susceptible sequences were identified among individuals from Massachusetts (one pair) and California (one pair from San Diego linked to another pair comprising one San Diego and one Los Angeles patient). The pair of patients identified from San Diego self-reported as a monogamous couple of whom it was previously believed that one had infected the other. No other linkages could be established from clinical records. Overall there was no evidence from the tree that reduced susceptibility was associated with the transmission of phylogenetically related viruses.

Analysis of amino acid sites conferring reduced susceptibility. Regression analysis was performed on amino acid identity at all 46 variable amino acid sites in the RT domain for the 110 RT sequences. Logistic regression on individual sites identified two sites as significantly associated with reduced susceptibility to nevirapine, amino acid (aa) 135 ($P = 0.008$) and aa 283 ($P = 0.001$), of which aa 135 was also significantly associated with reduced susceptibility to delavirdine ($P = 0.0002$) (Table 1).

A single variant amino acid was observed at position 283 in this data set, Leu→Ile (L283I). Isoleucine was present at this position in seven samples, five of which were from the 13 cases showing reduced susceptibility to nevirapine, and three of which were also among the 19 delavirdine cases. The mean

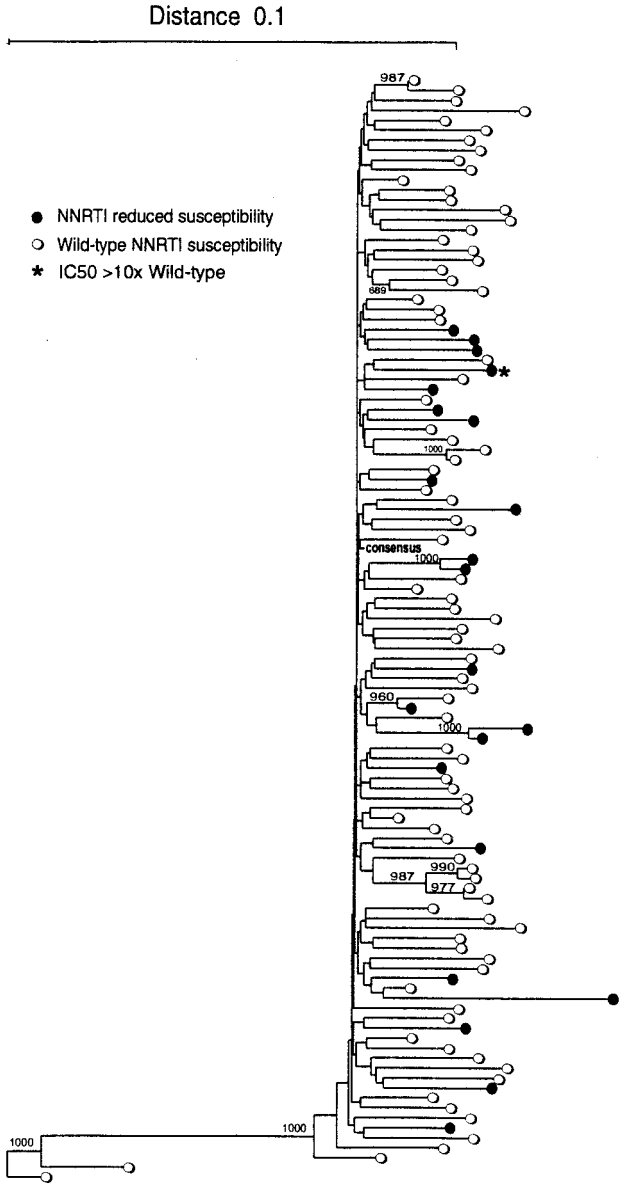

FIG. 1. Phylogenetic tree of RT sequences from patients with primary HIV infection. An unrooted maximum-likelihood phylogenetic tree for all 110 sequences analyzed is shown. The two outgroup sequences at the base of the tree belong to HIV-1 subtype C; all others are subtype B. Bootstrapped neighbor-joining trees gave essentially the same topology, and the number of bootstrap resamples supporting internal clusters (out of 1,000) is shown for all cases above 500. Symbols represent virus with wild-type (○) and reduced (●) susceptibility to NNRTI in the PhenoSense HIV assay. IC50, 50% inhibitory concentration.

changes in susceptibility for the seven strains with 283I were 2.5-fold for nevirapine and 2.35-fold for delavirdine.

