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pol Mutations Conferring Zidovudine and Didanosine Resistance with Different Effects In Vitro Yield Multiply Resistant Human Immunodeficiency Virus Type 1 Isolates In Vivo

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Specific mutations in the human immunodeficiency virus type 1 (HIV-1) *pol* gene that cause zidovudine (3′-azido-2′,3′-dideoxythymidine; AZT) and didanosine (2′,3′-dideoxyinosine; ddI) resistance were studied. The 50% inhibitory concentrations (IC_{50}s) of nucleosides for cloned viruses containing these mutations were compared with the IC_{50}s of the corresponding triphosphate analogs for mutant recombinant-expressed reverse transcriptases (RTs). Changes in ddATP inhibition of RNA-dependent DNA polymerase activity fully accounted for the ddI resistance of the virus caused by a Leu-74 → Val substitution in RT, including an augmentation by the AZT-selected substitutions Thr-215 → Tyr and Lys-219 → Gln in RT. In contrast, the AZT-selected substitutions studied did not cause as great a change in the IC_{50} of AZT-triphosphate (AZT-TP) for polymerase as they did in the IC_{50} of AZT for mutant virus. In addition, the mutation at codon 74 suppressed AZT resistance in the virus caused by the mutations at codons 215 and 219 but did not suppress the AZT-TP resistance of enzyme containing these same mutations in RT. The mutation at codon 74 was found in clinical isolates whether or not the patient had received AZT prior to starting ddI therapy. AZT resistance coexisted with ddI resistance following acquisition of Leu-74 → Val in three clinical isolates, indicating that the suppressive effect of Val-74 on the AZT resistance of the virus does not occur in all genetic contexts. When this suppression of AZT resistance was seen in the virus, Val-74 did not appear to cause mutually exclusive changes in AZT-TP and ddATP binding to RT in vitro. The results of the in vitro experiments and characterization of clinical isolates suggest that there are differences in the functional effects of these AZT and ddI resistance mutations.

The reverse transcriptase (RT) inhibitors zidovudine (3′-azido-2′,3′-dideoxythymidine; AZT), didanosine (2′,3′-dideoxyinosine; ddI), and zalcitabine (2′,3′-dideoxycytidine; ddC) are the principal antiretroviral therapies currently available for individuals with human immunodeficiency virus type 1 (HIV-1) infections (15). Each of these nucleoside analogs has been presumed to act by a similar mechanism in which the triphosphate form mediates premature termination of RT-catalyzed DNA synthesis (for a review, see reference 32). HIV-1 isolates with specific *pol* gene mutations that cause resistance to these nucleosides emerge during therapy (11, 13, 20, 27, 29, 30, 35–37, 40). However, the characterized AZT- and ddI-selected substitutions do not confer cross-resistance, which has been hypothesized to be due to the different effects of these mutations on one function: the RT binding of these structurally dissimilar inhibitors (13, 20, 27, 29, 30, 35, 37).

In some cases, resistance to different nucleosides overlaps, as would be expected if they shared a mechanism of action. The ddI-selected RT Leu-74 → Val or Met-184 → Val substitution causes both ddI and ddC resistance (13, 37). The Leu-74 → Val change also causes a reversal of the AZT resistance conferred by a Thr-215 → Tyr substitution in RT (37). This interactive effect of a combination of substitutions led to speculation that simultaneous treatment with both AZT and ddI may prevent the emergence of resistant virus variants in vivo (37). It also suggests that the same RT function, such as binding of AZT-triphosphate (AZT-TP), may be affected in different ways by these ddI- and AZT-selected substitutions. However, in one comparison of mutant enzyme containing AZT-selected substitutions with wild-type enzyme, AZT-TP inhibition of RT polymerase activity in cell-free assays was only minimally impaired (25). This observation raised the possibility that a function involved in reverse transcription other than AZT-TP binding may be altered in AZT-resistant viruses.

In the present study, clinical isolates from patients who received ddI therapy were evaluated by *pol* gene sequence analysis and drug susceptibility testing. The effects of specific combinations of AZT and ddI resistance mutations on HIV-1 drug susceptibility were defined in cells infected with cloned mutant virus by measuring the inhibition of intracellular reverse transcription by drug by the polymerase chain reaction (PCR) (6). The susceptibility of mutant recombinant-expressed RT polymerase activity to inhibitors was also evaluated in cell-free assays by using a heteropolymeric primer template. Comparative mutant susceptibility results from whole virus and enzyme assay systems, as well as a novel resistance phenotype characterized in the clinical isolates, are discussed in relation to the hypothesis that the

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specific mutations that we studied have different effects on RT function.

