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Resistance to 2′,3′-Dideoxycytidine Conferred by a Mutation in Codon 65 of the Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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A human immunodeficiency virus type 1 variant resistant to zalcitabine (2′,3′-dideoxycytidine [ddC]) was selected by sequential passage in the presence of increasing concentrations of ddC in peripheral blood mononuclear cell cultures. A mutation causing a lysine-to-arginine substitution was noted in reverse transcriptase (RT) codon 65 of this ddC-selected virus. A cloned mutant virus with this codon 65 mutation was constructed by using a novel PCR approach for site-directed mutagenesis. Characterization of this virus confirmed that the RT Lys-65→Arg substitution was necessary and sufficient for a fourfold increase in the ddC 50% inhibitory concentration, as well as for resistance to didanosine (2′,3′-dideoxyinosine [ddI]). Lys-65→Arg and virus resistance to ddC and ddI also developed during therapy in isolates from one ddC-treated patient and two ddI-treated patients. Recombinant-expressed codon 65 mutant RT enzyme was resistant to ddCTP and ddATP in cell-free polymerase assays. Results of mutant enzyme studies are consistent with Lys-65→Arg leading to changes in binding of the triphosphate forms of these nucleoside analogs to the RT. These data have implications for future studies of ddC resistance, particularly those aimed at defining its clinical relevance.

Human immunodeficiency virus type 1 (HIV-1) mutants that are resistant to reverse transcriptase (RT) inhibitors have been selected during virus propagation in the presence of drug both in vitro and in vivo. The RT genotypes observed in resistant viruses selected in vitro are similar to those seen in clinical isolates from patients treated with zidovudine (3′-azido-3′-deoxythymidine [AZT]) (11, 19, 23, 25, 27), didanosine (2′,3′-dideoxyinosine [ddI]) (7, 11, 13, 35), 2′,3′-deoxythiacytidine (9, 10, 34), and nonnucleoside RT inhibitors (29, 33). However, only limited information is available about resistance to 2′,3′-dideoxycytidine (ddC) (zalcitabine), a chain-terminating nucleoside analog in clinical use (8, 28).

A mutation in RT codon 74 that is selected by ddI therapy in vivo (7, 35) confers resistance to ddC. Mutations in codon 184 occur in vivo (34) and in vitro (9, 10, 13) and cause low-level ddI and ddC resistance, as well as high-level 2′,3′-deoxythiacytidine resistance. A mutation in RT codon 69 was identified in a DNA sample from peripheral blood mononuclear cells (PMBC) from a ddC-treated patient (8). This codon 69 mutation did confer a low level of ddC resistance to a mutant virus in vitro (8), but it is not clear how frequently it occurs in viruses isolated during ddC treatment. In order to further study genetic bases for ddC resistance, in vitro selection for ddC-resistant HIV-1 was undertaken by propagating wild-type virus in the presence of ddC. The mutations selected by ddI or 2′,3′-deoxythiacytidine therapy have been reported to cause resistance to the triphosphate forms of these nucleoside analogs in studies of recombinant-expressed mutant RT enzymes (7, 34). To determine whether ddC resistance may also be due to a change in the binding of ddCTP to the RT, the ddCTP susceptibility of a mutant recombinant-expressed RT was studied in a cell-free polymerase assay system.

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MATERIALS AND METHODS

Cells. PBMC from HIV-1-seronegative blood donors were used for cocultivation and propagation of clinical isolates (16, 18), as well as for drug susceptibility assays (17). Cloned mutant viruses were generated by transfection of cloned plasmid DNAs into COS-7 cells. Stocks of cloned mutant viruses were generated by passage of transfection supernatant fluids in MT-2 cells.

Viruses. Clinical isolates were obtained by primary coculture of patient PBMC with phytohemagglutinin-stimulated donor PBMC (7, 18). A previously characterized HIV-1 clinical isolate derived from an AIDS patient who had not yet received any antiretroviral therapy (isolate 14a-pre) (18) was used as the starting inoculum in the in vitro selection experiment. Cryopreserved PBMC from ddC-treated patients who had been enrolled in AIDS Clinical Trials Group protocol 012 were cocultivated with phytohemagglutinin-stimulated donor PBMC. Clinical isolates obtained following prolonged ddI therapy were available from a previous study of patients treated with investigational ddI therapy (7).

