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Human Immunodeficiency Virus Type 1 *pol* Gene Mutations in an AIDS Patient Treated with Multiple Antiretroviral Drugs

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Multiple mutations were found in the human immunodeficiency virus *pol* gene following treatment of an AIDS patient with antiretroviral drugs. After approximately 2.5 years of monthly alternating therapy with 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC), most of the *pol* sequences amplified from the patient's peripheral blood mononuclear cell DNA contained known AZT resistance mutations at codons 41, 67, and 215 and a putative ddC resistance mutation at codon 69 as well as other novel mutations. These mutations persisted for 6 months after the patient was switched to 2′,3′-dideoxyinosine monotherapy. Mutations known to be associated with 2′,3′-dideoxyinosine resistance did not occur during this time. Antiviral susceptibility testing of point mutants, introduced into the genetic background of laboratory strain NL4-3, showed that the codon 41 mutation antagonized ddC resistance when present with the codon 69 mutation. However, this antagonism was not found with a chimeric mutant containing the patient's *pol* gene sequence from codons 25 to 218, implying that other mutations compensated for the antagonism. Thus, alternating therapy with AZT and ddC resulted in the selection of viruses resistant to both drugs.

Clinical trials have shown that patients with AIDS usually benefit from nucleoside analog therapy, although these drugs frequently cause specific toxic side effects (4, 19, 30, 31). In most patients, $CD4^+$ cell counts increase and the frequency of opportunistic infections decreases (4, 19, 30, 31). Unfortunately, the beneficial effect in most cases is transient; symptoms eventually reappear, and the infections become refractory to therapy (3, 20).

A number of groups (1, 11, 13, 17, 22, 24) have now shown that viruses isolated from patients treated with 3′-azido-3′-deoxythymidine (AZT) for prolonged periods of time are less susceptible to AZT than viruses taken from the same patients prior to therapy. This resistance phenotype has been found to correlate with *pol* gene mutations which cause amino acid substitutions at five positions in the reverse transcriptase (RT), positions 41 (M→L), 67 (D→N), 70 (K→R), 215 (T→Y or F), and 219 (K→Q) (10, 15). Various combinations of these mutations have been found in AZT-treated individuals and appear to cause various levels of AZT resistance (14, 15, 23). Cross-resistance is restricted to nucleoside analogs containing a 3′-azido group (12).

St. Clair et al. (27) have described another mutation at position 74 (L→V) causing resistance to 2′,3′-dideoxyinosine (ddI). The ddI resistance mutation was found to cause cross-resistance to 2′,3′-dideoxycytidine (ddC) but antagonized AZT resistance. A mutation at codon 184 (M→V) likewise appears to cause resistance to both ddI and ddC (8).

In addition, we have previously described mutations at positions 69 (T→D) and 165 (T→I) in proviral DNA from a patient who had undergone long-term ddC therapy (6); neither is present in wild-type sequences in the human immunodeficiency virus (HIV) data base (21). When the T-69→D mutation was transferred to a laboratory strain of HIV, it caused a fivefold increase in the 50% inhibitory concentration (IC_{50}) of ddC but did not affect susceptibility to AZT or ddI. The T-165→I mutation had no effect.

Possible strategies for preventing or delaying the emergence of resistance include alternating or concurrent therapy with two or more drugs. Multidrug therapies may be equally important in preventing or reducing toxicities associated with each drug alone (26).

Here we report *pol* genotypes found in one individual at three time points during therapy. In this patient, alternating therapy with AZT and ddC resulted in the selection of viruses with mutations known to cause resistance to AZT and with the putative ddC resistance mutation T-69→D. These mutations persisted for at least 6 months, during which alternating therapy was discontinued and the patient was treated with ddI. We also report the phenotypes of viruses, constructed by site-directed mutagenesis, which contain combinations of these mutations and of a chimeric virus containing a portion of the RT-coding region derived from the patient's viruses.