MATERIALS AND METHODS

Subjects. Sixteen patients with AIDS or AIDS-related complex who had started prolonged ddI therapy during a phase I clinical trial were studied. These subjects each received different doses of a lyophilized preparation of ddI, ranging from 1.6 to 30.4 mg/kg of body weight once daily, during the trial (4). For all but one of the subjects, the regimens were changed to the sachet formulation of ddI at a dose of 375 mg twice daily in September 1990; one subject received 250 mg twice daily. Both of the ddI preparations used in the present study differed from the currently licensed chewable tablet formulation. Seven of the 16 patients had not received AZT prior to beginning ddI; the other 9 patients had received prior AZT therapy. None of the patients received any other antiretroviral therapy. An HIV-1 isolate was obtained from 15 of the 16 subjects prior to the initiation of ddI therapy. At least one additional isolate was obtained from each subject during ddI therapy.

Viruses and cells. Peripheral blood mononuclear cells (PBMCs) and plasma were separated from heparinized whole blood by Ficoll-Hypaque (Histopaque; Sigma Chemical, St. Louis, Mo.) density gradient centrifugation, and isolates were obtained by primary coculture with phytohemagglutinin (PHA; 10 μg/ml; Phytohemagglutinin-P; Difco Laboratories, Detroit, Mich.)-stimulated PBMCs from a single HIV-1-seronegative blood donor as described previously (18). Virus stocks were generated by passage of the supernatants from primary cocultures on PHA-stimulated PBMCs. Viruses were also derived from transfections of infectious molecular plasmid clones of HIV-1 (HxB2) into COS-7 cells. Either a plasmid containing a wild-type HIV-1 genome, pSP64-HxB2gpt (33) (generously provided by L. Ratner), or mutant infectious plasmid DNAs derived from pSP64-HxB2gpt by in vitro mutagenesis and molecular cloning were used in the transfections (see below). Virus stocks were generated by passage of these supernatants from the transfections in either PHA-stimulated PBMCs or the MT-2 cell line (14). The infectivity of a virus stock was determined by end point dilution (16) or by DNA quantitation by PCR (the PDQ method) (6).

Drugs. AZT and AZT-TP were generously provided by P. A. Furman (Burroughs Wellcome Research Laboratories, Research Triangle Park, N.C.), ddC was provided by I. Sim (Hoffmann-La Roche, Inc., Nutley, N.J.), and ddI was provided by C. MacLaren (Bristol Myers Squibb, Wallingford, Conn.). ddCTP, ddATP, and deoxynucleotide triphosphates (dNTPs) were obtained from Pharmacia (P-L Biochemicals, Milwaukee, Wis.).

HIV-1 susceptibility testing. Susceptibility testing was performed by the PDQ method, which determined the amount of reverse-transcribed HIV-1 DNA that accumulated at different drug concentrations during infections of PHA-stimulated PBMCs (6). The susceptibilities of cloned mutant viruses were measured by this PDQ method in infected PHA-stimulated PBMCs following virus passage in PBMCs (see Table 3). The cloned mutant viruses were also passaged, and their susceptibilities were tested by PDQ in infected MT-2 cells. Clinical HIV-1 isolates were passaged, and their susceptibilities were tested by the PDQ method only in infected PHA-stimulated PBMCs. For many of the clinical isolates, susceptibility testing was also done by using PBMC culture supernatant fluid HIV-1 core (p24) antigen enzyme-linked immunosorbent assay (DuPont-NEN Research Products, Billerica, Mass.) as the measure of virus replication (18). In a series of 30 isolates tested by both methods (including 17 tested in the present study and 13 tested earlier [6]), the correlation coefficient for 50% inhibitory concentrations (IC_{50}s) was 0.82 ($P < 0.05$). By either method, a uniform virus inoculum determined by infectivity titration was used for infections. For the p24-based drug susceptibility assay, the inoculum was 1,000 50% tissue culture infectious doses per 10^6 PBMCs. The inoculum used in the PDQ assay was operationally defined as the volume or dilution of virus that yielded a quantifiable, submaximal PCR signal (6). The IC_{50} was calculated by nonlinear regression analysis of the median effect equation (2, 6).

DNA sequencing of HIV-1 RT genes. A 761-bp fragment of the HIV-1 *pol* gene was amplified from infected control cell lysates of the susceptibility experiments or from cell lysates of stock generation cultures (6) by PCR (5′ primer, HIV-1 HxB2, sense strand, bases 2485 to 2504; 3′ primer, HIV-1 HxB2, antisense strand, bases 3246 to 3226). Stringent precautions to prevent carryover were followed in these amplifications and in the PDQ susceptibility experiments. Reagent controls were amplified in each PCR to control for potential contamination. The dominant sequence at each position in RT codons 35 to 235 was determined by direct sequencing of PCR products (Sequenase; U.S. Biochemical, Cleveland, Ohio) (1, 18). Mixtures at any position containing as little as 20% minority species could be reproducibly detected by this method (unpublished data).

