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In Vitro Selection of Variants of Human Immunodeficiency Virus Type 1 Resistant to 3′-Azido-3′-Deoxythymidine and 2′,3′-Dideoxyinosine†

QING GAO, ZHENGXIAN GU, MICHAEL A. PARNIAK, XUGUANG LI, AND MARK A. WAINBERG*

Lady Davis Institute-Jewish General Hospital, 3755 Chemin Cote Ste-Catherine, and McGill AIDS Centre, McGill University, Montreal, Quebec H3T 1E2, Canada*

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Drug-resistant variants of human immunodeficiency virus type 1 (HIV-1) have been isolated by in vitro selection. MT-4 cells were infected with either a laboratory strain (HIV-III_B) or a clinical isolate (no. 187) of HIV-1 and maintained in medium containing subeffective concentrations of the drugs 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI). By gradually increasing the drug concentration in the culture medium during propagation of the virus on fresh MT-4 cells, we were able to isolate variants of HIV-III_B and clinical isolate 187 which showed up to 100-fold increases in resistance to the drugs. The drug resistance phenotypes remained stable after propagation of the variants in the absence of drug pressure for over 2 months. However, variants resistant to one drug showed little or no cross-resistance to the other, suggesting that the genetic bases for resistance to the compounds differed. Genotypic analysis of these nucleoside-resistant variants by polymerase chain reaction (PCR) with primer pairs previously shown to correspond to mutations responsible for resistance to AZT was also carried out. A heterogeneity of genotypes was observed, with known mutations at *pol* codons 70 and 215 occurring in most of the AZT-resistant variants generated from either HIV-III_B or clinical strain 187. However, mutations in codons 67 and 219 were less frequently detected, and none of these changes were observed in each of four variants resistant to ddI. Cloning and sequencing studies of the reverse transcriptase coding region of two of the isolates were also performed and confirmed the PCR data that had been obtained. In addition to previously described mutation sites responsible for resistance to AZT, an HIV-III_B-resistant variant was shown to be mutated at positions 108 (Val → Ala) and 135 (Ile → Thr), while a resistant variant of strain 187 was mutated at positions 50 (Ile → Val) and 135 (Ile → Val).

A variety of antiviral agents are able to interfere in vitro in the life cycle of the human immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS (5, 21, 22).

Considerable emphasis has been placed on nucleoside analogs which function as chain terminators of viral reverse transcriptase (RT) activity. Certain analogs, such as 3′-azido-3′-deoxythymidine (AZT), have been used clinically and, despite side effects (8, 9), have been found to improve both the quality and the length of life of patients infected with HIV-1 (8, 9, 27, 40, 41).

The RT of HIV-1 (39) exhibits a considerable degree of infidelity during the replication of viral RNA (25, 28, 36). Therefore, genetic heterogeneity is characteristic of HIV-1 (6), and viral mutations with reduced sensitivity might be expected to be selected under drug pressure. Indeed, several groups have reported the isolation and partial characterization of AZT-resistant HIV-1 from patients on prolonged drug therapy (12, 14, 29). Specific mutations have been described in the *pol* sequences of certain of these AZT-resistant HIV-1 variants isolated from patients (12, 16).

The molecular characterization of resistance to antiviral nucleosides would be enhanced if strains of HIV-1 with reduced drug sensitivity could be generated in vitro and if such procedures could be utilized to give rise to HIV-1 variants resistant to compounds other than AZT. We now report the generation and characterization of several such variants resistant to either AZT or 2′,3′-dideoxyinosine (ddI). These variants were generated through in vitro selection procedures using both the III_B laboratory strain of HIV-1 and a clinical isolate.

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MATERIALS AND METHODS

Cells and viruses. MT-4 cells were provided by N. Yamamoto, Yamaguchi University, School of Medicine, Ube, Japan (23). Cells were mycoplasma negative as assessed by fluorescence microscopy with the dye 4′,6-diamine-2-phenylindole (DAPI). Cells were maintained in suspension culture (3×10^5 to 5×10^5 cells per ml) in RPMI 1640 medium (GIBCO Laboratories, Mississauga, Ontario, Canada) supplemented with 10% fetal bovine serum (Flow Laboratories, Toronto, Ontario, Canada), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The HIV-III_B laboratory strain was a gift of R. C. Gallo, National Institutes of Health, Bethesda, Md. Viral strain 187 was a clinical isolate obtained by coculture of cord blood lymphocytes with peripheral blood lymphocytes from an infected patient who had not been treated with antiviral agents. Both strains were propagated on MT-4 cells. Stock virus was

* Corresponding author.