(Some of these results were presented at the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy [5].)

MATERIALS AND METHODS

Amplification and analysis of *pol* sequences. Primers P5 and P7, described previously (7), were used in the polymerase chain reaction (PCR) to amplify a 654-bp *pol* gene fragment encoding RT amino acids 63 to 279. For some samples the primer P5up (5′ AAAGGTACCTGTCAACATAATTGGAAGA 3′) was used in combination with P7 to amplify an 865-bp *pol* gene

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TABLE 1. Oligonucleotide probes

Probe	Sequence	NL4-3 coordinates (21)
P41M	5′-CAGAATTGGAAAAGGA-3′	2665–2680
P41WT	5′-CAGAAATGGAAAAGGA-3′	2665–2680
P67/69WTRC	5′-TAGTACTATCTTTTTTCTT-3′	2757–2739
P67/69M	5′-AAAGAAAAAAAATAGTGATA-3′	2738–2757
P67M	5′-AAGAAAAAAAATAGTACTA-3′	2739–2757
P70WT	5′-TAGATGGAGAAAATTAGTA-3′	2756–2774
P70MRC	5′-TACTAATTTTCTCCATTTA-3′	2774–2756
P215M	5′-TGGGGATTTTACACACCAGAC-3′	3183–3203
P210WTRC	5′-CCACTTCAACAGATGTT-3′	3185–3169
P210M	5′-ACATCTGTGGAAGTGG-3′	3170–3185

fragment which includes codons 1 to 279. A *Kpn*I restriction site (underlined) facilitated cloning of the amplified fragment; P7 includes a *Pst*I restriction site. PCR amplification was performed as previously described (7). Clones carrying *pol* gene fragments were identified and analyzed by colony hybridization using oligonucleotide probes as previously described (7). Probes used in this study are listed in Table 1 with corresponding HIV type 1 NL4-3 coordinates. Sequencing was done with the Sequenase Version 2 Kit (United States Biochemical, Cleveland, Ohio). Sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (2).

Mutagenesis. Site-directed mutagenesis was performed by the overlap extension technique (9). To construct full-length HIV proviruses containing mutations at RT amino acid positions 41, 67, and 69 (and combinations thereof), we used the procedure described previously (6). In short, overlap extension was used to construct a 711-bp *Pst*I fragment containing the mutation(s). The *Pst*I fragment was substituted for the corresponding fragment of a plasmid containing the HIV type 1 NL4-3 *pol* gene (pJF1 [6]). Replacement of a unique *Bal*I (*Msc*I) fragment of the full-length proviral clone (pNL4-3) with that of the subclone resulted in the full-length mutant construct. This approach was also used to construct proviral clones with mutations at positions 210 and 215 except that a 507-bp *Eco*RV-*Age*I restriction fragment (see Fig. 2A) was constructed (the *Pst*I fragment does not include these codons).

The chimeric clone pJF138M1 is diagrammed in Fig. 2A. To construct this clone, we first amplified a 460-bp fragment from one of the late-AZT-ddC patient clones (obtained after 23 months of alternating AZT-ddC therapy). This region corresponds to bases 2744 to 3203 (RT amino acids 66 to 218) of the NL4-3 sequence. Adjoining upstream and downstream regions were then amplified from pJF1 (see above and reference 6). The amplified areas encompassed bases 2097 to 2766 and 3183 to 3549 of NL4-3; the upstream fragment included some vector sequence (67 bp of pUC118 [28]). Overlapping ends enabled joining of the three fragments in subsequent PCRs. The final product was digested with *Hin*dIII and *Age*I; this fragment then replaced the corresponding fragment of pJF1 to create pJF138A. A 526-bp fragment (corresponding to NL4-3 bases 2593 to 3118) was then amplified from a late-AZT-ddC patient clone with an upstream primer that included an *Xba*I site. This fragment was digested with *Xba*I and *Eco*RV and substituted for the *Xba*I-*Eco*RV restriction fragment of pJF138A to create pJF138A1. Replacement of the unique *Bal*I fragment of pNL4-3 with that of pJF138A1 then yielded pJF138M1. In this clone, the patient sequence replaced the NL4-3 sequence over the region encoding RT amino acids 25 (*Bal*I site) to 218. See Fig. 2B for a comparison of the NL4-3 and mutant amino acid sequences for this area.