Site-directed mutagenesis and HIV-1 provirus molecular cloning. Site-directed mutageneses were performed on a plasmid subclone containing either a *BalI* fragment (HxB2, bases 2620 to 4552) or an *SphI-SalI* fragment (HxB2, bases 1447 to 5786) of HIV-1 by the methods of Kunkel et al. (24). The complete RT-coding region of each plasmid subclone was sequenced to confirm each mutagenesis. The mutant restriction fragment was molecularly cloned into a plasmid containing the complete HxB2 genome. Transfection of infectious provirus plasmid DNA into COS-7 cells was performed by a DEAE-dextran method (23). Following virus stock generation, the PCR products of the RT genes were amplified from infected cell lysates and were directly sequenced to ensure genetic stability and to exclude cross-contamination.

Construction of mutant RT plasmid expression vectors. The subgenomic plasmid clones derived from pSP64-HxB2gpt and mutant HIV-1 constructs were used as templates for amplifications of RT-coding sequences by PCR in order to construct mutant RT plasmid expression vectors. Amplifications were done by using a 5′ primer containing an *NcoI* site along with a translational initiation codon and a 3′ primer containing a *HindIII* site along with a translation termination codon and cloned into the RT expression vector (pKRT2) as described previously (5). The intended changes were confirmed by DNA sequencing.

Preparation of recombinant-expressed RT. *Escherichia coli* JM 109 cells containing either the wild-type (HxB2) or the mutant RT expression vector were grown to the late logarithmic phase at 37°C in Luria broth with 100 μg of ampicillin (Sigma) per ml. RT expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 12 to 16 h. The cells were pelleted, washed, and frozen at −20°C. After thawing, cells were lysed in 50 mM morpholineethanesulfonic acid (MES; pH 6.0)–2 mM EDTA–0.02% Triton X-100–10% glycerol and were then sonicated. This crude lysate was used in polymerase assays to evaluate susceptibilities to ddATP

TABLE 1. RT genotype and drug susceptibility phenotypes of selected HIV-1 clinical isolates[a]

Subject no.	Time (mo) on ddI	RT amino acids[b]						IC_{50} (μM)[c]		
		AZT selected					ddI selected			
		Met-41	Asp-67	Lys-70	Thr-215	Lys-219	Leu-74	ddI	AZT	ddC
52	0	Leu	—	—	Tyr	—	—	0.8	0.07	0.009
	10	Leu	—	—	Tyr	—	Val			
	16[d]	Leu	—	—	Tyr	K > Q	Val	4.0	>1.00	0.10
27	0	—	—	Arg	T > Y	—	—	0.7	0.04	0.017
	16[d]	Leu	—	—	Tyr	—	Val	4.5	0.03	0.20
48	0	L = M	—	K = R	T = Y	—	—			
	7	Leu	—	—	Tyr	—	Val	11.1	0.23	0.58
50[e]	0	—	Asn	Arg	Phe	Gln	—	0.7	0.20	0.01
	8 (pb)	—	Asn	—	Phe	Gln	Val	2.1	0.02	0.02
	8 (pl)	—	Asn	Arg	Phe	Gln	Val	4.7		
45	7	—	—	—	—	—	Val	3.3	0.006	
1	0	—	—	—	—	—	—	0.3	0.007	
	14	—	—	—	—	—	Val	1.1	<0.01	
	24	—	—	—	—	—	Val	2.3	<0.01	
36[f]	0		—	—	—	—	—	1.4	0.003	0.02
	6		—	—	—	—	Val	5.5		0.04
44[f]	0	—	—	—	—	—	—	0.7		
	18		—	—	—	—	Val	2.3		
49	0	—	—	K > R	T > Y	—	—			
	10	—	—	—	T > Y	—	—			
15	0	—	—	K = R	—	—	—			
	15	—	—	—	—	—	—			

[a] Selected sequential isolates are listed. Isolates from three of the six subjects not listed here developed the Leu-74 → Val substitution during ddI therapy; this number included isolates from two subjects who did not have prior AZT therapy.

[b] —, the DNA sequence of the dominant product determined by PCR is identical to that of HxB2 at a particular position; blank space, genotype or phenotype was not determined for that isolate. The amino acid sequence is numbered relative to the amino-terminal proline of the native RT (HxB2). The single-letter abbreviations for the amino acids used are as follows: K, lysine; L, leucine; M, methionine; Q, glutamine; R, arginine; T, threonine; Y, tyrosine. Genotypic mixtures and the approximate relative proportion of each amino acid encoded are indicated if they were observed. Additional amino acid changes from the HxB2 sequence were noted in multiple isolates, some of which were obtained prior to ddI therapy. These substitutions, which were apparently independent of drug selection, included the following: Leu-214 → Phe (viruses from 15 subjects), Arg-211 → Lys (viruses from 8 subjects), Glu-122 → Lys (viruses from 5 subjects), Ser-162 → Leu (viruses from 3 subjects), and Pro-243 → Ala (viruses from 3 subjects).