† Dedicated to the memory of Randall Coates, a scientist who dedicated his most productive years to the conquest of AIDS and HIV-associated disease.

prepared from clarified culture media by ultracentrifugation and stored at −70°C. In addition, peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and infected with HIV-1 as previously described (30). A CD4(+) line of CEM cells was also employed in some studies and was replicated and infected by HIV-1 as described previously (38).

Drugs. AZT and ddI were gifts of Burroughs-Wellcome, Inc. (Research Triangle Park, N.C.) and Bristol-Myers-Squibb, Inc. (Wallingford, Conn.), respectively. Stock solutions of these drugs were prepared in RPMI 1640 medium and stored at −20°C until use.

Selection process. MT-4 cells (3×10^5 cells per ml) were preincubated for 30 min with the appropriate concentration of drug and then infected with HIV-III_B or clinical isolate 187 at a multiplicity of infection of 0.01 PFU per cell. Virus had first been titrated by plaque assay on MT-4 cells as described elsewhere (10). After a 3-h incubation at 37°C, the cells were washed twice to remove excess virus and then maintained in medium containing drug concentrations equivalent to those used in the preincubation. The initial concentration of AZT or ddI in the cell culture medium was slightly below the minimum effective concentration able to inhibit viral replication in the cells. Culture fluids were replaced twice weekly with fresh medium containing an appropriate drug concentration, which was gradually increased during propagation of the virus on fresh MT-4 cells in the following manner. During each cycle of infection, undiluted clarified culture supernatants (0.5 ml) obtained from HIV-infected MT-4 cells cultured in the presence of drug were used to infect fresh MT-4 cells (6×10^5 cells). Half of these newly infected cells were cultured in medium containing drug concentrations identical to those of the previous cycle of infection. The remaining cells were cultured in medium containing a higher drug concentration. Clarified culture fluids from infected cells grown in the presence of the higher drug concentrations were used as the source of virus for each subsequent cycle of infection.

Selection of drug-resistant variants of HIV-1. Preliminary studies to isolate drug-resistant variants by using HIV-1-infected MT-4 cells cultured in the presence of high concentrations of drug were unsuccessful. We therefore determined the minimum effective concentration of each of the drugs that was able to prevent the appearance of infectious virus over 14 days in culture supernatants of MT-4 cells infected with either HIV-III_B or our clinical isolate 187. We then chose concentrations of drug slightly below these minimum effective concentrations for use in the initial selection process. These concentrations were 0.015 μM for AZT and 17 μM for ddI. Infectious supernatants were obtained from HIV-1-infected MT-4 cells at 4 days postinfection during the initial selection at these low drug concentrations. As drug concentrations were subsequently increased during the selection process, the appearance of virus was delayed to 6 days postinfection, as assessed by both indirect immunofluorescence for p24 antigen and RT activity. Details of the in vitro selection process are summarized in Table 1.

Immunoblot analysis of viral proteins. Virus in clarified culture supernatants from infected MT-4 cells was concentrated by ultracentrifugation (100,000 × *g*, 1 h), suspended in 400 μl of TNE buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA), further purified by sucrose density gradient centrifugation, recentrifuged, and suspended in viral lysis buffer (TNE buffer containing 0.5% Nonidet P-40, 0.5% Triton X-100, aprotinin [2 μg/ml], leupeptin [2 μg/ml], pepstatin [1 μg/ml] and phenylmethylsulfonyl fluoride [20 μg/ml]) as previously described (2, 30). Viral proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with a 1:100 dilution of serum obtained from an HIV-1-positive patient (2, 37). The blots were washed, incubated at room temperature for 1 h with ^{125}I-labeled protein A (Amersham), rewashed, dried, and exposed to Kodak X-OMAT film for 16 h at −70°C with an intensifying screen.

TABLE 1. Details of procedure for in vitro selection of drug-resistant variants of HIV-1

Virus	Cycle of infection	Duration of infection cycle (days) in presence of:		Concn (μM) of drug employed in infection cycle	
		AZT	ddI	AZT	ddI
HIV-III_B	1	4	4	0.015	17
	2	5	5	0.0225	34
	3	5	6	0.0375	85
	4	6	7	0.375	169.5
	5	7	7	0.75	254
	6	7	7	1.5	339
	7	7	7	1.9	424
Clinical isolate 187	1	4	4	0.015	17
	2	5	5	0.0225	34
	3	7	7	0.0375	85
	4	7	7	0.375	169.5
	5	7	7	0.75	212
	6	7	7	1.5	254
	7	7	7	1.9	339
	8		7		424

Assays of viral replication. Viral RT assays and indirect immunofluorescence assays for detection of p24 were performed as previously described (2, 30). Antigen capture tests for determination of p24 levels in culture fluids were carried out by using kits purchased for this purpose from Abbott Laboratories (North Chicago, Ill.).