For mutagenesis, PCRs were run for 25 cycles as follows: 1 min at 94°C, 2 min at 45°C, and 2 min at 70°C, using either *Taq* polymerase (Perkin-Elmer Cetus, Emeryville, Calif.) or *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, Calif.). All HIV mutant constructs were verified by sequencing.

Plaque reduction assays. Transfection of HeLa $T4^+$ cells and preparation of virus stocks have been described previously (6). A quantitative plaque reduction assay (12) was used to assess viral susceptibility to ddC (Hoffmann-La Roche Inc., Nutley, N.J.), ddI (Sigma Chemical Co., St. Louis, Mo.), and AZT (Burroughs Wellcome Co., Research Triangle Park, N.C.). Plaque reduction assays were performed with HeLa $T4^+$ cells (16) at an initial cell density of 1.5×10^4 to 2×10^4 cells per well and a virus input of 100 to 300 PFU per well.

Nucleotide sequence accession numbers. Sequences in this paper have been submitted to GenBank (accession numbers: early sample, L08677 to L08682, L08684, L18776, and L18777; late-AZT-ddC sample, L18761 to L18770; post-ddI sample, L08683 and L18771 to L18775).

RESULTS

Three peripheral blood mononuclear cell (PBM) samples from a patient treated with multiple antiretroviral drugs were analyzed (Fig. 1). The first was obtained after 7 months of AZT therapy, which was sporadic because of toxicity, but prior to ddC therapy (the early sample); the second was obtained after 23 months of alternating AZT and ddC (the late-AZT-ddC sample); and the third was obtained after 6 months of ddI therapy (the post-ddI sample). Initially, a 654-bp fragment of the *pol* gene encompassing RT codons 63 to 279 was amplified from the first two samples. Additional PCR products, obtained later from these two PBM samples and from the post-ddI sample, included the area encoding RT amino acids 1 to 279. The amplified products were cloned, and several clones were sequenced from each sample. In addition, oligonucleotide probes corresponding to mutant and wild-type sequences were designed, and colony hybridizations were used to screen larger numbers of PCR-derived clones for the AZT resistance mutations M-41→L, D-67→N, K-70→R, and T-215→Y; the ddC resistance mutation T-69→D; and the L-210→W mutation (all prevalent in late-AZT-ddC and post-ddI clones but not in early clones). Table 2 summarizes colony hybridization and sequencing data obtained from each of the three PBM samples.

The majority of the clones from the early therapy PBM sample were wild type at all resistance loci analyzed. Approximately 10% (2 of 22) carried the AZT resistance mutation at codon 70. In contrast, the majority of the clones from the late-AZT-ddC sample had mutations at codons 41, 67, 69, 210, and 215.

Six clones obtained from the post-ddI PBM sample were sequenced over the area encoding amino acids 1 to 279; five had mutations at codons 41, 67, 69, 210, and 215. None carried the mutations at codons 74 (27) and 184 (8) which have been shown to cause ddI resistance and cross-resistance to ddC. See Fig. 3 for an alignment of the consensus amino acid sequences from each PBM sample over the area analyzed.

To explore the effects of the mutations at codons 41, 67, 69, 210, and 215 and possible interactions among them, we constructed molecular clones of HIV with combinations of these mutations and also a chimeric virus containing a portion of the RT coding region from a late-AZT-ddC patient clone (Fig. 2).