[c] Each ddI IC_{50} is a mean of two to six independent determinations, except the ddI IC_{50}s for the isolates from subject 48 (7 months) and subject 45 (7 months), which were each determined from a single experiment. Each independent experiment involved at least triplicate infections. The AZT IC_{50}s for isolates from the following subjects were determined from a single experiment: 52 (0 and 16 months), 48 (7 months), 1 (14 and 24 months), and 36 (0 month). The ddC IC_{50}s for isolates from the following subjects were determined from a single experiment: 27 (0 and 16 months), 48 (7 months), and 36 (0 and 6 months). The standard errors of the means for representative isolates with multiple IC_{50} determinations are shown in Table 2.

[d] For isolates obtained at these times, substitutions in addition to Leu-74 → Val developed on ddI therapy, as follows: for subject 52, Glu-40 → Phe, Lys-43 → Gln, and Leu-210 → Trp (the substitution Leu-210 → Trp has been reported to confer AZT resistance to an in vitro mutagenesis-derived virus [10]); for subject 27, Arg-211 → Gly.

[e] One isolate from subject 50 obtained following 8 months of ddI therapy was derived from PBMCs (pb), and the other isolate was derived from the plasma (pl) of the same blood sample.

[f] Subjects who had not received any AZT prior to the beginning of ddI therapy.

and ddCTP. Susceptibility to AZT-TP was evaluated by using RT enzymes that were further purified by a modification of the method of Kohlstaedt and Steitz (21). Crude cell lysates were centrifuged and extracted with a high-salt buffer (50 mM MES [pH 6.0], 50 mM KCl, 50 mM potassium phosphate, 0.02% Triton X-100, and 10% glycerol). Polyethyleneimine (0.05%; Polymin P) precipitation of the centrifuged extract removed nucleic acid prior to 60% ammonium sulfate precipitation and hydroxylapatite column chromatography of the ammonium sulfate precipitate. The hydroxylapatite column was equilibrated in high-salt buffer and was eluted with a step gradient from 50 to 250 mM potassium phosphate. Fractions containing RT activity were pooled and concentrated. The purification yielded an approximately equimolar ratio of p66 and p51 subunits (data not shown).

Cell-free RT polymerase assays. Crude lysates of recombinant-expressed RTs, purified recombinant-expressed RTs, and virion-associated RTs from two clinical isolates were tested for polymerase activity by using an 89-bp heteropolymeric, in vitro-synthesized RNA template primed by a 20-bp oligodeoxynucleotide (RT-DETECT; DuPont Medical Products, Billerica, Mass.; generously provided by R. Green).

Culture supernatants from the two clinical isolates were precipitated overnight with polyethylene glycol, centrifuged (2,100 × *g* for 45 min), and lysed in detergent-containing buffer (16.7 mM Tris HCl [pH 7.8], 3.3 mM dithiothreitol, 0.167 mM EDTA, 0.32% Triton X-100, 33% glycerol, and 533 μM KCl) prior to performing the polymerase assays.

The inhibition of equivalent levels of polymerase activity of the different RTs by ddATP and ddCTP at seven concentrations, from 0.5 to 250 μM, was determined in the presence of 500 μM (each) dNTP. The IC_{50}s of ddATP and ddCTP were determined from crude lysates. AZT-TP inhibition was determined at six concentrations, from 1.0 to 250 nM, by using 10 μM (each) dNTP. The AZT-TP IC_{50}s were determined with lower levels of dNTPs than were used for ddATP and ddCTP IC_{50} determinations because of the limited availability of AZT-TP. Following acid hydrolysis of the RNA template, the DNA product strand was hybridized in a streptavidin-coated well of a microtiter plate with both a

TABLE 2. Standard errors of the means of IC_{50}s for representative isolates for which there were multiple IC_{50} determinations

Subject no.	Time (mo) on ddI[a]	Standard error of the mean of IC_{50}s (μM)		
		ddI	AZT	ddC
52	0	0.5		0.001
52	16	2.2		0.07
27	0	0.3	0.01	
27	16	0.8	0.02	
50	0	0.6	0.07	0.001
50	8 (c)	2.2	0.007	0.02
50	8 (p)	4.5		
45	7		0.003	

[a] One isolate from subject 50 obtained following 8 months of ddI therapy was derived from PBMCs (c), and the other isolate was derived from the plasma (p) of the same blood sample.