PCR detection of relevant DNA sequences. For polymerase chain reaction (PCR), DNA was extracted according to published procedures (2, 32) either from 10^6 uninfected MT-4 cells or from MT-4 cells that had been infected with either parental-type HIV-1 (clinical isolate 187 or HIV-III_B) or AZT-resistant variants that had been selected and amplified by the procedures described above. When a double-amplification procedure was used, 1 μg of extracted DNA was initially used per PCR sample (100 μl), which contained 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 2.5 U of *Taq* polymerase, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, and 1 μM each of two previously described oligonucleotide primers, A and NEI, chosen to selectively amplify the region of the *pol* gene in which relevant mutations would be most likely to occur (16, 18). Samples were overlaid with 100 μl of light mineral oil and heated to 94°C for 5 min prior to being subjected to 30 thermal cycles of 1 min at 94°C for denaturation, 22 s at 45°C for annealing, and 1.5 min at 72°C for extension in a Perkin-Elmer Cetus Thermal Cycler. DNA thus generated (5 μl) was then subjected to further specific amplification with primers which recognize previously described mutations that account for AZT resistance in the viral *pol* gene, i.e. primer A paired with two wild-type or two mutated sequences or primer B paired with three wild-type or three mutated sequences (1 min at 94°C, 22 s at 45°C, and 30 s at 72°C for 30 cycles) to detect either wild-type or mutated

TABLE 2. Sensitivity of drug-resistant variants of HIV-1 to antiviral agents[a]

Variant	ID_{50} (μM) after:			
	Initial selection[b]		Additional propagation in absence of drug[c]	
	AZT	ddI	AZT	ddI
$HIV\text{-}III_B$	0.009 ± 0.0006	10.6 ± 0.85	ND	ND
AZT-resistant $HIV\text{-}III_B$	0.9 ± 0.1	10.6 ± 0.85	0.76 ± 0.075	ND
ddI-resistant $HIV\text{-}III_B$	0.0009 ± 0.0007	230 ± 9	ND	220 ± 10
ddI-resistant $HIV\text{-}III_B$	0.007 ± 0.0004	140 ± 16	0.0008 ± 0.0004	ND
Clinical isolate 187	0.01 ± 0.0006	10.6 ± 0.2	ND	ND
AZT-resistant 187	0.86 ± 0.075	11.0 ± 0.2	0.86 ± 0.075	ND
ddI-resistant 187	0.012 ± 0.0004	220 ± 12	ND	210 ± 11
ddI-resistant 187	0.012 ± 0.0001	170 ± 18	0.0092 ± 0.0006	ND

[a] Virus production was assessed by measurement of RT activity in clarified culture supernatants of infected MT-4 cells and by indirect immunofluorescence analysis for HIV-1 p24; the two methods gave identical results. Results were obtained 7 days postinfection. ID_{50} were determined by curve-fitting analysis and are reported as means ± standard deviations of three independent determinations.

[b] Data are from variants obtained after 8 weeks of our selection protocol, as described in Materials and Methods and in Table 1.

[c] Data are from the same resistant variants which had been further propagated on MT-4 cells for 2 months in the absence of drug following completion of the selection protocol. ND, not determined.

sequences at codons 70 and 215, respectively (16). Similar procedures were employed in the detection of mutated sequences corresponding to codons 67 and 219 (16). DNA was extracted with chloroform, precipitated with ethanol, electrophoresed on agarose gels, and visualized by ethidium bromide staining.

Cloning and sequencing. Cellular DNA was extracted as described above from MT-4 cells infected with AZT-resistant variants of either $HIV\text{-}III_B$ or clinical isolate 187. A 1,742-base segment containing the complete RT coding region, 34 bases of the 3′ end of the proteinase coding sequence, and 28 bases of the 5′ end of the integrase coding sequence was amplified by PCR as described above by using the primers 5′GTAGAATTCTGTTGACTCAGATTGG3′ and 5′GATAAGCTTGGGCCTTATCTATTCCAT3′ for the 5′ and 3′ ends, respectively. The PCR-amplified segments were purified from agarose gels, digested with *Hin*dIII and *Eco*RI (Pharmacia Fine Chemicals, Montreal, Canada), and ligated with digested M13mp19 (32). TG1 cells were transfected with the recombinants (32) and screened by digesting double-stranded DNA with restriction endonucleases. Single-stranded DNA was prepared from recombinant M13 clones, and nucleotide sequences were determined by using a TaqTrack sequencing kit (Promega Inc., Madison, Wis.).