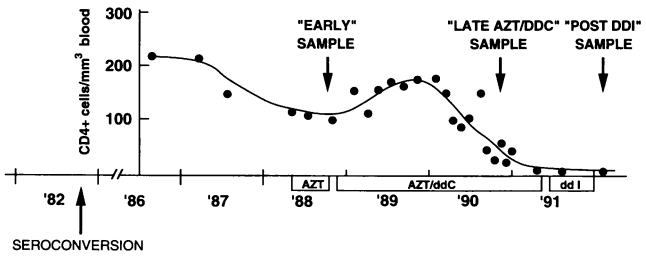

FIG. 1. Patient course. $CD4^+$ cell counts were obtained prior to and during antiviral therapy. The times of the first positive HIV serology and of PBM samplings are shown by arrows. Antiviral therapy regimens: AZT, AZT monotherapy (treatment was initiated at 1,200 mg/day but was sporadic because of anemia even though the dosage was lowered twice during this period to 600 and 400 mg/day); AZT/ddC, monthly alternating AZT (1,200 mg/day) and ddC (0.18 mg/kg of body weight per day); ddI, ddI monotherapy (334 mg/day).

After transfections, corresponding viruses were tested for susceptibility to AZT, ddC, and ddI.

Table 3 shows the IC_{50}s obtained. The codon 67 mutation was found to antagonize ddC resistance conferred by the codon 69 mutation (compare JF8 with JF4A), but this combination had no effect on AZT susceptibility. The latter result was expected; the codon 67 AZT resistance mutation exerts an effect only when present with other AZT resistance mutations (14, 15). Addition of the codon 215 mutation (JF14) returned the ddC IC_{50} to levels approximately fourfold higher than that for the wild type and increased the AZT IC_{50} ninefold. The codon 210 mutation did not affect AZT resistance either alone (JF10) or in combination with other mutations (JF20). Addition of the codon 41 mutation to JF20 was found to antagonize ddC resistance but further increased AZT resistance (JF26). The chimeric clone JF138M1 included post-AZT-ddC patient sequence from codons 25 to 218 with mutations at codons 41, 67, 69, 210, and 215 in addition to other differences from the wild-type clone, NL4-3 (Fig. 2). This virus expressed high-level AZT resistance and retained resistance to ddC in the presence of the codon 41 mutation.

None of the mutant viruses listed in Table 3 displayed a significant increase in the IC_{50} of ddI, nor did a variant of JF138M1 containing a mutation, T-39→A (Fig. 3), specific for the post-ddI sample (data not shown).

TABLE 2. HIV RT genotype analysis

Sample yielding clones	Amino acid[a] at RT position:						No. of clones
	41	67	69	70	210	215	
Before AZT-ddC therapy	—	d	t	k	l	t	14
	—	d	t	R	l	t	2
	—	d	t	k	Ind.	t	4
	—	d	t	Ind.	l	t	2
	m	—	—	—	—	—	12[b]
	Ind.	—	—	—	—	—	1[b]
Late AZT-ddC (23 mo)	—	N	D	k	W	Y	22
	—	N	D	R	W	Y	1
	—	N	t	R	W	Y	1
	—	d	D	k	W	Y	1
	—	d	t	k	l	Y	1
	—	N	D	k	Ind.	Y	1
	—	d	t	k	l	t	1[c]
	L	—	—	—	—	—	19[b]
	m	—	—	—	—	—	3[b]
	Ind.	—	—	—	—	—	2[b]
Post-ddI (6 mo)[b]	L	N	D	k	W	Y	5
	m	d	t	k	l	t	1[c]

[a] Mutant amino acids are in uppercase, and wild-type amino acids are in lowercase. "Ind." (indeterminate) indicates hybridization with neither mutant nor wild-type oligonucleotide for clones not sequenced. Clones that were indeterminate by hybridization and that were sequenced were found to have nearby, presumably neutral, mutations. —, loci that were not screened.