TABLE 3. Drug susceptibility phenotypes of cloned viruses

Cloned virus	Mean IC_{50} (μM)[a]		
	ddI	ddC	AZT
Wild type (HxB2)	0.8	0.06	0.002
Mutant Tyr-215 and Gln-219	1.1	0.09[b]	0.017
Mutant Val-74	2.0[c]	0.23[d]	0.003[b]
Mutant Val-74, Tyr-215, and Gln-219	4.0[e]	0.32[b]	0.004

[a] Each IC_{50} is the mean of two to seven PDQ susceptibility testing experiments (6) in which infections of PBMCs were performed in triplicate or quadruplicate at each of four drug concentrations, except as indicated otherwise.

[b] Only a single experiment was done.

[c] The mean ddI IC_{50} for Val-74 was greater than that for HxB2 ($P < 0.05$, one tailed).

[d] The mean ddC IC_{50} for Val-74 was greater than that for HxB2 ($P < 0.05$, one tailed).

[e] The mean ddI IC_{50} for Val-74, Tyr-215, and Gln-219 was greater than that for HxB2 ($P < 0.05$, one tailed) and that for Val-74 ($P < 0.05$, one tailed).

biotinylated oligodeoxynucleotide capture probe and a horseradish-peroxidase-labelled detection probe. After extensive washing, tetramethylbenzidine (TMBlue; Transgenic Sciences, Worcester, Mass.) was added to each well. The horseradish peroxidase-catalyzed color development was stopped after 1 h by the addition of hydrochloric acid to 0.5 N. The optical density at 450 nm was quantified by using a plate reader (Molecular Devices, Menlo Park, Calif.). IC_{50}s were determined as described above for virus susceptibility testing.

Statistics. Nonparametric tests of statistical inference (Wilcoxon signed rank test for correlated samples and Mann-Whitney U test for the independent samples in Tables 3 and 4) were used for IC_{50} comparisons.

RESULTS

RT Leu-74 → Val-associated ddI resistance. HIV-1 isolates with the RT Leu-74 → Val substitution that confers ddI resistance (37) were obtained from 11 of 16 subjects during ddI therapy (Tables 1 and 2). The mean ddI IC_{50} increased for paired isolates in which a Leu-74 → Val substitution developed during ddI therapy (0.8 versus 3.5 μM; $P = 0.03$, Wilcoxon signed rank test).

Infections of PBMCs with cloned mutant virus constructed by in vitro mutagenesis of HIV-1 HxB2 were assessed by the PDQ method. The codon 74 mutation alone conferred only a small degree of ddI resistance (0.8 μM for the wild type versus 2.0 μM for the Val-74 mutant; $P < 0.05$, Mann-Whitney U test) and ddC resistance (0.06 μM for the wild type versus 0.23 μM for the Val-74 mutant; $P < 0.05$, Mann-Whitney U test) (Table 3). Passage and PDQ susceptibility testing of cloned viruses in the MT-2 cell line, rather than in PBMCs, augmented the maximum increase in the IC_{50} of ddI to five- to eightfold above that for the wild-type virus (data not shown). ddI-resistant viruses containing only the Leu-74 → Val substitution without any accompanying AZT-selected substitutions were isolated during ddI therapy in vivo from subjects who had not previously been treated with AZT (Table 1). This observation is consistent with a selective advantage for such isolates.

The fact that the low degree of ddI resistance conferred by Leu-74 → Val alone was sufficient to provide a replication advantage to HIV-1 in the presence of ddI was also supported by the results of cell culture experiments. Equal mixtures of cloned viruses, one wild type and one mutant (Leu-74 → Val), were passaged once in vitro in PHA-stimulated PBMCs in the presence of ddI. After 17 days in culture, only the mutation encoding Val-74 was detected by direct DNA sequencing of PCR products, although equal mixtures of wild-type and mutant viruses were found at earlier time points. In contrast, in the absence of ddI, the relevant base in codon 74 persisted as an equal mixture of wild-type and mutant virus throughout 24 days in culture.

ddI resistance conferred by the Leu-74 → Val substitution of RT is augmented by Tyr-215 and Gln-219. The hypothesis that resistance to ddI conferred by the Leu-74 → Val substitution was augmented by specific AZT-selected substitutions was tested by using cloned viruses. Cloned mutant viruses rather than clinical isolates were used in order to avoid the potential influence of genetic heterogeneity on the IC_{50} of ddI. Numerous coding changes were present in the codons of clinical isolates other than those whose interactive effects were under study (Table 1). Codons with mixtures of wild-type and mutant bases were also often seen in these clinical isolates at the positions of interest (Table 1). The use of genetically homogeneous, cloned viruses allowed comparisons of only the mutations under study without any effects of other coding differences or mixtures at the position under study.