RESULTS

Variants of both $HIV\text{-}III_B$ and clinical isolate 187 with significant drug resistance were noted approximately 8 weeks after initiation of the selection process. These variants showed resistance to the drugs up to 100-fold higher than that of the parental drug-sensitive strains (Table 2).

Stability of the drug resistance phenotype. The drug-resistant variants described in Table 2 were, in some cases, further propagated on MT-4 cells in the absence of drug for 2 months. The subsequent replication of these viruses in the presence of AZT or ddI showed that these variants retained their resistance phenotype and exhibited a level of drug susceptibility virtually identical to that of virus which had been propagated in the continuous presence of drug (Table 2). No evidence of decreased sensitivity to AZT on the part of ddI-resistant isolates was detected in these studies (Fig. 1).

Infectiousness of drug-resistant variants of HIV-1. As illustrated in Fig. 2 and 3, when a multiplicity of infection of 0.01 was used in the absence of drug, the kinetics of infection of MT-4 cells by in vitro-selected drug-resistant variants were virtually identical to those of the parental drug-sensitive strains. A peak of virus production, as measured by RT activity in culture fluids, was observed at 4 days postinfection. In the presence of high drug concentrations (up to 100-fold greater than that normally required to inhibit viral replication), maximal virus production was delayed to about 7 days postinfection. Significant virus-induced cytopathology was seen in each case after 3 days; this was delayed to 5 days in the presence of high drug concentrations (not shown).

We further investigated the ability of our drug-resistant variants to infect CEM cells, MT-4 cells, and PBMC, as monitored by immunofluorescence assay, in the presence of drug, when a multiplicity of infection of 0.1 was used. Each of these viruses was able to cause infection in the presence of the same drug that had been employed in its selection (Table 3). Although these variants showed increased sensitivity to the drugs in PBMC and CEM cells compared with MT-4 cells, viral replication nonetheless occurred in the

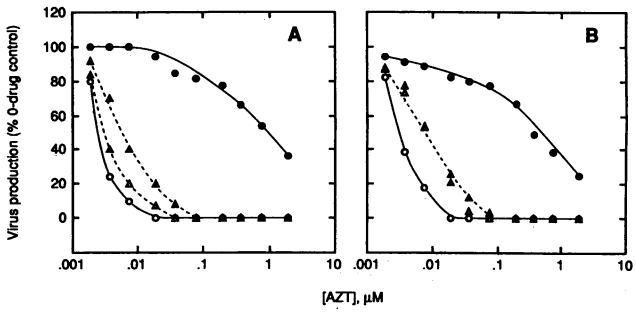

FIG. 1. Sensitivity of drug-sensitive and drug-resistant variants of $HIV\text{-}III_B$ (A) and clinical isolate 187 (B) to AZT. MT-4 cells were infected with HIV and cultured in the absence or presence of the indicated concentrations of AZT for 7 days. Virus production was then assessed by measurement of RT activity in aliquots of clarified culture supernatants. Symbols: ○, drug-sensitive parental virus; ●, AZT-resistant virus; △, ddI-resistant virus; ▲, second isolate of ddI-resistant virus.

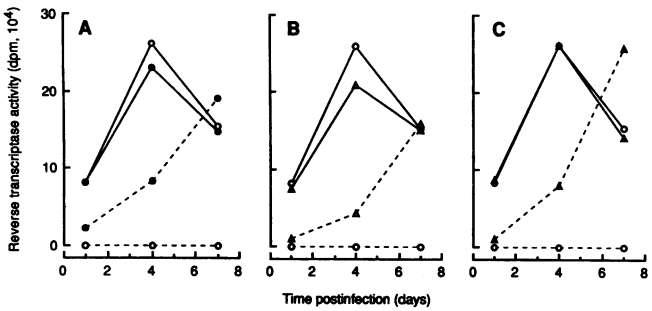

FIG. 2. Time course of virus production in MT-4 cells infected with variants of HIV-III$_B$ and cultured in the absence (——) or presence (– – –) of 1.9 μM AZT (A), 0.4 mM ddI (B), or 0.8 mM ddI (C). Virus production was assessed by measurement of RT activity in aliquots of clarified culture supernatants. Symbols: ○, drug-sensitive parental HIV-III$_B$; ●, AZT-resistant HIV-III$_B$; △, ddI-resistant HIV-III$_B$; ▲, second isolate of ddI-resistant HIV-III$_B$.

presence of concentrations significantly higher than those which abolished replication of the parental drug-sensitive strains.