Viruses with wild-type and mutant RTs were also derived from molecular plasmid clones of HIV-1 (HxB2). A molecular plasmid clone called pHxB2ΔBalI, which had the 1.8-kb *BalI* restriction fragment deleted from the polymerase (*pol*) gene, was constructed from the full-length, infectious molecular plasmid clone pSP65-HxB2gpt (32) (generously provided by L. Ratner). After pSP65-HxB2gpt was digested with *BalI*, the larger of the two fragments was religated following purification from an agarose gel. The deletion of HxB2 nucleotides 2620 to 5786 (GenBank accession number K03445) and the reconstruction of a *BalI* restriction digestion site were confirmed by restriction digestion and DNA sequencing of the region that included the new unique *BalI* site in pHxB2ΔBalI. Another

subclone derived from pSP65-HxB2gpt, called pHxB2 SphI-SalI, contained the 4.5 kb *Sph*I-*Sal*I fragment (HxB2 nucleotides 1447 to 5786) from pSP65-HxB2gpt (32) inserted into the corresponding restriction sites in pGEM 3Zf(−) (Promega, Madison, Wis.).

The method for generating cloned mutant viruses by cotransfection was adapted from an approach described by others (25). The pHxB2ΔBalI plasmid shares HxB2 nucleotides 1447 to 2620 and 4542 to 5786 with the mutant pHxB2 SphI-SalI plasmids constructed by site-directed mutagenesis and molecular cloning (see below). The sequence overlap facilitated homologous recombination and reconstruction of a full-length genome with the RT coding sequence derived from a mutant RT fragment. Both plasmids were cotransfected (10 μg of each) into COS-7 cells by using DEAE-dextran (20). As a control, transfections of only the pHxB2ΔBalI plasmid were done, and infectious virus was not produced. PCR products from cell pellets of second-passage stock generation cultures of cloned mutant viruses in MT-2 cells were directly sequenced to confirm the presence of the intended mutation(s).

The infectivity of virus stocks was determined by end point dilution and calculated by the method of Reed and Muench as cited in reference 18. Six replicates were performed at each dilution.

Drugs and chemicals. Drugs were obtained from the following sources: AZT was from Burroughs Wellcome, Research Triangle Park, N.C.; ddC was from Hoffman-LaRoche, Nutley, N.J.; ddI was from Bristol Myers Squibb, Wallingford, Conn.; foscarnet (phosphonoformate) was from Astra, Westborough, Mass.; nevirapine was from Boehringer-Ingelheim, Ridgefield, Conn.; and L697,661 was from Merck, West Point, Pa. A single lot of each drug was dissolved in phosphate-buffered saline at 1 μM, and aliquots were stored at −20°C until used. ddCTP, ddATP, and deoxynucleoside triphosphates (dNTPs) were obtained from Pharmacia (P-L Biochemicals, Milwaukee, Wis.). Oligodeoxynucleotides were synthesized with a DuPont Generator DNA synthesizer. Deoxyuridine phosphoramidite was from Cruachem, Herndon, Va.

Drug susceptibility assay. A previously published method was used to quantify viral replication in the presence of different concentrations of drugs by measuring HIV-1 core (p24) antigen in PBMC culture supernatant fluids by an enzyme-linked immunosorbent assay (ELISA) (DuPont-NEN Research Products, Billerica, Mass.) (17). Modifications included the use of an inoculum of 1,000 50% tissue culture infectious doses per million PBMC in 24-well plates in a volume of 1.5 ml. Four replicate cultures were performed in the absence of drug, and duplicate cultures were performed at each drug concentration. On day 3 of culture, 1.0 ml of resuspended cells was removed from each well and replaced with 1.5 ml of fresh medium. Cell-free supernatant fluid was harvested from each well and tested for p24 antigen by ELISA on day 7 of culture. The 50% inhibitory concentration (IC_{50}) was calculated by nonlinear regression analysis of the median effect equation (5).