For viruses which contained Leu-74 → Val, Thr-215 → Tyr, and Lys-219 → Gln substitutions, the IC_{50} of ddI was greater than that for viruses containing only Leu-74 → Val (4.0 versus 2.0 μM; $P < 0.05$, Mann-Whitney U test) (Table 3). During ddI monotherapy, most AZT-selected substitutions persisted in clinical isolates from five of the six subjects who had received prior AZT treatment (Table 1), which was consistent with the concept that the augmentation of ddI resistance confers a selective advantage during ddI therapy in vivo.

Effects of Val-74, Tyr-215, and Gln-219 in cell-free polymerase assays. The effects of the ddI- and AZT-selected substitutions Val-74, Tyr-215, and Gln-219 on the susceptibilities of nucleoside analog triphosphates were studied in cell-free assays in vitro by using recombinant-expressed RTs. Consistent differences in the IC_{50}s of AZT-TP or ddATP for the different RTs were not observed (data not shown) with homopolymeric oligodeoxynucleotide primer-RNA templates in a standard, tritium-based polymerase assay (17). However, a different assay with a heteropolymeric primer-template (see Materials and Methods) did

TABLE 4. Inhibition of RNA-dependent DNA polymerase activity of recombinant-expressed RTs

Recombinant-expressed RT	Mean IC_{50} (μM)[a]		
	ddATP	ddCTP	AZT-TP
Wild type (HxB2)	6.8	4.8	0.039
Mutant Tyr-215 and Gln-219	8.4	4.3	0.123[b]
Mutant Val-74	16.9[c]	9.4	0.031
Mutant Val-74, Tyr-215, and Gln-219	35.9[d]	16.2[e]	0.124[b]

[a] Each IC_{50} is a mean of 3 to 10 experiments in which polymerase assays (RT DETECT; DuPont Medical Products) were performed in duplicate at each of six nucleoside analog triphosphate inhibitor concentrations. Lysates of bacteria containing recombinant-expressed RTs were used for determination of ddATP and ddCTP IC_{50}s. The ddATP IC_{50}s determined from purified enzymes did not differ from those listed for either wild-type or mutant Val-74, Tyr-215, and Gln-219 enzymes (see text). AZT-TP IC_{50}s were determined for the wild type and each mutant by using purified enzymes.

[b] Mean AZT-TP IC_{50}s for Tyr-215 and Gln-219 and for Val-74, Tyr-215, and Gln-219 were each greater than that for HxB2 (for each comparison, $P < 0.05$, one tailed).

[c] The mean ddATP IC_{50} for Val-74 was greater than that for HxB2 ($P < 0.05$, one tailed).

[d] The mean ddATP IC_{50} for Val-74, Tyr-215, and Gln-219 was greater than that for HxB2 ($P < 0.05$, one tailed) and that for Val-74 ($P < 0.05$, one tailed).

[e] The mean ddCTP IC_{50} for Val-74, Tyr-215, and Gln-219 was greater than that for HxB2 ($P < 0.05$, one tailed).

discriminate between the susceptibilities of the different RTs.

The ddATP IC_{50}s for the recombinant-expressed RTs in bacterial lysates (Table 4) paralleled the ddI susceptibility pattern of virus replication (Table 3). The ddATP IC_{50} of the Val-74 mutant was significantly different from that of the wild-type enzyme. The increase in the IC_{50} of ddATP for mutant enzyme with Val-74, Tyr-215, and Gln-219 substitutions measured in a cell-free polymerase assay (Table 4) was similar to the augmentation in the IC_{50} of ddI for virus measured in infected cells in culture by a whole-virus phenotypic assay (Table 3). The IC_{50}s of ddCTP for mutant enzymes also paralleled the IC_{50}s of ddC for mutant viruses to ddC (Table 4). When purified enzyme was used in this cell-free polymerase assay, the IC_{50}s of ddATP for wild-type (7.1 μM) and mutant enzyme with Val-74, Tyr-215, and Gln-219 substitutions (34.2 μM) were equivalent to those seen in assays of crude lysates (Table 4).

The IC_{50}s of ddATP for the virion-associated RTs of two ddI-resistant isolates containing the Val-74 substitution (obtained from subject 44 after 18 months and from the plasma of subject 50 after 8 months of ddI therapy; see Table 1) were also determined. The degree of resistance of the virion-associated RT activities of these isolates to ddATP (range of ddATP IC_{50}s, 13.7 to 34.8 μM), relative to that of a ddI-susceptible isolate (ddATP IC_{50}, 6.2 μM), was also similar to the two- to fivefold magnitude of ddI resistance observed in infected cells (Tables 1 to 3).