Amino acid site 135 was associated with reduced susceptibility to both nevirapine and delavirdine. At this site the consensus amino acid among B-subtype strains of HIV-1 is isoleucine, and four variant amino acids were observed in this data set (Table 2). One strain, which had a substantially greater reduction in susceptibility to both drugs than any other, had a methionine at this site, which was not observed in any other strains. This strain also had the only example of a mutation at position 138 (138A). Because there were too few variant strains, 138 was not included in this analysis. Although the phenotype of the 138A mutation has not been described, other mutations at this site are known to be associated with highly reduced susceptibility to nonnucleoside drugs (23), and so this strain was not included in further analyses. The second most common amino acid at position 135, threonine (I135T), was observed in 26 individuals (24%) and on its own was associated with mean fold changes of 2.2 to nevirapine and 2.5 to delavirdine. The 135T mutation was found in 11 of the 19 instances of reduced susceptibility to delavirdine and in 8 of the 13 nevirapine cases.

TABLE 1. Individual amino acid sites associated with reduced susceptibility to antiretrovirals in subjects with primary HIV infection[a]

Antiretroviral	Amino acid	Odds ratio	*P*	Exact test *P*
Nevirapine	283	28.1	0.001	<0.003
	135	5.5	0.008	0.005
Delavirdine	135	8.5	0.0002	<0.0001

[a] All sites where *P* was <0.01 are shown; in view of the number of tests performed, probability values higher than 0.01 were not considered significant.

The five alternative amino acids at 135 and two at 283 specify a potential 10 different genotypes at the pair of sites, of which 7 were observed in this data set. Using data at both sites in the analysis gave further information about susceptibility. Thus, of the seven strains with 283I, the three that were wild type at 135 had a mean fold change of 2.0 for nevirapine, while those with 135T had an almost 50% greater reduction in mean susceptibility (2.9-fold; $n = 4$) (Table 3). Strains that were 135T and wild type at 283 also showed a mean susceptibility reduction of 2.0-fold. However, not all mutations at these sites were associated with reduced susceptibility, and the susceptibilities to the two drugs were not always concordant. In particular, 135V showed little change in susceptibility to delavirdine but an increased susceptibility to nevirapine relative to the wild-type control (0.5-fold; $n = 5$) (Table 3). Taken together, genotype at aa 135 and 283 explained 15% of the variation in susceptibility to nevirapine and 9% of the variation in susceptibility to delavirdine in this data set.

Phenotypic testing of site-directed mutants. RTVs containing mutations at the amino acid sites that were identified by the regression analysis, both alone and in combination, were constructed in vitro. The isoleucine (I) at 135 of RT in NL4-3 was mutated to each of the five different amino acids that were found in patient viruses, leucine (L), threonine (T), methionine (M), arginine (R), and valine (V). RTVs containing M, L, or T at 135 alone showed very small reductions in susceptibility to the NNRTIs tested, while RTVs containing R or V at 135

TABLE 2. Mean fold change in susceptibility to nevirapine and delavirdine associated with variant amino acids at residue 135 in the RT coding region

Amino acid	*n*[a]	%	Mean fold change	
			Nevirapine	Delavirdine
I[b]	72	66	1.03	1.4
T	27	25	2.20	2.52
V	6	5.5	0.66	1.80
L	3	2.8	1.09	1.69
M[c]	1	0.9	6.96	20.28

[a] Total scored = 109.
[b] Amino acid in NL4-3.
[c] This strain also had 138A in RT.

TABLE 3. Mean fold change for each genotype defined by amino acid at positions 135 and 283 for nevirapine and delavirdine[a]

Genotype		Mean fold change		*n*
283	135	Nevirapine	Delavirdine	
L[b]	I[b]	0.98	1.37	67
	T	2.08	2.58	22
	V	0.53	1.83	5
	L	1.09	1.69	3
I	T	2.89	2.30	4
	I	2.01	2.45	3

[a] Four strains were not scored for aa 283. Only genotypes for which *n* was ≥3 are shown.
[b] Genotype of NL4-3 at this site.

showed a slight increase in NNRTI susceptibility. RTVs containing the L283I substitution alone also showed no significant decrease in susceptibility to NNRTIs. However, those containing the L283I substitution in combination with the I135M or -L substitution showed a four- to fivefold decreases in susceptibility to all three of the NNRTIs tested (Table 4), while the 135T + 283I constructs showed a smaller but still significant decrease. These results confirm the role of the mutations identified from the regression analysis in conferring reduced susceptibility of HIV-1 RT to NNRTIs when present in combination.