In vitro selection of ddC-resistant virus. PBMC were infected at a multiplicity of infection of 1,000 50% tissue culture infectious doses per 10^6 cells at each passage. The cell-free supernatant fluids and cell pellets were harvested at the peak of infection in each passage as assessed by p24 antigen ELISA. The duration of each passage varied between 7 and 10 days in culture. Culture fluids were replaced twice weekly with fresh medium containing an appropriate ddC concentration. The concentration of ddC was increased with serial passage. Every passage was initiated at each of two ddC concentrations; the supernatant fluid from the culture maintained in the higher drug concentration was used for the next passage, as long as an adequate amount of virus for the subsequent inoculum was obtained. The first two passages were performed in the presence of 0.005 or 0.05 μM ddC, and the concentrations were doubled every few passages to reach 0.75 and 1.5 μM ddC in passages 15 through 34. As a control, the same virus was passaged in parallel under the same conditions in the absence of any drug. Every 10 passages, the IC_{50}s of ddC, ddI, and AZT were determined and RT sequence information was obtained for ddC-selected and control viruses.

Site-directed mutagenesis and molecular cloning of mutant HIV-1. A modification of recombinant PCR (14, 15) was used to introduce specific mutations into the HIV-1 RT coding sequences of HxB2 (32) (Fig. 1). A 1.2-kb fragment that included the relevant RT sequences (from the *Bgl*II restriction site at nucleotide 2096 to nucleotide 3245, including the *Eco*RV restriction site at HxB2 nucleotide 2979) was amplified from pSP65-HxB2gpt (32). Two primer pairs were used, including overlapping, internal mutagenic primers which had deoxyuridine incorporated in place of deoxythymidine (Fig. 1 and Table 1). Stringent precautions to prevent carryover were used for all amplifications. To control for potential contamination, reagent controls were included. The PCR products were mixed with pAMP vector DNA and digested with uracil deglycosylase, which allowed annealing of the strands complementary to the internal primers, as well as annealing of the flanking ends of each PCR product to the complementary strands of pAMP vector DNA (Fig. 1) (CloneAmp System; Gibco-BRL, Gaithersburg, Md.). Annealed pAMP and PCR product DNAs were used to transform bacteria, thereby cloning the PCR products into pAMP without ligase (30). A similar application of PCR and uracil deglycosylase to site-directed mutagenesis has been reported by others (31). The 883-bp fragment derived from PCR was sequenced, and the *Bgl*II-*Eco*RV restriction fragment was then directionally cloned into the larger fragment of *Bgl*II- and *Eco*RV-digested pHxB2 SphI-SalI by standard ligase-mediated methods.

DNA sequencing of HIV-1 RT. A 761-bp fragment of the HIV-1 *pol* gene was amplified by PCR (5′ primer, HIV-1 HxB2, sense strand, bases 2485 to 2504; 3′ primer, HIV-1 HxB2, antisense strand, bases 3245 to 3226 [3 CA 3245 in Table 1]) from cell lysates from various sources. These sources included (i) the in vitro selection experiment, (ii) stock generation cultures of cloned mutant viruses and ddI-treated patient isolates, and (iii) primary cocultivations of PBMC from patients treated with ddC. The dominant sequence at each position in RT codons 1 to 225 (HxB2 nucleotides 2549 to 3223) was determined by direct sequencing of PCR products (Sequenase; U.S. Biochemical, Cleveland, Ohio) (4).

Construction of mutant RT plasmid expression vectors. The pHxB2 SphI-SalI plasmids constructed by site-directed mutagenesis and molecular cloning were used as templates for PCR amplification in order to construct mutant RT plasmid expression vectors. Amplifications were done with a 5′ primer containing an *Nco*I site and a translation initiation codon, while the 3′ primer contained a *Hin*dIII site and a translation termination codon. The amplification products were digested with both *Nco*I and *Hin*dIII and then cloned into the larger *Nco*I and *Hin*dIII restriction digestion fragment of an RT expression vector (pKRT2), as previously described (6, 7). DNA sequencing of the entire RT was performed to confirm the presence of the intended changes and to rule out errors introduced during PCR.