The correlation between the AZT-TP susceptibilities of recombinant-expressed mutant RTs in cell-free polymerase assays (Table 4) and the AZT susceptibilities of the corresponding virus in infected cells (Table 3) was not as consistent as that observed for ddATP and ddI. An increase in the IC_{50} of AZT-TP from that for the wild type was apparent for the mutant RT with Tyr-215 and Gln-219 substitutions on a heteropolymeric primer-template (Table 4). This change was of a smaller magnitude than the increase in the AZT IC_{50} measured for the mutant virus in infected cells with Tyr-215 and Gln-219 substitutions (Table 3). Moreover, the AZT-TP IC_{50} for the mutant recombinant-expressed RT with Val-74, Tyr-215, and Gln-219 substitutions was not different from that for mutant recombinant-expressed RT with Tyr-215 and Gln-219 substitutions (Table 4). In contrast, in infected cells the addition of Val-74 to Tyr-215 and Gln-219 decreased the AZT IC_{50} from the elevated level for the mutant virus with substitutions at Tyr-215 and Gln-219 back to the level for the wild-type virus (Table 3).

AZT resistance in isolates obtained during ddI therapy. The effects of RT gene mutations seen in clinical isolates obtained during ddI therapy on AZT resistance were also characterized. At the beginning of ddI therapy, six subjects had viruses with an AZT-resistant RT genotype. Except for the Lys-70 → Arg substitution, AZT-selected substitutions persisted in viruses isolated during ddI therapy (Table 1). Four of these isolates were found to contain the ddI-selected Val-74 substitution; three of these remained resistant to AZT and acquired resistance to ddI and ddC (Tables 1 and 2). Despite the discontinuation of AZT, the virus from one subject (subject 52) developed a higher level of AZT resistance and virus from another subject (subject 27) maintained an intermediate level of AZT resistance. A third isolate (from subject 48) obtained during ddI therapy after stopping AZT therapy also had ddI, ddC, and high-level AZT resistance. Each of these three isolates had a different level of AZT resistance and some genetic differences, but each contained Met-41 → Leu and Thr-215 → Tyr substitutions (Tables 1 and 2). A fourth isolate (from subject 50) contained the mutation at codon 74 and persistent AZT resistance mutations in codons other than codon 41. It showed a decrease in AZT resistance coincident with the appearance of ddI resistance (Table 1).

DISCUSSION

The effects of specific combinations of AZT- and ddI-selected mutations in RT codons 74, 215, and 219 on drug and inhibitor susceptibilities were compared in cloned mutant viruses and recombinant-expressed enzymes. Changes in virus susceptibility to ddI and ddC caused by Val-74 are reflected in the enzyme assays and may result from a change in the enzyme's ability to bind to ddATP and ddCTP. A similar correlation was not seen for the susceptibility of virus to AZT and enzyme to AZT-TP conferred by the Tyr-215 and Gln-219 substitutions. Val-74 also suppressed AZT resistance in virus with Tyr-215 and Gln-219 substitutions because of a functional alteration in addition to changes in AZT-TP binding. In some clinical isolates, the combined effect of Val-74 and AZT resistance mutations was multiple nucleoside resistance. The effects of Val-74 are discussed further below.

Resistance to ddI has previously been documented only for patients who received AZT therapy before starting ddI therapy (31, 34, 37). Viruses resistant to ddI because of the mutation at codon 74 occured in individuals who were receiving ddI but who had not received prior antiretroviral therapy, although the degree of ddI resistance attributable to the Val-74 substitution alone was small. The two- to fourfold increases in the IC_{50} of ddI noted here for both clinical isolates and cloned viruses appear to provide a selective advantage in vivo and a growth advantage in cell culture over wild-type virus in the presence of ddI.

A potentially clinically relevant effect of combining ddI- and AZT-selected substitutions was defined here for the first time. The AZT resistance mutations at codons 215 and 219 augment the ddI resistance conferred by the mutation at codon 74. These and other AZT resistance mutations may

persist during ddI therapy, perhaps because they provide a selective advantage to virus replication in the presence of ddI after acquisition of the mutation at codon 74. In contrast to the persistence of AZT resistance mutations in isolates from five of six subjects, isolates from 5 of 15 subjects who stopped AZT therapy without starting any alternate anti-HIV-1 therapy were reported to have reverted to AZT sensitivity (26). Neither AZT-resistant clinical isolates obtained at the initiation of ddI therapy (Tables 1 and 2) nor cloned mutant virus containing only the mutations at codons 215 and 219 (Table 3) were resistant to ddI. These AZT-selected substitutions did not increase the IC_{50} of ddI unless they were present in combination with the ddI-selected substitution at position 74. Therefore, preexisting AZT resistance may lead to more pronounced increases in the resistance of virus to ddI. This augmentation of ddI resistance was evident in the IC_{50}s of ddATP for mutant enzymes measured in cell-free polymerase assays. The resistance of virus to ddC conferred by the mutation at codon 74 was also reflected in the IC_{50}s of ddCTP for the enzyme.