HIV-1 viral proteins. To rule out the possibility that phenotypic mixing between human T-cell lymphotropic virus type I (HTLV-I), known to be present in MT-4 cells, and HIV-1 contributed to the observed drug resistance, drug-sensitive and -resistant viruses were concentrated from the culture fluids of drug-treated, infected MT-4 cells and analyzed by immunoblot, using serum from an HIV-1-seropositive individual. No differences in the patterns of viral proteins detectable in the different viral strains were observed and the only proteins consistently detected were those of HIV-1 (Fig. 4). Nor were differences observed in the patterns of viral proteins after these strains had been propagated in the absence of drug in MT-4 cells (data not shown).

PCR detection of relevant DNA sequences. Figure 5 illustrates a typical experiment on the detection of previously described mutations in HIV-1 variants selected in vitro for resistance to AZT. Results obtained with in vitro-generated AZT-resistant variants derived from clinical strain 187 and the III$_B$ laboratory strain of HIV-1 are shown. We found that mutated codon 70, which is associated with AZT drug

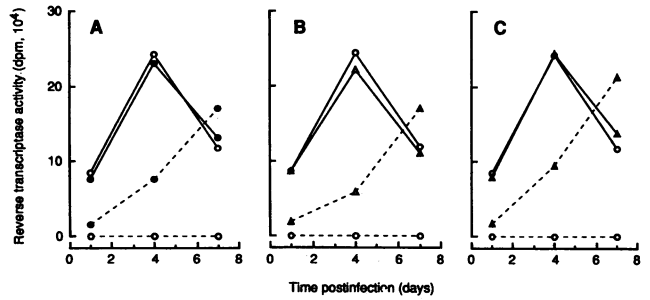

FIG. 3. Time course of virus production in MT-4 cells infected with variants of chemical isolate 187 and cultured in the absence (——) or presence (– – –) of 1.9 μM AZT (A), 0.4 mM ddI (B), or 0.8 mM ddI (C). Virus production was assessed by measurement of RT activity in aliquots of clarified culture supernatants. Symbols: ○, drug-sensitive parental clinical isolate 187; ●, AZT-resistant clinical isolate 187; △, ddI-resistant clinical isolate 187; ▲, second isolate of ddI-resistant strain 187.

TABLE 3. Infectiousness of drug-resistant HIV-1 on different cell types in the presence of drug

Cell type and HIV-1	% p24(+) cells[a] with:			
	AZT		ddI	
	0.037 μM	0.19 μM	21.2 μM	106 μM
MT-4				
AZT-resistant HIV-III$_B$	100	100	5	0
AZT-resistant 187	100	100	0	0
ddI-resistant HIV-III$_B$			100	100
ddI-resistant 187			100	100
CEM				
AZT-resistant HIV-III$_B$	17	0	0	0
AZT-resistant 187	10	0	0	0
ddI-resistant HIV-III$_B$	0	0	16	0
ddI-resistant 187	0	0	14	0
PBMC				
AZT-resistant HIV-III$_B$	17	0	0	0
AZT-resistant 187	12	0	0	0
ddI-resistant HIV-III$_B$	0	0	43	0
ddI-resistant 187	0	0	40	0

[a] Virus production was assessed 7 days postinfection by indirect immunofluorescence assay for HIV-1 p24. Values are reported as percentage of that noted with the same drug-resistant isolate propagated in the absence of drug, which was generally close to 100% of actual cell numbers present for MT-4 cells, 75% for CEM cells, and 10% for PBMC. No virus production was noted in any of the cell types in the presence of the indicated concentrations of drug when parental drug-sensitive HIV-III$_B$ or clinical isolate 187 was used.

resistance, was present in proviral DNA obtained from MT-4 cells that had been infected with either of these viral variants. As expected, the nonmutated codon 70, associated with sensitivity to AZT, was not detected in either of these cases. In contrast, studies on codon 215 revealed that the mutated form, previously shown to be associated with AZT resistance, was present in the proviral DNA of amplified resistant strain 187 but not of AZT-resistant HIV-III$_B$. Proviral DNA from cells infected with the latter variant continued to express wild-type or sensitive-type codon 215.