Preparation of recombinant-expressed RT. *Escherichia coli* JM 109 containing either a wild-type (HxB2) or mutant RT expression vector was grown to late logarithmic phase at 37°C

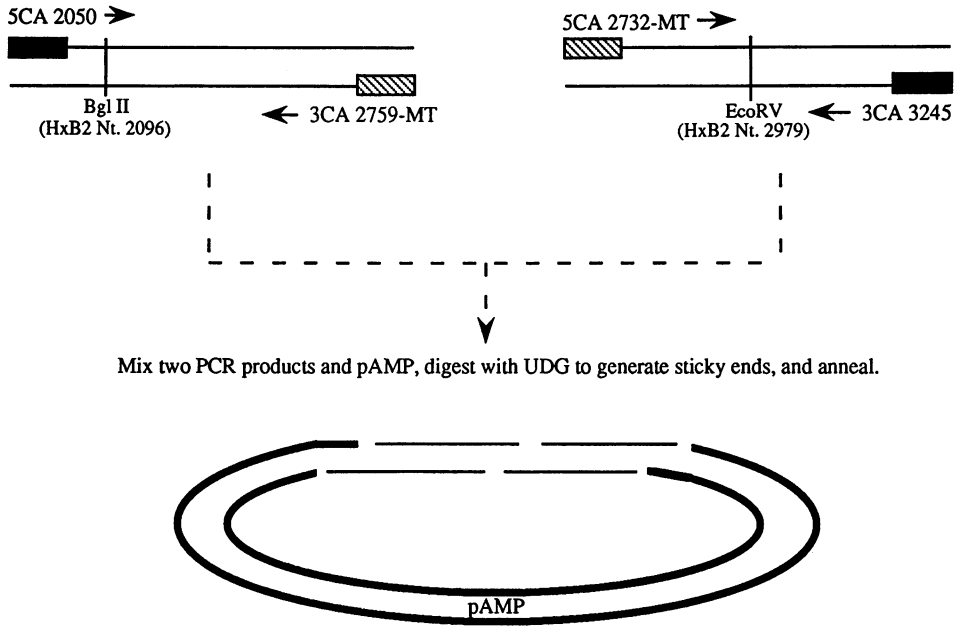

FIG. 1. Outline of site-directed mutagenesis by recombinant PCR. Two separate PCR amplifications were performed. As depicted in the upper portion of the figure, 5 CA 2050 and 3 CA 2759-MT series oligonucleotides (Table 1) were used as primers for one PCR. The 5 CA 2732-MT series and 3 CA 3245 oligonucleotides (Table 1) were used as primers for the other amplification. RT Lys-65→Arg was encoded in the primers 5 CA 2732-MT (65) and 3 CA 2759-MT (65) (Table 1). Both RT Lys-65→Arg and Ser-68→Gly were encoded in primers 5 CA 2732-MT (65+68) and 3 CA 2759-MT (65+68) (Table 1). The 5 CA 2050 and 3 CA 3245 oligonucleotides contained sequences complementary to the single-stranded ends of pAMP vector DNA (CloneAmp System; Gibco-BRL) (30). The two PCR products were mixed with each other and pAMP vector DNA, digested with uracil deglycosylase (UDG) to generate sticky ends by cleavage at the deoxyuridine residues in the primers that have been incorporated into the PCR products, and annealed. Bacteria were then transformed with the annealed molecules depicted in the lower portion of the figure in order to generate molecular clones of the recombinant PCR products.

in Luria broth with 100 μg of ampicillin (Sigma, St. Louis, Mo.) per ml. Expression of RT was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 to 16 h. The cells were pelleted, washed, and frozen at −20°C. After being thawed, cells were lysed in 50 mM morpholineethanesulfonic acid (MES) (pH 6.0)–2 mM EDTA–0.02% Triton X-100–10% glycerol and then sonicated.

