Human Immunodeficiency Virus Type 1 Reverse Transcriptase Genotype and Drug Susceptibility Changes in Infected Individuals Receiving Dideoxyinosine Monotherapy for 1 to 2 Years

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The genetic mechanisms of human immunodeficiency virus type 1 (HIV-1) resistance to dideoxyinosine (ddI) in vivo have been described based on data from primary HIV-1 isolates. To better define the spectrum of HIV-1 reverse transcriptase (RT) changes occurring during ddI therapy, we determined the genotype and ddI susceptibility of the RT gene of HIV RNA isolated from the plasma of 23 patients who had received 1 to 2 years (mean, 87 ± 16 weeks) of ddI monotherapy. Population-based sequencing of plasma virus showed that 12 of 23 (52%) patients developed known ddI resistance mutations: L74V (7 patients), K65R (2 patients), L74V with M184V (3 patients), and L74V with K65R (1 patient). Five patients developed one or more known zidovudine resistance mutations (at codons 41, 67, 70, 215, and/or 219) during the study. Other amino acid substitutions were found, but only S68G and L210W occurred in more than one patient. Studies of sensitivity to ddI were performed on population-based recombinant-virus stocks generated by homologous recombination between a plasmid containing an HXB2 clone with the RT gene deleted and RT-PCR products of the RT genes from patients' plasma RNA. The sequences of the virus stocks produced by this procedure were typically identical to the sequence of the input PCR product from plasma RNA. Both an MT-2 cell-based culture assay and a cell-free virion-associated RT inhibition assay showed that viruses possessing an L74V and/or M184V mutation or a K65R mutation had reduced sensitivity to ddI. Viruses without these specific mutations had no change in sensitivity to ddI. The results presented here show that the spectrum of RT mutations in a population of patients on ddI monotherapy is more complex than previously described. The development of multiple mutational patterns, including those that confer resistance to other nucleoside analogs, highlights the complexity of using the currently available nucleoside analogs for antiretroviral therapy.

Resistance of human immunodeficiency virus type 1 (HIV-1) to reverse transcriptase (RT) inhibitors has been associated with the development of specific amino acid changes encoded by the RT gene. The most frequently described mutation in patients receiving dideoxyinosine (ddI) is L74V (16, 28). Experiments using site-directed mutagenesis have shown that the L74V mutation confers a 5- to 10-fold decrease in sensitivity to ddI as determined by in vitro HeLa-CD4 cell assays (28). Other mutations in the RT gene that have been similarly shown to confer decreased susceptibility to ddI in both in vitro and in vivo studies are K65R (5, 7, 8) and M184V (7, 14, 33). Each of these mutations has also been shown to reduce the susceptibility of HIV-1 isolates to other nucleoside analogs, such as dideoxycytosine (ddC) (5, 7, 8, 28, 33) and 3'-thiacytidine (3TC) (7-9). As the number of drugs available to treat HIV infection increases, the impact of prior therapy

and the resistance mutations that develop during these therapies become greater factors in patient care.

New information regarding the benefits of ddI monotherapy or of ddI in combination with other drugs (13) and the Food and Drug Administration approval of ddI for use as an initial therapy in HIV-infected individuals have led to an increased interest in factors affecting its use. The frequency of mutations other than L74V appearing in vivo that confer ddI resistance has not been examined systematically with direct clinical samples. In addition, studies examining ddI resistance in patient specimens have primarily used proviral DNA or cocultured virus stocks (5, 7, 33), which may underrepresent the frequency of mutations (17, 26, 27, 31) or misrepresent selected subpopulations (11, 18, 30). In this study, the frequency of genotypic changes in the RT genes of asymptomatic patients receiving 1 to 2 years of ddI monotherapy was examined by direct sequencing of cDNA from plasma virion RNA. Also examined was the relationship of the genotypic changes observed before and after therapy to the in vitro phenotypic sensitivity to ddI of recombination-derived virus stocks measured by two different in vitro assays.

MATERIALS AND METHODS

Patients. Subjects in this study were HIV-1-seropositive, asymptomatic individuals with CD4 lymphocyte counts of 200 to 500 cells/ μ l at study entry (mean ± standard deviation, 329 ± 85 cells/ μ l) who were randomized to the ddI-alone arm (250 mg twice a day) in AIDS Clinical Trials Group protocol 143. Thirty-four patients were originally randomized into this arm of the study; eight patients left the study in the first year. Among the remaining 27 patients, samples were

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mined by nonlinear regression. IC_{50} s for each patient isolate were determined from two to four different assays. Statistical differences between pretherapy and posttherapy IC_{50} s for each group were determined by a two-tailed paired Student *t* test. Differences between changes in IC_{50} were determined by a two-tailed unpaired Student *t* test.