The data in Table 4 provide further details with regard to a variety of nucleoside-resistant variants of HIV-1 that have been generated in vitro. A mutation at position 70 was detected in each case of AZT resistance studied, but changes at codons 67, 215, and 219 were found in only some of the AZT-resistant isolates tested and never in viruses resistant to ddI. No correlation was apparent between degree of resistance to AZT (as measured by 50% inhibitory dose [ID$_{50}$]) and the presence or absence of mutated codons 67, 70, 215, and 219. These results indicate the heterogeneity of genotypes, which may account for resistance to AZT, and make clear that not all AZT-resistant strains of HIV-1 can be expected to have all or most of the previously described mutations. These PCR analyses are not quantitative and thus may not adequately reflect the broad range of quasispecies that may be present in each of the isolates chosen for examination.

Cloning and sequencing. To confirm and extend the data obtained by PCR analysis, we used primer pairs previously shown to correspond to mutation sites responsible for resistance to AZT to amplify a 1,742-base segment of the HIV-1 genome containing the entire RT coding region from the same AZT-resistant variants of each of the HIV-III$_B$ and 187 strains of HIV-1 described in Fig. 4. Cloning into TG1 cells was performed as described above. Sequencing results re-

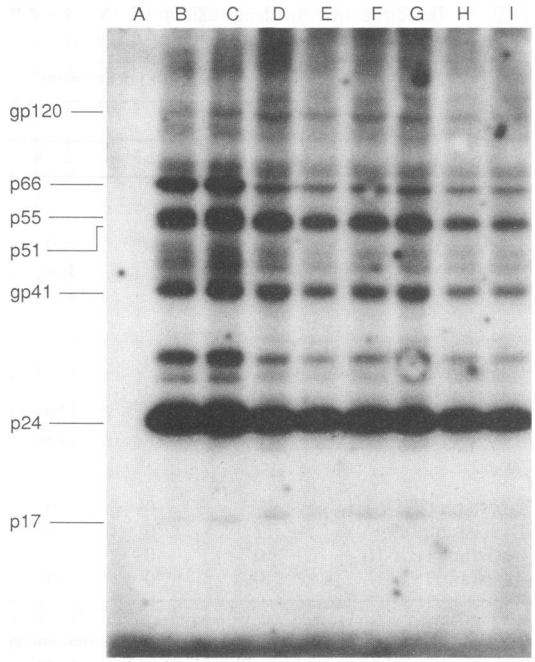

FIG. 4. Immunoblots of viral preparations harvested from uninfected or infected MT-4 cells. Lanes: A, uninfected cells; B, cells infected by HIV-III_B; C, clinical isolate 187; D, AZT-resistant HIV-III_B; E, AZT-resistant strain 187; F, ddI-resistant HIV-III_B; G, ddI-resistant strain 187; H, second strain of ddI-resistant HIV-III_B; I, second strain of ddI-resistant strain 187.

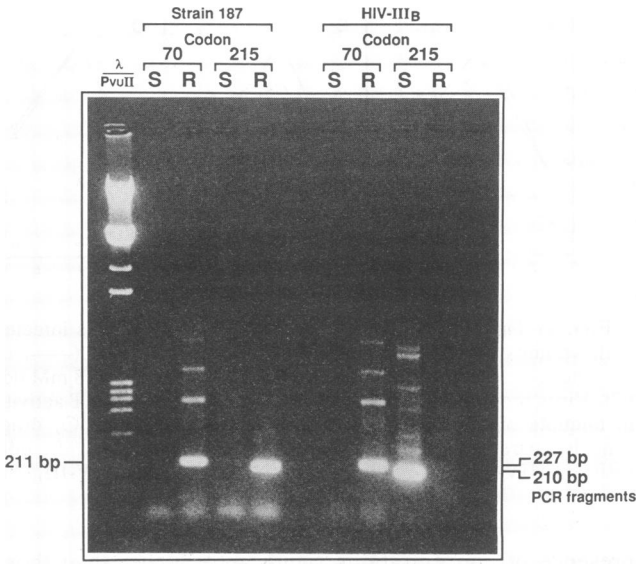

FIG. 5. Detection of sensitive wild-type (S), or resistant mutated (R) sequences corresponding to codons 70 and 215 of the HIV-1 *pol* gene by PCR with AZT-resistant variants of clinical strain 187 and HIV-III_B generated by in vitro selection. These are the same AZT-resistant viruses described in Table 2.