TABLE 1. Oligodeoxynucleotides used in site-directed mutageneses

Designation[a]	Nucleotide sequence[b]
5 CA 2050	5′-*CUACUACUACU*ACCAAATGAAAGATTGTAC-3′
3 CA 2759-MT (65)	5′-ATTUAGUACU**A**UCTTUT**C**UCTUTAUGGC-3′
5 CA 2732-MT (65)	5′-GCCAUAAAGA**G**AAAAGA**U**AGUACUAAAT-3′
3 CA 2759-MT (65 + 68)	5′-ATTUAGUAC**CA**UCTTUT**C**UCTUTAUGGC-3′
5 CA 2732-MT (65 + 68)	5′-GCCAUAAAGA**G**AAAAGA**UG**GUACUAAAT-3′
3 CA 3245	5′-*CAUCAUCAUCAU*CATAACCCATCCAAAGGAAT-3′

[a] The four-digit number in each designation indicates the flanking terminal position in the HxB2 nucleotide sequence (GenBank accession number K03455). For 5′ primers (indicated here as 5 CA) this is the most 5′ base on the positive strand, and for 3′ primers (called 3 CA) this is the most 3′ base on the negative strand.

[b] The italicized sequences are complementary to the single-stranded ends of the pAMP vector DNA and can anneal to them following uracil deglycosylase digestion of the PCR product into which these primers are incorporated. The boldface, underlined bases represent the specific mutation(s) introduced in RT codon 65 (AAA→AGA, changing coding from lysine to arginine) by the MT (65) primers or in both RT codon 65 and RT codon 68 (AGT→GGT, changing coding from serine to glycine) by the MT (65 + 68) primers. The underlined bases that are not in boldface are mutations which do not lead to an amino acid change in codon 67 (GAC→GAT, both encoding aspartate).

Cell-free RT polymerase assays. Lysates of recombinant-expressed RTs 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). RT was incubated with primer-template at 37°C for 1 h. Following acid hydrolysis of the RNA template, the DNA product strand was hybridized in a streptavidin-coated microtiter plate well with both a biotinylated oligodeoxynucleotide capture probe and a horseradish-peroxidase-labelled detection probe. After extensive washing, tetramethylbenzidine (TMBlue; Transgenic Sciences, Worcester, Mass.), a chromogenic substrate for horseradish peroxidase, was added to each well. After 1 h, the reaction was terminated by addition of hydrochloric acid to 0.5 N. The optical density at 450 nm was quantified with a plate reader (Molecular Devices, Menlo Park, Calif.).

The polymerase activity of mutant RT lysates was compared with that present in wild-type RT lysates, using equivalent amounts of total protein from each lysate. 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. IC_{50}s were determined as described for virus susceptibility testing.

Statistics. The Mann-Whitney U test for independent samples was used for statistical evaluation of IC_{50} comparisons. A one-tailed test was used for the comparisons of the cloned mutant and wild-type viruses because higher ddC and ddI IC_{50}s were hypothesized on the basis of the IC_{50}s of the

TABLE 2. Virus susceptibility results

Virus	RT genotype	IC_{50} (mean ± SEM) (μM) of:		
		ddC	ddI	AZT
In vitro selected virus 14a-pre, 10th passage				
No drug	Wild type	0.06 ± 0.02		
ddC	Lys-65→Arg	0.25 ± 0.14		
Cloned site-directed mutant viruses				
HxB2	Wild type	0.07 ± 0.03	0.5 ± 0.1	0.003 ± 0.0003
HxB2-MT 65	Lys-65→Arg	0.31 ± 0.02[a]	4.3 ± 0.3[a]	0.003 ± 0.001
HxB2-MT 65+68	Lys-65→Arg Ser-68→Gly	0.44 ± 0.06[b]	2.4 ± 1.3[b]	0.005[c]

[a] The difference between the IC_{50}s indicated for HxB2 and HxB2-MT 65 viruses was significant ($P < 0.05$ by the Mann-Whitney U test [one tailed]).
[b] The difference between the IC_{50}s indicated for HxB2 and HxB2-MT 65+68 viruses was significant ($P < 0.05$ by the Mann-Whitney U test [one tailed]).
[c] Only one determination was performed.

ddC-selected virus (see Table 2). Two-tailed tests were used for polymerase assay data (see Table 3).

RESULTS

RT Lys-65→Arg confers ddC resistance to HIV-1. In order to maximize the potential for genetic heterogeneity in the initial inoculum, a clinical isolate, 14a-pre (18), was used for the in vitro selection of ddC-resistant virus in PBMC. Following 10 passages of this virus in increasing concentrations of ddC, the IC_{50} of ddC was elevated fourfold compared with that for the virus grown in the absence of drug (Table 2). The RT coding sequences of the ddC-selected variant contained only a single base change in the region from codon 1 to 230, which encodes most of the polymerase domain. This AAA→AGA change in RT codon 65 leads to substitution of an arginine for a lysine.