DISCUSSION

The majority of cases of drug-resistant HIV arise de novo in the treated patient as a consequence of the error-prone nature of viral replication and the high replication rate seen in the viral population. However, transmission of drug-resistant virus has been described in many studies ranging from ZDV resistance when ZDV monotherapy was widespread to the transmission of multidrug-resistant virus described in recent studies. Within the context of potent antiretroviral therapy, the transmission of drug-resistant virus remains a significant issue as the success of combination antiretroviral regimens in the management of HIV disease is prejudiced if the patient has been infected with a virus which is already resistant to one of the component drugs. Virological failure of combination therapy is known to develop more rapidly in the presence of resistance to component drugs (4).

In smaller early surveys of the prevalence of drug resistance in newly infected individuals (16, 19), it was suggested that up to 10% of individuals might be infected with ZDV-resistant virus. Four recently published large studies of the prevalence of resistance to a number of drugs suggest that the frequency of transmission of strains showing high levels of drug resistance may be of the order of 5% for each of the major classes of antiretrovirals (2, 13, 21, 26). However, in addition to this lower prevalence of strains with >10-fold reductions in susceptibility, Little et al. recently showed, using a highly reproducible phenotypic assay, a much higher prevalence of strains with susceptibility 2.5- to 10-fold lower than that of the control, especially to NNRTIs and PR inhibitors (13). Where equivalent data were available, similar observations were obtained in other studies (2, 26).

TABLE 4. Susceptibility to NNRTIs of NL4-3 constructs containing mutations at 135 and 283

Construct	Fold change in susceptibility		
	Delavirdine	Nevirapine	Efavirenz
283I	1.5	1.6	1.4
135L	3.0	2.7	2.6
135M	2.3	2.6	2.2
135R	0.5	0.8	ND[a]
135T	1.9	2.0	1.6
135V	0.7	0.8	ND
135L + 283I	5.0	4.2	4.1
135M + 283I	4.0	4.5	3.2
135T + 283I	2.8	3.4	2.5

[a] ND, not determined.

The reduced susceptibility described was not associated with mutations at amino acid sites that were previously known to affect susceptibility to NNRTIs, and its genotypic basis remained unclear. We therefore extended the analysis to all 46 amino acid sites that showed significant variation in a data set of 110 HIV RT sequences from patients with primary infection, using logistic regression to detect associations between individual sites and susceptibility. This approach identified two novel sites, aa 135 and 283, with strong associations with NNRTI susceptibility which were then tested by in vitro mutagenesis. This confirmed that specific combinations of nonwild-type amino acids at these two sites reduced susceptibility to NNRTIs (Table 4). While this established that the phenotypic susceptibility differences are due to genetic variation at these sites, it is not known by what mechanism the phenotypic effect is mediated. It is likely that mutations at aa 135 affect NNRTI susceptibility by virtue of their proximity, in p51, to the NNRTI binding site (9, 11), but aa 283 is located near the tip of the thumb domain in RT, and at present its role is unclear.

The high prevalence of reduced susceptibility to NNRTIs in newly infected individuals raised questions about whether there were particular clusters or transmission networks within which individuals were becoming infected with these strains at high frequency. A molecular epidemiological analysis of the sequence data showed no clustering of strains with reduced susceptibility (Fig. 1), although a few small clusters were identified. In most cases, due to the prevalence of individuals reporting anonymous sex as a risk activity, these pairs could not be explained from the available clinical data, but one pair of sequences derived from the two members of a long-term sexual partnership. From this finding we conclude that there was sufficient phylogenetic information in the data for a cluster of strains with reduced susceptibility to have been detected if it had existed.

It has only recently become possible to detect phenotypic susceptibility differences in the range discussed here, and so knowledge of their historic prevalence is limited. In particular, it is not currently possible to determine whether variation at these sites is related to the use of NNRTIs in the treatment of HIV in the United States since 1995. However, we note that in some other HIV clades, the amino acid variants that we have found to be associated with NNRTI susceptibility are common (12). Thus, it seems likely that the variation at these positions among B-subtype sequences represents natural polymorphism and predated NNRTI therapy. The clinical consequences of this naturally occurring variation in susceptibility for treatment response remain to be determined.

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