RESULTS

Genotypic analysis. The sequence analysis of the RT genes from codons 1 to 242 for all patients is shown in Table 1. Nine patients had prior AZT experience ranging from 1 to 12 months when they entered the study. At baseline, the K70R mutation was found in 4 of the 23 patients. No other drug resistance mutations were found in any of the baseline specimens. After 1 to 2 years of ddI therapy, the ddI resistance mutation L74V was found in the RNA of 11 of the 23 (48%) patients. Three of these patients also developed the M184V mutation, while one patient developed the K65R mutation in addition to the L74V mutation. Two patients (9%) developed the K65R mutation alone, both of whom also had S68G. All three patients whose virus possessed the K65R mutation were in the AZT-experienced group (3 of 9), while none of the previously untreated patients developed this mutation (0 of 14). Two patients (no. 9 and 1) had minority populations of virus possessing the K65R mutation. The plasma RNA from 10 patients showed no previously described ddI resistance mutations in their RT genes at the end of the study.

A number of changes encoded by codons other than 65, 74, and 184 were also seen and are shown in Table 1. Five patients developed virus populations possessing AZT resistance mutations (at codons 41, 67, 70, 215, and/or 219) during ddI monotherapy: two had prior AZT experience (2 and 12 months), and three had not reported prior AZT experience. The virus from one of these five patients also developed a ddI resistance mutation (L74V). Two of the four patients with the K70R mutation at baseline reverted to the wild type by the end of the study. Of the other changes observed, only S68G and L210W were seen in more than one patient.

Recombinant viruses. Plasma for preparation of RT-PCR products for recombination from baseline and posttherapy pairs was available from 21 of the 23 patients. Virus stocks were generated by cotransfecting RT-PCR products from patients with an HXB2 plasmid clone with a deletion of the RT gene and were grown to high-titer stocks in the absence of ddI. Sequencing of the RNA from the resulting virus stocks showed that 15 of the 21 (71%) stocks maintained the genotype that was present in the RT-PCR product generated from the plasma. For all positions where amino acid changes were seen following stock generation, mixtures of different bases at the specific position affected were evident in the sequencing data of the DNA used for the transfection. Two of the six stocks that had genotypic changes were affected at ddI resistance mutation positions: stock from patient 11 lost the K65R mutation, and stock from patient 9 lost the L74V mutation. The other four stocks had one change (two stocks) or two changes (two stocks) at positions not conferring ddI resistance (codons 70, 106, 177, 178, 200, or 210).

A second set of transfections were performed on the samples that had lost ddI resistance genotypes after transfection, after which the virus stock was expanded in cultures without drugs and in the presence of 1 μ M ddI. Sequencing of these pairs of cultures showed that for patient 11 (who had relatively equal mixtures of mutations at codons 65 and 74) the stock generated in the absence of ddI again lost the K65R mutation but retained the L74V mutation. The stock generated for this patient in the presence of ddI maintained both K65R and L74V. For patient 9 (who had a mixture of predominantly wild

FIG. 1. ddI sensitivity of recombinant HIV-1 patient strains was assessed by either a cell-free virion-associated RT inhibition assay (A) or an in vitro MT-2 cell dye reduction assay (B). BL, baseline; END, end of study; SD, standard deviation. Data for the L74V-with-M184V group is from patients 1 through 3, data for the L74V group is from patients 4 through 11, data for the K65R-with-S68G group is from patients 12 and 13, and data from the "none" group is from patients 14 through 16 and 18 through 22.

type at codon 65 and a mixture of predominantly mutant at codon 74), the stock generated in the absence of ddI again lost the K65R minority and L74V majority populations, while the stock generated in the presence of ddI had relatively pure populations of both K65R and L74V.

Phenotypic assays for ddI sensitivity. The virus stocks generated by the recombinant method were assayed for ddI sensitivity by a cell culture assay and a cell-free RT inhibition assay. Results from both assays are shown in Fig. 1. In the cell culture assay, the mean IC_{50} for the posttherapy isolates from the L74V-with-M184V group was approximately threefold higher than the mean baseline (mean posttherapy IC_{50} versus mean baseline IC_{50} , $P = 0.03$, $n = 3$), that for the L74V group was 1.5-fold higher than the mean baseline ($P = 0.01$, $n = 8$), and that for the K65R-with-S68G pair was 2.8-fold higher than the mean baseline. There was a 1.1-fold difference between the pre- and posttherapy IC_{50} s in the group with no ddI mutations ($P = 0.42$, $n = 8$). There was no difference in the susceptibilities of the isolates from patients (patients 14 to 16) who developed only AZT resistance mutations (the pretherapy IC_{50} was $5.01 \pm 1.4 \mu$ M, while the posttherapy IC_{50} was $5.05 \pm 0.76 \mu$ M; $P = 0.97$).