In order to test whether the RT Lys-65→Arg change was necessary and sufficient to cause ddC resistance, a cloned mutant virus containing only this mutation in RT was generated from HxB2 plasmids. The degree of increase in the ddC IC_{50} was similar to that observed in the in vitro-selected virus (Table 2). An increase in the ddI IC_{50} was also observed (Table 2). No changes were noted in susceptibility to AZT (Table 2), phosphonoformate (wild type, 23.8 μM; RT Arg-65 mutant, 25.4 μM), nevirapine (wild type, 0.3 μM; RT Arg-65 mutant, 0.2 μM), or L697,661 (wild type, 0.04 μM; RT Arg-65 mutant, 0.02 μM).

The possibility that the Lys-65→Arg substitution may be selected in vivo was also investigated. Virus was isolated from patients without any drug selection pressure. Two isolates from ddI-treated patients were found to have acquired low-level ddI and ddC resistance, in association with development of two genetic changes from their respective pre-ddI therapy isolates, Lys-65→Arg and Ser-68→Gly (AGT→GGT). Cloned mutant virus with these mutations in codons 65 and 68 did not differ appreciably in ddC or ddI IC_{50} from virus with only the codon 65 mutation (Table 2). One patient's paired isolates had a 30-fold increase in ddI IC_{50} (from 0.2 μM ddI pretherapy to 5.9 μM ddI posttherapy) and an 11-fold increase in ddC IC_{50} (from 0.007 μM ddC pretherapy to 0.08 μM ddC posttherapy). The other patient's isolates had a fivefold increase in ddI IC_{50} (from 0.8 μM ddI pretherapy to 3.8 μM ddI posttherapy) and a threefold increase in ddC IC_{50} (from 0.03 μM ddC pretherapy to 0.1 μM ddC posttherapy).

Paired HIV-1 isolates from four patients who received ddC monotherapy for 10 to 16 weeks were studied. One member of each pair of viruses was derived from cryopreserved PBMC collected before ddC monotherapy, and the other was derived from those collected during ddC monotherapy. None of the pre-ddC viruses contained the RT Lys-65→Arg substitution. In a virus derived from PBMC obtained during the 12th week of ddC therapy from one of these four patients, the codon 65 mutation was present as a mixture with the wild-type base. A mutation was not present in RT codon 68, 74, 69, or 184, or in any of the positions associated with AZT resistance, in any of these isolates from ddC-treated patients. Neither the ddI-treated patients nor the ddC-treated patients studied here had received AZT therapy prior to starting investigational ddI or ddC monotherapy.

RT Lys-65→Arg causes ddCTP resistance in recombinant-expressed enzyme. The Lys-65→Arg mutant RT had a degree of polymerase activity similar to that of wild-type RT (Table 3). The degree of resistance seen in mutant viruses was paralleled in mutant recombinant-expressed RTs. The codon 65 mutation caused a fourfold increase in ddCTP IC_{50} and a fivefold increase in ddATP IC_{50} (Table 3). The combination of mutations in both codons 65 and 68 also caused an increase in ddCTP IC_{50} compared with that of the wild type (Table 3).

DISCUSSION

HIV-1 RT Lys-65→Arg was selected by in vitro passage of virus in PBMC in the presence of ddC. This codon 65 mutation

TABLE 3. Polymerase assay results

RT	Genotype	% of wild-type polymerase activity	IC_{50} (mean ± SEM) (μM) of:	
			ddCTP	ddATP
HxB2	Wild type	100	5.6 ± 0.6	6.2 ± 0.9
HxB2-MT 65	Lys-65→Arg	88	23.4 ± 3.4[a]	29.9 ± 8.7[a]
HxB2-MT 65+68	Lys-65→Arg Ser-68→Gly	100	12.6 ± 2.6[b]	