vealed that the HIV-III_B variant was mutated at positions 67 (Asp → His), 70 (Lys → Arg), 108 (Val → Ala), and 135 (Ile → Thr). Strain 187 was found to be mutated at positions 50 (Ile → Val), 70 (Lys → Arg), 135 (Ile → Val), and 215 (Thr → Tyr). Parental drug-sensitive strains of HIV-III_B and clinical isolate 187 were found not to be mutated at any of these positions when compared with previously published sequences of the RT coding region (15, 16, 18).

DISCUSSION

Previous studies have described the isolation from patients on prolonged AZT therapy of HIV-1 variants resistant to this drug (12, 14, 29). We now report that in vitro selection involving continuous propagation of HIV-1 in increasing concentrations of drug can also give rise to such resistant variants. We have isolated HIV-1 variants resistant to AZT and ddI from a clinical isolate of HIV-1 and the III_B laboratory strain.

TABLE 4. Detection of specific mutated sequences in individual nucleoside-resistant variants of HIV-1

HIV-1 variant	ID_{50} (μM) of:			Mutation at codon:			
	AZT	ddI	ddC[a]	67	70	215	219
HIV-III_B	0.05	5.52	0.35	–	–	–	–
HIV-III_B–AZT-1	1.4	1.65	0.55	–	+	+	–
HIV-III_B–AZT-2	2.7	1.45	0.60	–	+	–	–
HIV-III_B–ddI-1	0.06	45.7	0.75	–	–	–	–
HIV-III_B–ddI-2	0.04	53.8	0.66	–	–	–	–
187	0.06	2.75	0.40	–	–	–	–
187–AZT-1	5.2	1.65	0.65	+	+	+	–
187–AZT-2	4.8	3.75	0.75	–	+	+	–
187–ddI-1	0.05	26.3	0.45	–	–	–	–
187–ddI-2	0.06	38.2	0.65	–	–	–	–

[a] ddC, dideoxycytidine.

The drug resistance phenotype was stable if these viruses were grown in the absence of drug pressure for 2 months. This indicates that alterations in the HIV genome were probably responsible for the observed decrease in drug susceptibility. The lack of cross-resistance to other drugs and the differing levels of resistance observed suggest that different genomic alterations may have been responsible for resistance to AZT and ddI.

Previous studies have implicated mutations in the *pol* gene, which encodes viral RT, as responsible for the emergence of HIV-1 resistance to AZT in patients treated with this drug for protracted periods (12, 16). We have shown that our in vitro-generated AZT-resistant variants have mutations in some but not all of these same sequences, suggesting that the problem of HIV-1 resistance to AZT and other nucleosides is likely to be genetically more complex than initially believed. Our cloning and sequencing studies indicate the existence at positions 50, 108, and 135 of additional mutation sites responsible for resistance to AZT. Sequence analysis by other investigators has also indicated the existence at positions 125, 142, and 294 of previously undescribed mutation sites responsible for resistance to AZT (12). Furthermore, the latter study reported that certain variants of HIV-1 that were resistant to AZT were also relatively insusceptible to ddI (12), a finding consistent with earlier observations on the possibility of HIV drug cross-resistance (29).

One problem with previous observations may be that HIV-1 isolation from patients on prolonged AZT therapy was carried out directly on MT-2 cells, a procedure successful in the isolation of HIV-1 only about 30% of the time (15, 16). This process may have contributed to selection of a

relatively small subpopulation of AZT-resistant variants possessing relevant mutations only at positions 67, 70, 215, and 219. Of these last, mutations at codons 70 and 215 are presumably of greatest importance, since when inserted singly into drug-sensitive strains of HIV-1, they can induce development of AZT resistance (15). In contrast, insertion of mutated codons 67 and 219 did not yield similar results. This is the reason that the previously described mutations at codons 70 and 215 were analyzed by PCR in the present study.

It had been hoped that PCR amplification of known mutated sequences responsible for resistance to AZT or other drugs might be performed on blood samples from patients on prolonged anti-HIV therapy. The detection of such sequences might be possible long before the isolation of viruses possessing a resistance phenotype from these same individuals. Thus, recommendations for alternative therapy could be considered prior to the emergence of clinically significant burdens of resistant viruses. The fact that the number of mutation sites associated with AZT resistance is larger than first believed may complicate attempts to establish the molecular diagnosis of HIV drug resistance by PCR and similar techniques. Of course, the new mutation sites described here may not occur under clinical conditions. Efforts to validate the existence of these mutated codons in clinical material is now being conducted by PCR with newly generated primer pairs.