In the RT inhibition assay, the mean IC_{50} for the posttherapy isolates from the L74V-with-M184V group was approximately fivefold higher than the mean baseline ($P = 0.03$,

TABLE 2. Clonal analysis of patients with multiple ddI resistance mutations

Patient	Mutations encoded by plasma genotype	No. of clones	Amino acid encoded by codon ^a :			
			65	68	74	184
1	K65R, L74V, M184V (1 year)	5	R	S	L	M
		2	K	S	V	V
		1	R	S	L	V
11	K65R, L74V	2	R	S	L	M
		8	K	S	V	M
		1	K	S	L	M
9	K65K(R), L74V	4	R	G	L	M
		6	K	S	V	M

^a Boldface indicates a mutation.

$n = 3$), that for the L74V-alone group was threefold higher than the mean baseline ($P = 0.01$, $n = 8$), and that for the K65R-S686 pair was sixfold higher than the mean baseline ($n = 2$). All of the virus stocks that did not possess the L74V or K65R mutation were sensitive to ddI (1.2-fold difference from the baseline; $P = 0.42$, $n = 8$). There was no difference in the susceptibilities of the isolates from patients (patients 14 to 16) who developed only AZT resistance mutations (the pretherapy IC_{50} was $1.49 \pm 0.53 \mu M$, while the posttherapy IC_{50} was $2.21 \pm 0.77 \mu M$; $P = 0.13$).

Linkage of mutations. A number of patients had two ddI resistance-conferring mutations or showed evidence of mixtures of clones with mutations at two or three of these codons. Clonal analysis was performed to assess the relationship between these mutations on individual viral genomes (Table 2). Patients 1 and 9 had minority populations of viruses possessing K65R along with L74V with M184V and L74V, respectively. Patient 11 had predominant populations of viruses possessing K65R and L74V. Analysis of 7 to 10 clones from each patient showed that the K65R mutation was not linked with the L74V mutation: 12 of 12 clones containing the K65R mutation did not have the L74V mutation, while 16 of 16 clones containing the L74V mutation did not contain the K65R mutation. The M184V mutation was seen in clones containing either the K65R or L74V mutation. All clones from patient 9 that had the K65R mutation also had the S68G mutation, while all clones that were wild type at codon 65 were also wild type at codon 68. All clones from patients 1 and 11 (either wild type or mutant at codon 65) were wild type at codon 68.

DISCUSSION

The amino acid changes that were encoded between codons 2 and 242 in the RT genes of a group of patients receiving 1 to 2 years of ddI monotherapy were examined by population-based sequencing. Viral RNA isolated from plasma was used for these experiments, since mutations tend to appear in the plasma before peripheral blood mononuclear cells (PBMC) (17, 23, 26, 27, 31) and isolates from PBMC cocultures may misrepresent subpopulations selected during the culture process (11, 18, 30). Mutations were defined as amino acid changes compared to each patient's own baseline sequence.

The results of the study presented here show that different pathways of genotypic resistance appear in patients receiving ddI monotherapy. The most frequent mutation seen in this group of patients was L74V, which was found in 48% of the patients. Most of these patients were previously found to have

the L74V mutation by selective PCR after 1 year of therapy (23). The L74V mutation was seen in conjunction with other ddI resistance-conferring mutations, K65R and M184V. The frequency of the L74V mutation in this study was slightly less than that in a previous study of 64 ddI-treated patients with lower starting CD4 cell counts, which showed by selective PCR techniques that 56% of patients developed the L74V mutation within 6 months of ddI monotherapy (16). Another study with clinically advanced patients showed the L74V mutation appearing in the PBMC proviral DNA of 69% of the patients (4). Studies have also shown that L74V confers resistance to ddC (28) and 1592U89 (29).

While the significance of the L74V mutation in conferring ddI resistance in patients has been established (16, 28), the overall importance of the K65R mutation in the patient population is not firmly established. The results presented here indicate that K65R is found in approximately 10% of patients receiving ddI monotherapy. Gu et al. (7) found that 4 of 11 patients receiving more than 6 months of ddC therapy developed the K65R mutation. Other studies of K65R have focused on its effect on drug resistance and enzyme function in viral isolates or proviral DNA (5, 7) and have not assessed the relative prevalence of this mutation in the plasma of patient populations. These studies have shown, however, that K65R also confers resistance to ddC (33) and 1592U89 (29).