[b] The third (post-ddI) sample was amplified to include RT codons 1 through 279. The first two samples were initially amplified to include codons 63 through 279. Clones from these PCR products were the source of the tabulated data for RT positions 67, 69, 70, 210, and 215. Subsequently, amplifications of the first two samples were performed to yield the larger segment, and clones were scored for residue 41 by colony hybridization.

[c] These clones were sequenced and contained frameshifts or stop codons which preclude encoding active RT.

DISCUSSION

The patient studied here could not tolerate AZT for prolonged periods because of anemia (4, 19, 30, 31). Alternating therapy with AZT and ddC reduced AZT toxicity to tolerable levels, but after 23 months, there were few sequences that did not carry resistance mutations and the vast majority had resistance mutations at codons 41, 67, 69, and 215.

The fact that this patient, like a ddC-treated patient studied previously (6), harbored the D-69 mutation only after ddC therapy is consistent with its being a ddC resistance mutation; D-69 is otherwise absent or very rare in nature (21). In a recent study (25), this mutation was found in only one of four patients after long-term alternating AZT-ddC therapy. However, those *pol* gene sequences were obtained from virus isolates grown in uninfected-donor PBMs in the absence of AZT and ddC. Such cultivation may tend to select against some resistance muta-

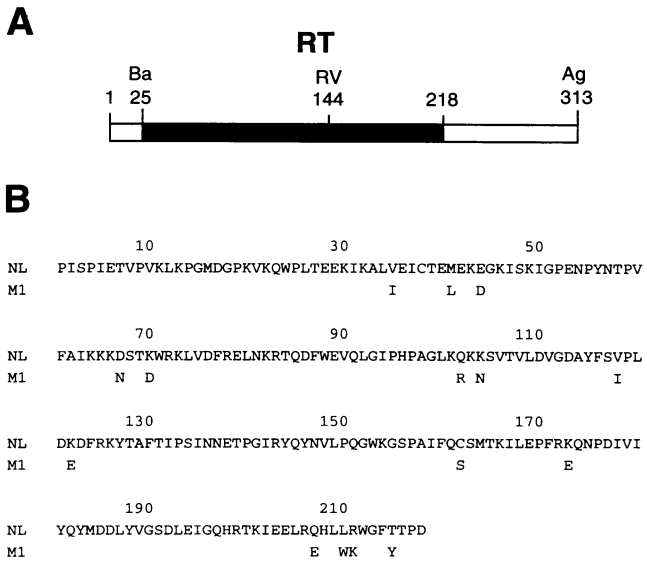

FIG. 2. Diagram of the chimeric mutant JF138M1. (A) 5′ RT coding region of clone JF138M1. The portion of the coding region derived from patient sequence is shaded, and that from NL4-3 is not. Codon numbers and restriction sites are indicated (Ba, *Bal*I; RV, *Eco*RV; Ag, *Age*I). (B) Alignment of the N-terminal region of the RT of viruses NL4-3 (NL) and JF138M1 (M1). Only amino acids differing from the NL4-3 sequence are shown.

TABLE 3. Susceptibility phenotypes

Virus construct	RT residue[a] at position:					IC_{50} (μM ± SD)[b] of:		
	41	67	69	210	215	AZT	ddC	ddI
NL4-3	m	d	t	l	t	0.026 ± 0.002	0.41 ± 0.03	2.3 ± 0.2
JF4A	m	d	D	l	t	0.028 ± 0.003	2.15 ± 0.17	2.6 ± 0.1
JF8	m	N	D	l	t	0.026 ± 0.008	0.59 ± 0.04	2.3 ± 0.5
JF14	m	N	D	l	Y	0.23 ± 0.09	1.63 ± 0.13	2.3 ± 0.5
JF18	m	N	t	l	Y	0.21 ± 0.07	0.53 ± 0.18	3.1 ± 0.6
JF10	m	d	t	W	t	0.044 ± 0.013	0.63 ± 0.15	1.8 ± 0.3
JF20	m	N	D	W	Y	0.26 ± 0.02	1.48 ± 0.23	3.5 ± 0.3
JF26	L	N	D	W	Y	2.55 ± 0.69	0.39 ± 0.12	1.9 ± 0.3
JF138M1[c]	L	N	D	W	Y	1.75 ± 0.49	1.50 ± 0.14	2.7 ± 0.4