It is important that the PCR analyses discussed here not be over interpreted. For one thing, they are not quantitative and may not reflect the broad range of quasispecies potentially present in the samples which were amplified. The failure to find a given change could be due to the presence of nonmutated viral subpopulations which were amplified, while the presence of a given change may not have biological significance if it was due to the presence of a subpopulation that did not contribute to resistance.

An important characteristic of HIV-1 is its genomic diversity, due in part to the infidelity of viral RT in transcription of viral nucleic acid (25, 28, 36). We are uncertain whether the drug-resistant viral strains identified in this study existed among the heterogeneity of viral quasispecies prior to in vitro selection or whether relevant mutations occurred during replication under drug pressure. In view of the shortness of the period (i.e., <8 weeks) required for drug-resistant variants to appear in our study, it is not unlikely that these variants in fact preexisted and that the gradient of drug pressure employed resulted in the elimination of drug-sensitive strains. However, in previous studies, we failed to isolate drug-resistant forms of either HIV-III_B or clinical isolate 187 in vitro when we began with moderate to high initial concentrations of AZT (30). In the current study, the resistant variants generated possessed up to 100-fold increased resistance to this compound.

The current studies were performed with MT-4 cells because of the ability of HIV-1 to rapidly propagate in this cell line. However, the MT-4 line is also infected with HTLV-I (10, 23). The possibility that phenotypic mixing between HTLV-I and HIV-1 contributed to the observed drug resistance in this study was ruled out by immunoblot analysis that showed no differences between drug-sensitive and -resistant viral isolates. This does not rule out the possibility that interactions between HTLV-I and HIV-1 contributed to enhanced HIV-1 replication and *pol* gene mutations in this study, e.g., through the effects of the HTLV-I-encoded transactivator protein (*tax*) on the HIV-1 long terminal repeat sequence (33). Nor can a role for other coinfecting viruses in the development of HIV-1 clinical resistance to AZT be excluded (20, 34). In this context, it is important to note that the drug-resistant variants of HIV-1 described in this study were able to replicate in the presence of high drug concentrations in cells other than MT-4. Of course, it would be useful to know whether drug-resistant strains of HIV-1 could be selected in PBMC or other types of primary cell culture.

Viral drug resistance has, of course, been described in a variety of other systems, including herpesviruses and acyclovir (3, 4, 13, 17), cytomegalovirus and ganciclovir (1, 7), and influenza virus and amantadine (11, 19). In each case, it has been possible both to isolate viruses which possess a resistance phenotype from subjects receiving prolonged antiviral therapy and to generate such viruses through the types of in vitro selection procedures utilized in our study. AZT and similar drugs have also been used to antagonize the replication of murine (31) and feline immunodeficiency-inducing viruses (24) in both tissue culture and animals; in some cases, it has been possible to demonstrate drug resistance in virus-infected animals that have been treated with antiviral drugs (26).

It is therefore not surprising that in vitro selection of nucleoside-resistant variants of HIV-1 should now have been demonstrated. However, it is noteworthy that a number of groups, including our own, have previously attempted to demonstrate the in vitro selection of such variants without success (14, 18, 30, 35). The use in our study of a cell line that replicates HIV-1 more efficiently than those used previously (30) and the tedious schedule of increasing drug concentrations described in Table 1 probably both played important roles in our success.

Current studies indicate that no single drug is sufficient to attenuate viral replication in infected cells over protracted periods. The technique described here of in vitro selection of nucleoside-resistant variants of HIV-1 may be useful in predicting the likelihood of emergence of HIV-1 drug resistance in clinical trials. Current efforts are aimed at determining whether combinations of different antiviral drugs, used either simultaneously or sequentially, will also yield drug-resistant variants of HIV-1 under tissue culture conditions.

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ADDENDUM

Since acceptance of the manuscript, two reports of a related nature have appeared in the literature (J. H. Nunberg, W. A. Schleif, E. J. Boots, J. A. O'Brien, J. C. Quintero, J. M. Hoffman, E. A. Emini, and M. E. Goldman, J. Virol. **65**:4887–4892, 1991; and B. A. Larder, K. E. Coates, and S. D. Kemp, J. Virol. **65**:5232–5236, 1991).

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