A number of patients in this study developed AZT resistance mutations despite being randomized to ddI monotherapy. A recent report by Demeter et al. showed similar findings (3). Two patients had reported prior AZT therapy of 2 to 12 months, and the continued emergence of AZT resistance mutations despite changes in drug therapy has also been reported previously (10). Four patients, however, reported no prior AZT experience and had no preexisting AZT resistance mutations, yet they developed AZT resistance mutations in the absence of ddI resistance mutations. It has been shown that patients receiving combination AZT-ddI therapy rarely develop ddI resistance mutations but do develop AZT resistance mutations (15, 23). While surreptitious use of AZT during this study is unlikely for one patient (24), it cannot be ruled out for the other four patients whose viruses developed AZT resistance mutations. In contrast, evidence that some AZT resistance mutations may confer partial resistance to ddI does exist (2, 19). Phenotypic results presented here show that those patients who developed solely AZT resistance mutations did not show reduced sensitivity to ddI in our assays. Further studies with sensitive tests for measuring ddI resistance in a larger number of well-defined patients will be needed to further investigate the relationship between AZT resistance mutations and ddI sensitivity.

Generating virus stocks by homologous recombination allows susceptibility testing to be performed on virus strains from patients who are culture negative when tested by conventional methods. This technique also produces a virus stock with the virus' RT gene on a homogeneous background, which isolates changes in susceptibility or growth rate to the introduced RT-coding sequence. Recombinant virus stocks for phenotypic testing in this study were generated from plasma virus RNA. Most of the virus stocks generated by this technique had the same genotype as the plasma-derived PCR product from which they were generated. Two virus stocks lost known ddI resistance mutations (K65R and L74V) when expanded in the absence of ddI, but when the stocks were regenerated in the presence of ddI, the original genotypes were maintained. Analysis of the sequence of the PCR product used for transfection showed that genotypic mixtures were present before transfection. The resulting loss of one viral species during this process

may be due to a difference in replication rates between the two species in the absence of drug pressure during *in vitro* growth; however, growth competition experiments are needed to assess this hypothesis. The maintenance of ddI resistance mutations by growth in the presence of ddI provides evidence that the mutant genotypes have a selective advantage under drug pressure and that drug pressure during culture may be necessary at times to maintain the original genotype. This, however, may lead to a bias in favor of resistant virus. When this recombinant technique is used, the routine comparison of the sequence of the virus stock to the PCR product used for transfection will be necessary to verify the genotype and thus to effectively analyze any phenotypic results.

Phenotypic assessments were performed by a cell culture-based assay and a cell-free biochemical assay. Results showed that both methods were able to detect changes in ddI sensitivity conferred by viruses containing the L74V and/or M184V mutation. The cell-free RT inhibition assay was more sensitive than the MT-2 cell assay, in that it provided consistently greater changes between baseline and posttherapy isolates containing known ddI resistance mutations. In the RT inhibition assay, ddATP is added directly to the assay, while cell culture systems depend on the conversion of ddI in the medium to ddATP. This phosphorylation step has been shown to be poor in activated cell lines, which are typically used for susceptibility tests, compared to that in nonactivated (resting) cell types (6, 25). The lack of a cell-dependent phosphorylation step probably accounts for the increased sensitivity of the RT inhibition assay in measuring ddI resistance in our study. The relatively modest increases in IC_{50} in our phenotypic assay with MT-2 cells compared to IC_{50} s obtained by St. Clair et al. (28), who used HeLa-CD4 cells, are also likely due to differences in phosphorylation activity between the two cell types.

Analysis of clones from three patients with both K65R and L74V showed that the two mutations comprised separate viral quasispecies, because viruses with K65R (with or without S68G) did not have L74V. The drug resistance provided by both of these quasispecies would thus be additive. The lack of viruses possessing both K65R and L74V suggests that this combination renders the RT dysfunctional, but site-directed mutagenesis studies will be necessary to evaluate this premise. The association of L74V with M184V was seen in approximately 13% of the patients in this study. There appeared to be no restriction on linkage with the other ddI resistance mutations, as clones with M184V alone, or with K65R or L74V, were found. Other studies have identified the emergence of M184V *in vivo*. Zhang et al. (33) found M184V in 4 of 11 clinical isolates from patients who received more than 6 months of ddC therapy, and Gu et al. (8) found M184V by selective PCR in 5 of 7 isolates from patients who received more than 6 months of ddI therapy, one of which also had K65R. The M184V mutation has been shown to confer resistance to ddI, ddC (5, 7), and 1592U89 (29) and has also been found to develop after passage of virus in 3TC (5), in patients receiving 3TC (14, 22), and in patients receiving oxathiolanecytosine analogs (21).

The results of this report show that most patients receiving ddI monotherapy develop resistance to ddI. This resistance is primarily mediated by a mutation at codon 74, but changes at codons 65 and 184 also contribute. Isolates from these patients showed reduced sensitivity to ddI. A number of patients also developed AZT resistance mutations, but the origin of these mutations is unclear. The frequency and type of mutations seen in this study indicate the complexity of a change of nucleoside therapies for clinically failing patients. The coresistance provided by a relatively small number of mutations to a

relatively small number of drugs complicates the choice of alternative therapies. As the use of combinations of antiretroviral drugs continues to increase, careful and complete studies will be needed to assess the changes in mutation patterns in patients who move from one therapy to another.

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