[a] The difference between the IC_{50}s indicated for HxB2 and HxB2-MT 65 RTs was significant ($P < 0.05$ by the Mann-Whitney U test [two tailed]).
[b] The difference between the IC_{50}s indicated for HxB2 and HxB2-MT 65+68 RTs was significant ($P < 0.05$ by the Mann-Whitney U test [two tailed]).

was sufficient to cause an increase in ddC and ddI IC_{50}s, on the basis of studies of HxB2-derived site-directed mutant virus, and was documented to emerge in vivo following ddC or ddI treatment of HIV-1-infected patients. It was observed in clinical isolates from one patient treated with ddC and two patients treated with ddI. These isolates were obtained without in vitro drug selection, which might have made it difficult to distinguish whether such mutants arose in vivo or during in vitro isolation. Lys-65→Arg was present as a mixture with the wild-type codon in the isolate from the patient who received ddC therapy for a brief duration of 12 weeks, and only the mutant was detected in directly sequenced PCR products from cells infected with the isolates from patients who had received more long-term ddI therapy. Although Lys-65→Arg can develop in patients following ddC or ddI treatment, it is not possible to extrapolate from these data to predict the prevalence of Lys-65→Arg among ddC- or ddI-treated patients. However, Gu et al. have also independently identified this same mutation in a few additional post-ddC therapy clinical isolates and have documented that these isolates have a 5- to 15-fold higher ddC IC_{50} than do isolates lacking this mutation (12).

Only low levels of ddC resistance were noted here. Further passage of the ddC-selected virus is under way to identify whether additional mutations contribute to ddC resistance. It is noteworthy that AZT resistance does increase with the sequential accumulation of multiple mutations during treatment over many months in vivo (1). The ddI and ddC resistance caused by the ddI-selected RT Leu-74→Val substitution can also be augmented by certain AZT resistance mutations (7).

Another substitution (Ser-68→Gly) in addition to the codon 65 mutation was noted in the two isolates from the ddI-treated patients discussed here. The addition of this codon 68 mutation to the codon 65 mutation did not appreciably change the virus or enzyme susceptibility phenotype from that observed with only the codon 65 mutation. However, further characterization of the effects of Ser-68→Gly as a solitary substitution is necessary in order to exclude a role for it as a cause of ddC or ddI resistance.

The study of recombinant-expressed codon 65 mutant RT was undertaken here for two reasons: to evaluate whether this mutation rendered the RT nonfunctional, as others had observed by using different methods (2, 3), and to further explore the hypothesis that some nucleoside resistance mutations may affect nucleoside analog triphosphate binding (7). While the viability of the cloned codon 65 mutant virus strongly suggested that the codon 65 mutant RT was functional, this was confirmed by the results of polymerase assays of the recombinant-expressed codon 65 mutant RT. It is important to note that the comparison between wild-type and mutant enzymes here is based on comparing lysates containing equivalent amounts of total protein. A rigorous comparison of polymerase activities would require purification of the different RTs to homogeneity.

The enzyme studies reported here also support the hypothesis that some nucleoside resistance mutations affect binding of nucleoside analogs to RT. Mutations that alter inhibitor susceptibility in polymerase assays include mutations in codons 65 (this report), 74 (7), 184 (34), and perhaps 215 (26) and the combination of 215 and 219 (7). In contrast, some combinations of multiple AZT resistance mutations do not seem to alter AZT-triphosphate binding to RT (7, 22).

The need to differentiate only a fourfold difference from the wild-type ddC IC_{50} in drug susceptibility assays of clinical isolates represents a challenge, in addition to the lengthy and laborious process of isolating, expanding, titrating, and then testing the drug susceptibility of a patient's virus. These data suggest other approaches to monitor for ddC resistance in clinical isolates, however. Either a selective PCR assay for the codon 65 mutation, similar to those described for AZT resistance mutations (21, 24) and used in the studies of Gu et al. (12), or a polymerase activity assay of RT, such as used in the present study, may be more rapid and reproducible than drug susceptibility assays of a virus isolate. If the study of a larger number of patient samples in additional studies indicates that Lys-65→Arg is a common cause of ddC resistance, such assays might expedite obtaining an answer to the important question of whether ddC-resistant virus predicts disease progression during therapy.

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