[a] Wild-type residues are shown in lowercase; mutant residues are in uppercase.
[b] Each value is the average from at least two separate assays.
[c] Patient genetic background from amino acid 25 to 218 (see text).

tions. Additional ddC-treated patients must be studied to determine the clinical significance of the D-69 mutation.

Antiviral susceptibility testing of mutant constructs demonstrated that the codon 69 mutation did not affect the AZT IC_{50} either alone or in combination with two AZT resistance mutations. Other workers have found the D-69 mutation in an occasional patient believed to have been treated only with AZT (18, 29), causing speculation that this mutation contributes to AZT resistance (29); the present results provide no support for this.

The codon 41 mutation was found to antagonize ddC resistance when present in combination with mutations at codons 67, 69, 210, and 215 in the laboratory strain NL4-3 (JF26). This antagonistic effect was not expressed by the chimeric clone JF138M1, which contained all of the resistance mutations present in JF26. The genetic background of this patient's HIV population apparently allowed expression of the ddC resistance phenotype. Certain discrete mutations found in the late-AZT-ddC clones but not in the early clones may have compensated for the antagonism of ddC resistance caused by the codon 41 mutation. These include mutations at codons 35, 44, 102, 104, 118, and 122 (Fig. 3). Studies of other patients treated with a similar therapeutic regimen and of additional mutant constructs might help determine which, if any, of these mutations are important in regard to resistance.

Our results with the codon 210 mutation and previous results with a mutation at codon 165 (6) indicate that some mutations observed only after prolonged therapy do not cause or contribute to resistance. Perhaps these mutations exert an effect only in the context of a particular genetic background.

Our most important findings are that in this patient alternating therapy with AZT and ddC resulted in the selection of viruses with mutations causing resistance to both drugs. The genetic background of the patient's virus appeared to be involved in expression of the ddC resistance phenotype when the codon 41 mutation was present. None of the mutations reverted to the wild type during 6 months of ddI therapy, but mutations known to cause ddI resistance (8, 27) did not appear. Thus, although the virus could apparently become resistant to both AZT and ddC, ddI resistance might demand a set of mutations which could not be tolerated by an RT containing AZT and ddC resistance mutations (27). In vitro studies to determine the viability of mutants constructed with AZT, ddC, and ddI resistance mutations will help resolve this question.

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          10                  30                  50
Early     PISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALVEICTEMEKEGKISKIGPENPYNTPV
Late                                    I    L  D
Post                                    I  A L  D

          70                  90                  110
Early     FAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPL
Late        N D                                R N              I
Post        N D                                  N              I

          130                 150                 170
Early     DKDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPDIVI
Late       E
Post       E

          190                 210                 230
Early     YQYMDDLYVGSDLEIGQHRTKIEELREHLLKWGFTTPDKKHQKEPPFLWMGYELHPDKWT
Late                                  W   Y
Post                                  Y W   Y

          250                 270
Early     VQPIVLPEKDSWTVNDIQKLVGKLNWASQIYAGIKVKQL
Late
Post
```

FIG. 3. Alignment of consensus amino acid sequences from the three PBM samples: early, late AZT-ddC (Late), and post-ddI (Post). For the early and late-AZT-ddC samples, the region encoding amino acids 1 to 62 was derived from two sequenced clones. The remainder of these two sequences and the post-ddI sequence were derived from at least five sequenced clones. Residues not previously reported in any wild-type isolates are shown in boldface.

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