We also confirmed that the addition of a mutation at codon 74 to certain combinations of AZT resistance mutations (at codons 215 and 219) can reverse the AZT resistance phenotype (37). However, the suppressive effect of the Val-74 substitution on AZT resistance caused by the Tyr-215 and Gln-219 substitutions was not observed in polymerase assays in vitro. The lack of reversal of AZT-TP resistance by the mutation at codon 74 suggests that a function that is not measured in the cell-free polymerase assay mediates the suppressive effect of the mutation at codon 74 on the AZT resistance phenotype.

The effects of the Tyr-215 and Gln-219 substitutions on AZT-TP susceptibility were seen in cell-free assays of polymerase activity on a heteropolymer oligodeoxynucleotide primer-RNA template. However, the effect of these mutations at codons 215 and 219 on AZT-TP resistance (Table 4) was less than half that seen on AZT resistance of virus in infected PBMCs, as measured by the PDQ assay (Table 3). Additional preliminary experiments also indicated that a mutant enzyme containing AZT-selected substitutions at positions 67, 70, 215, and 219 was not as resistant to AZT-TP as expected on the basis of previous reports of the high-level AZT resistance seen in viruses with these mutations (30, 37, 38, 40). The lack of the expected resistance of this mutant enzyme to AZT-TP confirmed the observations of Lacey et al. (25). Nucleoside analog triphosphate binding is measured in such cell-free polymerase assays (12, 25). It appears that an RT function that is not measured in these cell-free polymerase assays is involved in the AZT resistance of virus in infected cells. In contrast, the function(s) measured in the cell-free polymerase assay accounted for the ddI resistance phenotypes of the mutations examined in the present study. The synergistic inhibition seen in vitro with a combination of AZT and ddI (18) or AZT and ddC (7) is consistent with the hypothesis that AZT and ddI resistance mechanisms involve different RT functions.

A third effect of a combination of AZT- and ddI-selected substitutions on drug susceptibility was observed. Several AZT-resistant clinical isolates that acquired the mutation at codon 74 during ddI therapy following AZT withdrawal maintained some degree of AZT resistance. These data document the fact that reversal of AZT resistance does not always occur with acquisition of the mutation at codon 74. Each of the isolates which exhibited some degree of AZT resistance (Table 1, subjects 52, 27, and 48) contained the mutation at codon 74 in combination with the mutations at codons 41 and 215. Studies of cloned mutant viruses are necessary to confirm the hypothesis that the phenotype of multiple nucleoside resistance is due to a particular combination of mutations (e.g., at codons 41, 74, and 215), and such mutants have not yet been reported (28, 37). The multiple nucleoside-resistant clinical isolates characterized in the present study demonstrate that caution must be used in generalizing about drug susceptibility effects beyond the specific mutations studied in cloned mutant viruses.

The fact that AZT resistance does not invariably decrease with the addition of the mutation at codon 74 of RT to an AZT-resistant HIV-1 *pol* gene is consistent with the functional differences in the AZT and ddI resistance mechanisms indicated by our enzyme studies. On the basis of the data presented here, it is unlikely that the Leu-74 → Val substitution causes mutually exclusive changes in AZT-TP and ddATP binding to RT in vitro. In a structural model of the RT-nevirapine complex, it is also of interest that amino acid residues 74 and 215/219 are not physically approximated to the polymerase active site or to each other (22). Speculation has centered on AZT resistance involving another factor(s) that is present in a RT replication complex in the infected cell but that is not assessed in cell-free RT polymerase assays (25). However, RT polymerase assays on heteropolymeric primer-templates do detect some degree of AZT-TP resistance (12, 25) (see above). Further characterization of the interactive effects of mutations which either maintain or suppress AZT resistance (28, 37) may facilitate identification of the aspect of RT function that contributes to AZT resistance. Such correlations of RT structure and function may help to design improved inhibitors that are less likely to allow the emergence of resistance.

Regimens of currently available nucleoside analogs may be less effective for individuals initially infected with multiply resistant HIV-1 isolates like the ones described here. Efficacy was demonstrated in earlier studies among subjects who were presumably infected with viruses susceptible to each of these nucleosides (8, 9, 19, 39). AZT resistance has been associated with disease progression during therapy (38). If nucleoside resistance is a cause of treatment failure, it may become a priority to optimize treatment strategies that limit the development of multiply resistant viruses. In developing approaches to limiting drug resistance, various strategies warrant rapid evaluation in clinical trials: comparison of different drug combination regimens (3), administration of monotherapies or combinations in various sequences, and early treatment. A more rapid assay to screen for the emergence of ddI resistance caused by the mutation at codon 74, such as the virion RT enzyme ddATP susceptibility test used in the present study, may also prove to be clinically useful.

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